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Based on the Triangular Prism Method for Computing Fractal Dimension: An Index of Nerve Regeneration Factor Promotes Growth of Dorsal Root Ganglions in Neonatal Rat

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Abstract: In order to investigate the relationship between neurite growth of Dorsal Root Ganglions (DRGs) in neonatal rat and different concentration of Nerve Regeneration Factor (NRF) cultured, We applied Triangular Prism Method (TPM) to calculate the fractal dimension of neurite outgrowth of rat DRGs which were obtained after DRGs were treated with different concentrations of NRF. Our results showed that fractal dimension of neurite outgrowth of rat DRGs changed with the amount and length of neurite positively, therefore, it can be used as an important index for quantitative measurement of the neurite growth of rat DRGs cultured by NRF.

Key words: Nerve regeneration factor, dorsal root ganglion, morphology, fractal dimension, triangle prism method

INTRODUCTION

Fractal geometry developed by Mandelbrot (1967) to model natural outline or surface as well as other complex forms that traditional Euclidean geometry fails to analyze. A major application of fractal geometry in the biology and medicine has been the use of fractal dimension to characterize the form of the studied phenomena at different scales. As development of imaging technique, fractal analysis appears to provide considerable potential for characterizing morphology in biomedical images. The fractal dimension is a central construct of fractal geometry. It is called fractal dimension because it is a fractional (or non-integer) number. A biomedical image can be viewed as a hilly terrain surface whose "elevation" is proportional to the image gray-level values. Typically, a fractal dimension of terrain surface may be a non-integer value between 2 to 3. A grayscale image can be interpreted as a 3D space where the x, y coordinates represent 2D position on the image plane and the z coordinate represents the gray-level values. Most biological and medical images are spatially complex and then, the fractal dimension appears to be a useful index for measuring the surface roughness (i.e., brightness differences) (Lopes and Betrouni, 2009). Several studies have used fractals to characterize textures and features in biomedical

images. For example, Zioupos *et al.* (2006) concentrated primarily on the variation of toughness of ageing bone with age and then examined the fracture profile morphology of the various samples by fractal analysis. Kalmanti and Maris (2007) applied fractal dimension as an index of brain cortical changes throughout life. Mashiah *et al.* (2008) indicated that measurement of fractal dimension seems to be a sensitive method to assess the hematological cell phenotype and to define a clinical group. Iftekharuddin *et al.* (2009) exploited the effectiveness of two novel fractal and fractalwavelet features to segment and classify tumor regions from non-tumor regions in both single and multimodality pediatric brain MR images. Oczeretko *et al.* (2010) described fractal analysis of dental radiographic images in the irregular regions of interest. Sun *et al.* (2011) made use of fractal dimension analysis on multidetector CT images for quantifying the morphological changes of the pulmonary artery tree in patients with pulmonary hypertension. Parvu *et al.* (2012) performed a study of complex morphological features that characterize cells and tissues of healthy human periodontium utilizing the fractal geometry. Karperien *et al.* (2013) indicated that the techniques of fractal analysis have been used for quantitating microglial morphology, to categorize gross differences but also to differentiate subtle differences.

Previous research found that Nerve Regeneration Actor (NRF) has effects of promoting nerve growth and preventing neuron apoptosis; the effect of NRF on primary cultured neurons remains unclear (Qi *et al.*, 2006). Here we investigated the effects of NRF on primary cultured Dorsal Root Ganglions (DRGs) in neonatal rat. The neurite outgrowth of rat DRGs has complex branching structures, its morphological complexity differs according to concentration of NRF, conventional digital image analysis techniques, based on diameter, length and perimeter, is not fitted into identify morphology of neurite outgrowth of rat DRGs. One way of tackling this question is by fractal analysis technique for the digital microscope images. The neurite outgrowth is found to exhibit fractal behavior and the derived fractal dimension gives a good description of its morphological complexity. We demonstrate the application of adopting the Clarke's Triangle Prism Method (TPM) (Clarke, 1986), to compute fractal dimension of neurite outgrowth of DRGs and illustrate relationship between neurite outgrowths of DRGs induced by NRF and different NRF concentration with fractal dimension.

MATERIALS AND METHODS

Materials: Neonatal Sprague-Dawley (SD) rats were provided by the Experimental Animal Center of Nantong University (EACNU). NRF was prepared by Key Laboratory of Neuro regeneration, Nantong University. Rabbit anti NF-H polyclonal antibody was from Sigma. Rhodamin goat-anti-rabbit IgG was from Santa Cruz. DRGs were harvested from neonatal SD rats.

Explanted DRGs were plated on a poly-L-lysine coated 96-well plate (Falcon) with L15 media containing 1% FBS and maintained at 37°C, in a 5% CO₂, 95% air atmosphere. The cultured DRGs explants were treated and classified into 5 groups: Low, medium and high-concentration groups with 0.1, 0.5 and 2.0 µg mL⁻¹ of NRF added to the media respectively, a NGF group treated with 0.05 µg mL⁻¹ NGF added to the media (used as positive control) and a negative control group treated without any additives to the media. After DRGs explants had been cultured for 5 days immunofluorescence staining was performed. Primary antibody was rabbit anti NF-H poly-clonal antibody, (1:200) and secondary antibody was Rhodamin goat-anti-rabbit IgG (1:200). By utilizing Leica DMIRB inverted fluorescence microscope, the immunofluorescence neurite images were acquired.

Images preprocessing: Visually, the background illumination of resulted images was uneven and it usually

appears as a brighter area towards the center of the image and a darker area towards its edges. It is essential that uneven illumination background on microscopic images should be reduced before applying image analysis. Our correction method is based upon assumption that the uneven illumination is an additive low frequency signal (Leong *et al.*, 2003). Therefore, low pass filtering can be used to extract it from an image. This filtering may be achieved by convolving the image with a Gaussian kernel. The purpose is to smooth the image until it is devoid of features but retains the weighted average intensity across the image and corresponds to the underlying illumination pattern. Our correction method of uneven illumination in digital microscopy image is the following:

$$\hat{U}(x, y) = N(x, y) - \text{LPF}\{N(x, y)\} + c$$

where $N(x, y)$ presents the vignetting image captured from a digital camera, $U(x, y)$ defined the uniform intensity image, $\hat{U}(x, y)$ presents the corrected image obtained after applying image processing techniques. Convert a copy of the original 24 bit color vignetting image to an 8 bit grayscale image and set up the size of the digital image to 1024×1024.

Fractal dimension calculation based on triangular prism

method: Fractal Dimension (FD) is a real number that describes the fractal property of the object. Unlike the dimensions in Euclidian geometry, the FD is not restricted to be an integer; instead, an object's FD is usually a real number whose value depends on the property of the object. Different FD values indicate different texture structures in the image.

Generally, the more complex the geometric structure is, the higher its FD value will be. In this study, we exploit the TPM algorithm to calculate FD on the microscopic image of neurite outgrowth of rat DGRs induced by NRF and compare various outcomes under different culture condition (i.e., different concentration of NRF). TPM allows estimation of fractal dimension with use of Richardson's plot. It is based on estimation of top of prism field for 5 points. In a synthetic representation, the fractal dimension calculation of grayscale image based on TPM is implemented in six steps (Iftekharuddin *et al.*, 2009).

Step 1: Divide 2D image into equal-sized rectangular sub images with each sub-image has a side length of r . Find the intensity values at four corners of this rectangular such as p_1 , p_2 , p_3 and p_4

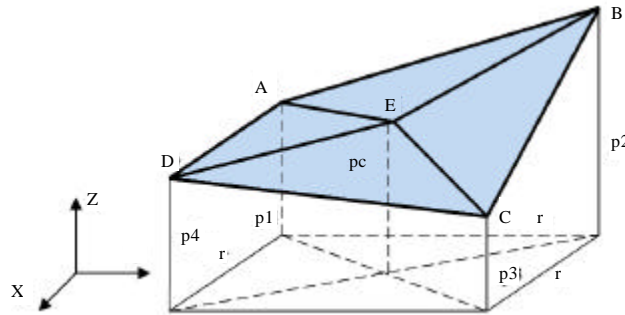


Fig. 1: Triangular prism method

Step 2: Find average intensity value of these four corner pixels pc, it is considered as the height in the third dimension for the center pixel of this sub-image. These five intensity values form four triangles such as ABE, BCE, CDE and DAE

Step 3: Calculate the surface area of each triangle respectively and calculate the sum of the surface area of these four triangles ($S_{i,j} = S_{ABE} + S_{BCE} + S_{CDE} + S_{DAE}$)

Step 4: Calculate total triangular prism surface area:

$$S(r) = \sum_{i,j=1}^{N(r)} S_{i,j}$$

where $N(r)$ represents the required number of rectangular($r \times r$) that covers whole 2D image

Step 5: Record $\log(S(r))$ and the corresponding $\log(r)$

Step 6: Determine whether iteration number is reached, if yes, find the best fit line for $\log(S(r))$ and $\log(r)$, FD is the slope of this line, else if no, change the rectangular size r , repeat STEP1 to Step 5

Statistical analyses: The mean and standard deviation were used to describe all data for subgroups of experiments. We used paired t-test to compare differences between NRF or NGF group and the control group. For this tests, a significance level of $p < 0.05$ was considered. Statistical analysis was performed with STATA 10 software package (Stata Corp).

RESULTS

For all the five experiment types studied, we found that whole DRGs explants grew very slowly and no obvious neurite outgrowth could be detected until 5 days of culture in the negative control group, a little neurite

formation showed on day 5 in the low-concentration NRF group, more neurite outgrowth appeared from day 2 and the neurites formed in culture were most extensive in number and radial-like in shape on day 5 in the high-concentration NRF group. In regard to the increasing number of neurites and their length, the high-concentration NRF group showed little difference with NGF group. Figure 2 showed typical neurite outgrowth images of DRGs under different culture conditions. Clearly, these five neurite outgrowth exhibit significant difference in morphological complexity, the DRGs cultured by high-concentration NRF showed the most complex appearance and the DRGs cultured by low-concentration NRF exhibiting the least neurite features. The fractal dimension for these 5 culture conditions is found to be 2.58, 2.67, 2.74, 2.76 and 2.79, respectively, increasing with concentration and correlate well with the length and number of neurite on each rat DRGs cell possesses. Based TMP algorithm, we calculated fractal dimension for 5 group images under in each condition and performed pair t-test; the results are shown in Fig. 3. We concluded that the fractal dimension of the medium-concentration group as well as the high-concentration group was significantly different from the value of the control group. In contrast, the difference between these two groups and NGF group was not significant.

To evaluate the influence of microscope magnification on calculation of fractal dimension of neurite outgrowth image, the experiment was observed for each group with $\times 10$, $\times 20$ and $\times 40$ optical magnifications, respectively. The fractal dimension of neurite image was calculated at different optical magnification. The outcomes of calculation of the medium-dose group ($0.5 \mu\text{g mL}^{-1}$) under three conditions were shown in Table 1. It can be seen that the fractal dimension of neurite image was nothing to do with optical magnification of the microscope.

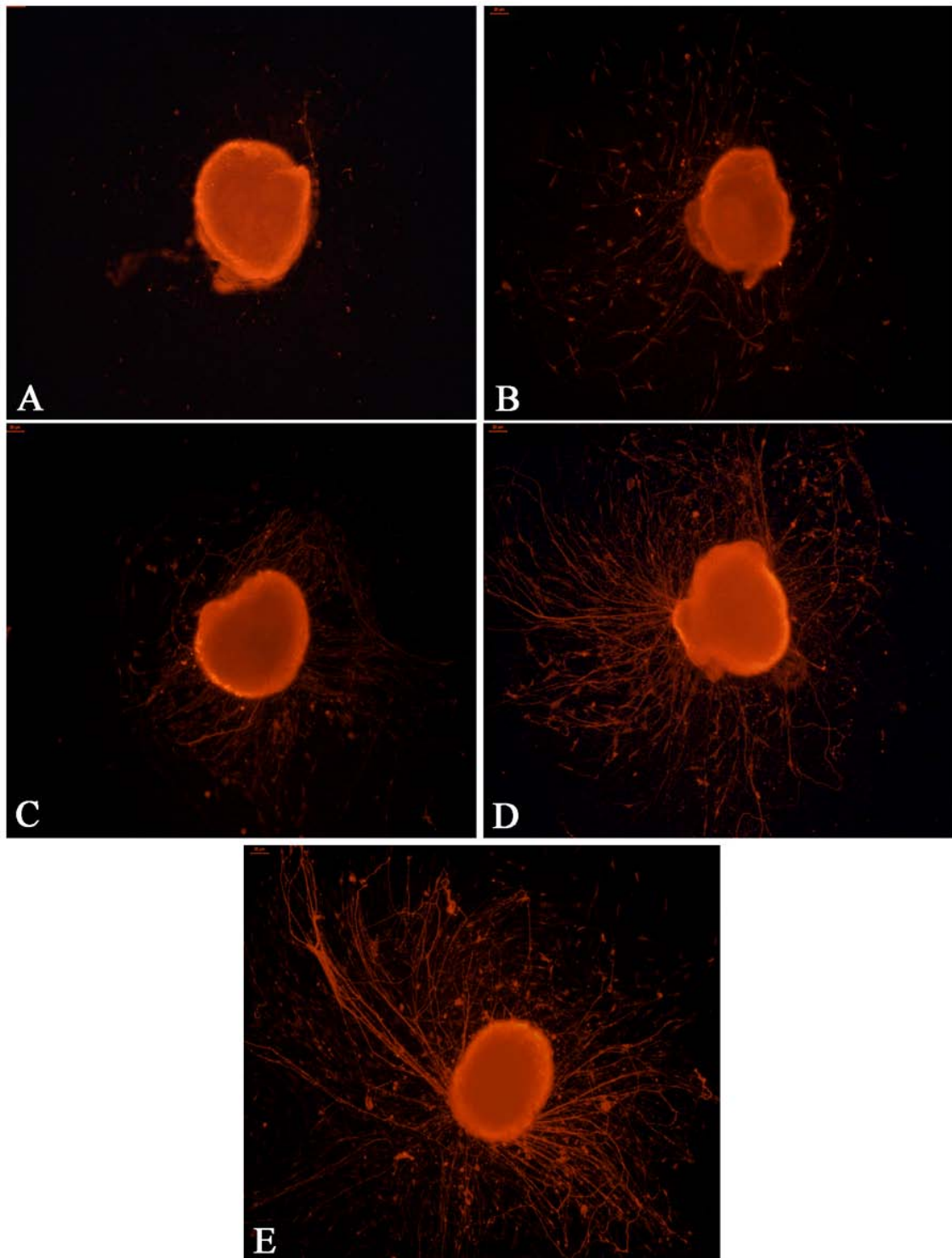


Fig. 2(a-e): Effect of different concentrations of NRF on neurite outgrowth of Cultured DRGs ($\times 40$), (a) Control, (b) NRF ($0.1 \mu\text{g mL}^{-1}$), (c) NRF ($0.5 \mu\text{g mL}^{-1}$), (d) NRF ($2.0 \mu\text{g mL}^{-1}$) and (E) NGF ($0.05 \mu\text{g mL}^{-1}$)

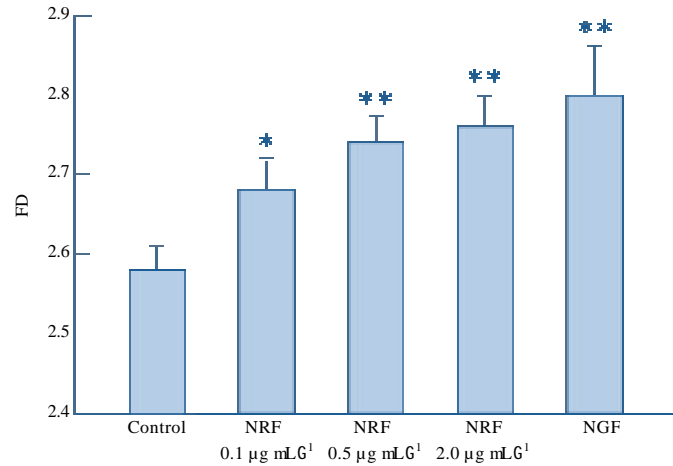


Fig. 3: Fractal dimension of cultured DRGs in 5 different concentration groups (Means±SD, n=10). *p<0.05 and **p<0.01 vs. the negative control

Table 1: Relationship between fractal dimension of neurite outgrowth and magnification of objective

Magnification	Mean	Std. deviation	Std. error
×10	2.7487	0.08742	0.03657
×20	2.7459	0.08581	0.03675
×40	2.7482	0.08675	0.03646

DISCUSSION

This study observed the neurite outgrowth in the whole DRGs explant culture treated with NRF by immunofluorescent staining. The results showed that NRF could promote the neurite outgrowth of rat DRGs. Considering that the neurite outgrowth of rat DRGs under such culture condition was complicated, especially the number and length of neurite being hardly measured with conventional image analysis techniques, we introduced triangle prism method to compute fractal dimension of neurite outgrowth of rat DRGs to quantitatively describe that the promotion function of NRF on neurite growth was concentration-dependent.

The concept of fractal geometry introduced by Mandelbrot (1982) has been applied in biology and medicine. It provides a mathematical model for the description of many complex biological geometric structures. For biomedical grayscale image, fractal dimension generally takes a number between 2 and 3. The larger the fractal dimension is, the more complex biological geometric structure becomes. Thus, fractal dimension can be applied to describe the irregularity of neurite outgrowth quantitatively. The fractal dimension has better performance that reflect objects occupying whole 2D image plane than conventional method based on Euclidean geometry. Our study indicated that the fractal

dimension of neurite outgrowth applying TPM algorithm coincide with the surface roughness of neurite images.

Till now, many methods exist to compute fractal dimension: Box-counting (BC), Differential Box-counting (DBC), Fractional Brownian Motion (fBm) method, Triangular Prism Method (TPM) and so on. Each method has its own theoretic basis, stability, effect and computation efficiency. However, one proper method should be selected in special application. The BC method is the most frequently used for measurements in various biomedical application fields because of its simplicity and easy to develop. There are main drawbacks of the BC method, firstly, it requires signal binarization and secondly, the computational accuracy of the FD is box size sensitive. The TPM is one of area measurement methods that use structuring elements of various scales and compute the area of the objective intensity surface at scale. TPM compares the surface areas of triangular prisms with the pixels area (step size squared) in log-log form. The method derives a relationship between the surface area of triangular prisms defined by the grey-level values of the image and the step size of the grid used to measure the prism surface area. Although TPM was also found to be sensitive to noise or extreme gray-level values, TPM method is the fastest and gives more accurate results than the BM method. This was why we choose TPM to compute fractal dimension of the neurite.

The cultured rat DRGS explants in vitro contains irregular biological structures and it is manifested as self-similarity features to a certain extent (Romero *et al.*, 2009). An exact calculation of the neurite contour by means of conventional morphological analysis is

impossible since it is dependent on the magnification used, therefore it is promoting neurite outgrowth of rat DRGs in difference concentrations. Fractal dimension is independent of the scale of magnification when dealing with biomedical image (Kalmanti and Maris, 2007). From a visual point of view, the neurite length and number increased with increasing NRF concentration and the neurite growth of DRGS accelerated, the complexity of the geometry of neurite enhanced. According to the calculation results, the fractal dimension of neurite outgrowth of DRGs corresponding to medium and high-concentration group also increases, showed significant concentration-dependent relationship. Hence, fractal concept and calculation of FD can play important role in quantitative evaluation of neuronal behavior, not only for characterizing various annotations of neurite complexity and morphology but for describing the growth and evolution of rat DRGs cell. Fractal dimension can be used as an important quantitative index of 2D microscopic image analysis in the field of nerve-regeneration research. Finally, the fractal dimension of neurite outgrowth of rat DRGs has no obvious change no matter what magnification; this result just reflects that the characteristics of the objects with fractal features have self-similarity or stench symmetry.

CONCLUSION

Fractal analysis of digital microscopic images of neurite outgrowth of rat DRGs, using the triangle prism method was presented.

Neurite outgrowth of rat DRGs induced by medium-concentration and high-concentration group tend to have a higher fractal dimension as compared to which induced by low-concentration group.

Fractal analysis can detect subtle morphologic changes in neuritis outgrowth of DRGs and can provide detailed information of rat DRGs cell induced by different concentrations of NRF.

The application of the fractal analysis is very valuable for measuring dimensional properties and spatial parameters of irregular biological structures.

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