Growing axons analysis by using Granulometric Size Distribution

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Abstract. Neurite growth (neuritogenesis) in vitro is a common methodology in the field of developmental neurobiology. Morphological analyses of growing neurites are usually difficult because their thinness and low contrast usually prevent to observe clearly their shape, number, length and spatial orientation. This paper presents the use of the granulometric size distribution in order to automatically obtain information about the shape, size and spatial orientation of growing axons in tissue cultures. The results here presented show that the granulometric size distribution results in a very useful morphological tool since it allows the automatic detection of growing axons and the precise characterization of a relevant parameter indicative of the axonal growth spatial orientation such as the quantification of the angle of deviation of the growing direction. The developed algorithms automatically quantify this orientation by facilitating the analysis of these images, which is important given the large number of images that need to be processed for this type of study.

1. Introduction

Analysis of neurites outgrowth from explants of developing nervous tissue in culture media is a common experimental strategy to analyze the factors influencing or regulating neuritogenesis (dendro- and axonogenesis), the directed growth cone migration, the directed neurite growth and the establishment of projection pathways and/or local neuronal circuits in the developing central nervous system [1][2]. Developing neurites are subtle dynamic structures. Their changing morphological characteristics make them difficult to measure and quantify. Thus, their relevant features, i.e. shape, thickness, length, spatial orientation etc, are usually described qualitatively. However, 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 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 (ilumination), 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 require 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
To evaluate the results of the application of the granulometric size distribution, in a first step we used synthetics images, and in a second step we used images corresponding to different patterns of axons distribution commonly found in tissue cultures analyzed with the light microscope.

For the first step, we generated 30 synthetic images, 10 with a white background, 10 with light gray background and 10 with dark gray background. For each background, rectangles of different thicknesses with orientations of 0 °, 45 °, 90 ° and 135 °. The number of rectangles in each image was also variable. In Figure 1 we can see some examples of synthetic images.

In the second step we analysed 16 images of growing axons in tissue culture. Some of those images show clear preferential spatial orientations; others images show irregularly distributed spatial orientations. Images without axons were also analyzed. Figure 2 shows three cases representative of the growth of axons.

All algorithms were implemented using Matlab R14.

Figure 1. Synthetic Images

Figure 2. Growing axons Images
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 \( A \subset \mathbb{R}^2 \) by a structuring element \( E \subset \mathbb{R}^2 \) 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:

\[
A \oplus E(x) = \{ x \in A : E_x \cap A \neq \emptyset \}
\]

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:

\[
A \circ E(x) = (A \Theta E) \oplus E
\]

Closing is defined as dilation followed by erosion, that is:

\[
A \bullet E(x) = (A \oplus E) \Theta E
\]

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:

\[
G(\lambda) = 1 - \frac{\Omega(A \circ E_x)}{\Omega(A)}
\]

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:

\[
f \Theta g(s,t) = \min_{(x,y) \in D_g} \{ f(s + x, t + y) - g(x, y) \}
\]

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:

\[
f \oplus g(s,t) = \max_{(x,y) \in D_g} \{ f(s - x, t - y) + g(x, y) \}
\]
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 \cite{14}-\cite{15}.

\begin{equation}
G(\lambda) = 1 - \frac{\Omega(A \circ E_\lambda)}{\Omega(A)}
\end{equation}

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
The granulometric size distribution function was calculated for test images using structuring elements in different orientations. The variability of the moments was analysed in function of the background color, the number of rectangles, the width of the rectangles and their orientation.

A. Analysis of the variation of mean with respect to the variation of gray level of the background
In synthetic images with different gray levels for the same kind of rectangles (Fig.1), mean values of the granulometric size function were measured. This parameter showed a high variability without following any specific behaviour. Some of the results are shown in Table I.

| structuring element | white background 4 thin rectangles at 45° | light gray background 4 thin rectangles at 45° | dark gray background 4 thin rectangles at 45° |
|---------------------|------------------------------------------|---------------------------------------------|------------------------------------------|
| line 0°             | 0.3864                                   | 0.5416                                      | 0.9499                                   |
| line 90°            | 0.6249                                   | 0.6233                                      | 0.8805                                   |

| structuring element | white background 17 thin rectangles at 45° | light gray background 17 thin rectangles at 45° | dark gray background 17 thin rectangles at 45° |
|---------------------|--------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| line 0°             | 0.8593                                     | 0.8175                                       | 0.8749                                       |
| line 90°            | 0.4689                                     | 0.4345                                       | 0.3664                                       |

| structuring element | white background 7 thick rectangles at 0° | light gray background 7 thick rectangles at 0° | dark gray background 7 thick rectangles at 0° |
|---------------------|-------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| line 0°             | 0.5054                                    | 0.3987                                       | 0.5439                                       |
| line 90°            | 0.891                                     | 0.8423                                       | 0.9139                                       |

B. Analysis of the variation of the mean with respect to the number and thickness of rectangles.

The mean value calculated from the size distribution function increases with the number and thickness of the rectangles in all test images analyzed. However, we cannot distinguish whether this increase is due to the increase in the number or in the thickness. Only this function could be used to assess the occupancy of axons in the image, ie the ratio between the area occupied by neurons and the background area. Table II shows these results.
TABLE II
MEAN VALUE OF THE GRANULOMETRIC SIZE DISTRIBUTION FUNCTION IN IMAGES WITH DIFFERENT NUMBER OF AXONS. (WITH A LINEAR STRUCTURING ELEMENT AT 0°)

| White background  | Light gray background | Dark gray background |
|-------------------|-----------------------|---------------------|
| 4 thin rectangles at 45° | 4 thin rectangles at 45° | 4 thick rectangles at 135° |
| 0.5406            | 0.5416                | 0.8912              |
| 8 thin rectangles at 45° | 8 thin rectangles at 45° | 8 thick rectangles at 135° |
| 0.6536            | 0.6521                | 0.9021              |

C. Analysis of the variation of the mean with respect to the orientation of the rectangles and the orientation of the structuring element used

For the different synthetic images the granulometric size distribution was calculated with linear structuring elements with different orientations.

The results showed that when the structuring element used corresponds to the inclination of the rectangles of the images, the average value of the function is lower than in the other case. This is because when performing an opening with a structuring element which coincides with the shape of objects in the image, these objects do not appear in the resulting image, so that the measured volume is smaller and therefore the associated moment.

Table III shows some examples. This table highlights the values of the structuring element whose size distribution function coincides with the orientation of the rectangles. It is worth to note that this result was confirmed for the 30 test images.
D. Analysis of the images of growing axons

Having analyzed the results for synthetic images, we evaluated the statistical on images of growing axons in tissue culture (Fig. 2). As in previous studies, the calculation of the granulometric size distribution function with different 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 mean value calculated from the granulometric function also increases with the number and the thickness of the growing axons.

The granulometric function was calculated using a square structuring element. When there is no definite orientation of the axons, the average value calculated from the granulometric function is lower than in the other case. This feature allows the characterization of axonal networks where axons are irregularly distributed without any preferential orientation, since this structuring element analyzes all directions simultaneously.

### Table IV

**Mean value of the granulometric size distribution function in images with growing axons.**

| Inclination of the rectangles in the image | Linear structuring element at 0° | Linear structuring element at 90° | Linear structuring element at 135° |
|------------------------------------------|---------------------------------|----------------------------------|----------------------------------|
| 90°                                      | 0.8175                          | 0.4345                           | 0.8319                           |
| 90°                                      | 0.8153                          | 0.6014                           | 0.7284                           |
| 135°                                     | 0.7671                          | 0.5714                           | 0.5497                           |
| 90°                                      | 0.8749                          | 0.3664                           | 0.9057                           |
| 90°                                      | 0.9078                          | 0.4639                           | 0.8846                           |
| 135°                                     | 0.8912                          | 0.8788                           | 0.8075                           |
| 90°                                      | 0.8924                          | 0.3735                           | 0.8657                           |
| 90°                                      | 0.9064                          | 0.4814                           | 0.8813                           |
| 90°                                      | 0.8593                          | 0.4689                           | 0.8789                           |
| 90°                                      | 0.6673                          | 0.4669                           | 0.7698                           |
| 0°                                       | 0.5054                          | 0.891                            | 0.8658                           |
| 0°                                       | 0.3987                          | 0.8423                           | 0.8615                           |
Tables IV and V show the discrimination power of the mean value calculated from the granulometric function, in images of growing axons.

5. Conclusions
The present paper is a preliminary attempt to design a reliable method for digital image analysis with the ability to easily identify growing axons and to quantify 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 in tissue culture.

The 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.

This utility is not relevant when the growing axons fasciculate (parallel growing direction and close contact between adjacent axons) displaying a clearly defined preferential growth direction.

The granulometric size distribution function, however, can be advantageously applied (with great utility) in those cases when the growing axons do not display a preferential growth direction but they spatially arrange as irregular complex axonal 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 of axons displaying none preferential spatial orientation.

The proposed automatic algorithms simplify the analysis of these images, which is important due to the large number of images that need to be processed for a robust study.

As future work we plan to implement the algorithms developed to characterize, in vivo, the degree of order or variability of growing axons with respect to the intrinsic spatial reference axis of the developing central nervous system (cephalic-caudal, dorsal-ventral and radial axes) as well as the time or stage-dependent changes in these parameters during the central nervous system 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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