Mathematical morphology: detection and characterization of directed axonal growth in vitro

M A Gonzalez 1, V L Ballarin 1, M Rapacioli 2,3, A Rodríguez Celín 2,3, V Sánchez 2,4 and V Flores 2,3,4
1 Measurement and Signal Processing Laboratory, School of Engineering, UNMdP, Juan B. Justo 4302, Mar del Plata, B7608FQD, ARGENTINA
2 CONICET (Comisión Nacional de Investigaciones Científicas y Tecnológicas), Rivadavia 1917, Ciudad Autónoma de Buenos Aires, C1033AAJ, ARGENTINA.
3 Interdisciplinary Group in Theoretical Biology, Department de Biostructural Sciences, UF. ARGENTINA.
4 Institute of Cell Biology and Neurosciences “Prof. E. De Robertis”, U.B.A. ARGENTINA.
E-mail: mazulgonzalez@fi.mdp.edu.ar

Abstract. Neurite growth in vitro, neuritogenesis, is a widespread methodology in the field of developmental neurobiology. Morphological analysis of growing neurites is usually a difficult task because of their thinness and their low contrast that makes it difficult to observe clearly their shape, number, length and spatial orientation. This paper presents the use of the granulometric size distribution function to obtain, automatically, information about the shape, size and spatial orientation of growing axons in tissue cultures. The results presented show the efficiency of the granulometric size distribution for this application. The automatic detection of growing axons and the precise characterization of a relevant parameter, indicative of the axonal growth spatial orientation, was obtained by this morphological tool. The developed algorithms facilitates the analysis of these images by automatically quantifying the angle of deviation of the direction of growth, which is important given the large number of images that need to be processed for this type of study.

1. Introduction
In vitro culture of developing neurons is a common strategy to analyze dendro- and axonogenesis regulation. It is known that the specificity of the peripheral-central connections and the establishment of local circuits within the central nervous system depends in part on the directed growth cone migration [1][2]. Developing neurites are highly dynamic and subtle structures. Given the difficulty to measure and quantify their relevant features, i.e. shape, thickness, length, spatial orientation etc, they are usually described qualitatively. A relevant aspect in developmental neurobiology is the statistical analysis of neuritogenesis and this requires precise records of the above mentioned characteristics [3]. This situation reveals the need for designing and implementing algorithms with the ability to precisely reveal some of these neurite features.

The main object of this work was the application of digital image processing algorithms to images obtained from cultures of explants obtained from a specific superior area of the chick central nervous system in order to automatically characterize the shape, size and spatial orientation of the growing axons.

Images obtained from this kind of tissue cultures are usually characterized by noisy background, non-homogeneous gray level (illumination), groups of neuronal bodies, isolated migrating neurons and a large number of neurites with different characteristics depending on the embryonic stage and on the area under analysis. Axons usually differ in shape, gray level, texture, size, orientation etc. All these features greatly difficult the acquisition of a binary image [4].
Conventional image processing methods for analysis of shapes of objects requires a binary image and a subsequent calculation of factors roundness, shape, area, etc. [4][5]. Unlike standard techniques, morphological techniques are based on concepts of geometry, algebra, topology and set theory, to characterize structural properties in images [6]-[10]. The central idea of these techniques is to examine the geometric structures in an image overlaying them with small patterns called structuring elements. Of all mathematical morphology analysis techniques, the most appropriate tool for discriminating shapes is the granulometric size distribution. [11][13].

This paper proposes the computation of the granulometric size distribution with structuring elements of various shapes and sizes to quantify the number and orientation of the growing axons and the subsequent statistical analysis.

2. Materials
We used microscope images of different patterns of growing axons distribution. We analysed 16 images of growing axons. The images were converted to grayscale (255 levels). The color was not providing information to assist the study. Some of those images show clear preferential spatial orientations; others images show irregularly distributed spatial orientations. Images without axons were also analyzed. Figure 1 shows three cases representative of the growth of axons.

All algorithms were implemented using Matlab R14, especially image processing libraries.

3. Methods

3.1. Mathematical Morphology applied to binary images
The basic operations of binary morphology are erosion and dilation [6]. These operations compare subsets of the binary image with a pattern element called structuring element. The structuring element is moved over the whole image [8]-[10].

Formally, the erosion of a set $A \subseteq \mathbb{R}^2$ by a structuring element $E \subseteq \mathbb{R}^2$ is defined as:

$$A \Theta E(x) = \{x \in A : E_x \subseteq A \}$$

which represents the translation of the set $E$ at $x$. The result of erosion is a binary image where pixels are white if the structuring element is included within the subset of the analyzed image and black otherwise.
Similarly, dilation of a set \( A \subset \mathbb{R}^2 \) by a structuring element \( E \subset \mathbb{R}^2 \) is defined as:
\[
A \oplus E(x) = \{ x \in A : E_x \cap A \neq \emptyset \}
\] (2)
The result of dilation is a binary image where pixels are white if at least exists one pixel of intersection between the subset of the original image and structuring element and black when the intersection is empty. Dilation is an operation of expansion while erosion is a contraction operation. These basic operations define new morphological operators. For example, opening is defined as erosion followed by dilation:
\[
A \circ E(x) = (A \Theta E) \oplus E
\] (3)
Closing is defined as dilation followed by erosion, that is:
\[
A \bullet E(x) = (A \Theta E) \Theta E
\] (4)
From these operators, the granulometric size distribution is defined. This mathematical morphologic function is a tool for characterization of shapes and sizes of objects in an image. Given the family \( \{A \circ E_x\}_{x \in \lambda} \) obtain by the application of successive openings with structuring elements of increasing size is defined by the name of granulometric size distribution function is defined by:
\[
G(\lambda) = 1 - \frac{\Omega(A \circ E_x)}{\Omega(A)}
\] (5)
where \( \Omega \) is a measure of the resulting image. For the case of binary images, \( \Omega \) results to be the area.

### 3.2. Mathematical Morphology applied to gray level images
As in mathematical morphology for binary images, the basic morphological operations for gray level images are erosion and dilation [8]-[10].

Given two images \( f \) and \( g \), with domains \( D_f, D_g \subset \mathbb{R}^2 \), erosion of image \( f \) by structuring element \( g \) is defined as:
\[
f \Theta g(s,t) = \min_{(x,y) \in D_g} \{ f(s + x, t + y) - g(x, y) \}
\] (5)
For each pixel of the image, the erosion operation is defined as the minimum difference between the intensities of the shifted structuring element and the corresponding intensities of the original image. Similarly, the dilation of image \( f \) by structuring element \( g \) is defined as:
\[
f \oplus g(s,t) = \max_{(x,y) \in D_g} \{ f(s - x, t - y) + g(x, y) \}
\] (6)
Dilation of each pixel of the image is defined as the maximum value of the sum between the intensities of the subset of the original image and the intensities corresponding structuring element.
The opening operator and the granulometric size distribution are defined similarly to the binary operators [14]-[15].
\[
G(\lambda) = 1 - \frac{\Omega(A \circ E_x)}{\Omega(A)}
\] (7)
Successive openings are made to the original image with increasing structuring elements. Then a measure \( \Omega \) is calculated for each opening. In the case of gray level images, \( \Omega \) is the volume. This result is normalized to the original image.
After calculating the granulometric size distribution function, which is actually a probability distribution function, mean, standard deviation and energy are calculated.

4. Results

We evaluated the statistical on images of growing axons in tissue cultures. The minimum mean value calculated from the granulometric function using lineal structuring elements increased with the number and the thickness of the growing axons. The plot of the percentage of area occupied by the axons versus the minimum mean value obtained illustrates this relationship (Figure 2). The area occupied by neurons was determined by counting the number of pixels that are included in them and then dividing this value by the total number of pixels. Although this analysis cannot determine the number of axons, it showed a linear relationship and a good approximation of the area occupied by axons. The mean value obtained with other structuring elements did not show linear relations.

![Figure 2: Area Vs, Minimum Mean Value obtained with oriented linear structuring element](image)

The calculation of the granulometric size distribution function with linear structuring elements permitted to know the orientation of the objects in the images. The mean value determined from this function is lower when the axon has the same orientation as the structuring element used in the calculation of the granulometric function. The minimum mean value obtained by means of this function allows a precise quantification of the angle of deviation of the axons longitudinal axes which constitute a reliable parameter indicating the preferential spatial orientation or the preferential growth direction (see figure 3).
5. Conclusions
The present paper is a preliminary approach to design a reliable method for digital image analysis with the ability to identify and to quantify some relevant parameters of axonal growth indicative of their plasticity, dynamism and sensitivity to different factors during neuritogenesis (growth factors, extracellular matrix components, axon-neuron interactions, axon-glial cell interactions etc).
Our results show that the granulometric size distribution function is a useful tool for the characterization of the spatial orientation of objects both in synthetic images and in real images of growing axons. This utility is not relevant when the growing axons fasciculate (close contact between parallel adjacent growing axon) displaying a defined preferential growth direction. The granulometric size distribution function, however, can be successfully applied in those cases when growing axons do not display a preferential growth direction but spatially arrange as irregular complex networks. Even in these cases the granulometric function allows the detection and quantification of axons displaying different growth direction. This is a remarkable utility of the granulometric function since the most frequent images of axons growing in tissue culture correspond to complex networks with none preferential spatial orientation.

As future work, a similar algorithm able to analyze in vivo the degree of order of growing axons will be implemented. Reliable data to analyze in vivo the establishment of peripheral and central neural circuits requires a precise recording about how growing axons orientate and direct their growth with respect to intrinsic spatial reference axis (cephalic-caudal, dorsal-ventral and radial axes) as well as the time or stage-dependent changes in these parameters during development. These data could help to better understand the neuritogenic process and the development and stabilization of the neuronal circuits.

6. References
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