FRACTAL METHODS IN CELLULAR AND NUCLEAR MORPHOLOGY

Loretta ICHIM[1]

Articolul propune utilizarea a două metode de evaluare a dimensiunilor fractale în scopul determinării modificărilor de distribuţie a mărimii nucleilor celulari, pentru aprofundarea unor mecanisme de transformare sub acţiunea infecţiei virale. S-a determinat distribuţia mărimii nucleilor celulari prin măsurarea şi prelucrarea spectrelor de difuzie a luminii în conformitate cu teoria Mie. Rezultatele experimentale obţinute indică faptul că dimensiunile fractale prezintă valori mult mai mari în cazul culturii de celule infectate cu virus VHS, în raport cu cele neinfectate. Acest fapt permite o discriminare clară între culturile biologice infectate şi neinfectate cu virus.

This paper propose the using of two fractal methods to determinate changes in size distribution of cell nuclei for deeply study same transformation mechanisms under viral infection. It was determined size distribution of cell nuclei by measuring and processing light scattering spectra according to Mie theory. The obtained results indicate that fractal dimension values are obviously bigger for HSV virus infected biological samples compared to non-infected biological samples. This allows us clearly discriminate between virus infected and non-infected biological samples.

Keywords: light scattering spectroscopy; Mie scattering; spectra; signal analysis; fractal dimension.

1. Introduction

Fractal analysis is frequently used in biomedical signal processing [1,2] and has also been applied at the cell and subcellular level [3-5].

The important advantage of fractal analysis is the ability to quantify the irregularity and complexity of the curve (signal) with a measurable value, which is called the fractal dimension [6]. Signal complexity can be analyzed either directly in time domain, or in frequency domain, or in the phase space. Analysis in frequency domain requires Fourier or wavelet transform of the signal, while analysis in the phase space requires the data in a multi-dimensional space.

[1] Researcher scientist, Dept. of Automatic and Industrial Informatics, University POLITEHNICA of Bucharest and "Ştefan S. Nicolau" Institute of Virology, Romanian Academy, Bucharest, ROMANIA; iloretta@yahoo.com

It is in the space were chaos meets fractals, since strange attractors have fractal dimension. However, estimating the fractal dimension of this attractor involves a large computational burden. An embedded system has to be constructed from the original time-domain signal, based on the method of delays. But to analyze events of brief duration it is necessary to make calculations directly in time domain.

We have to stress that fractal dimension in this case is always between 1 and 2 ($1 \leq D \leq 2$), since it characterises complexity of the curve representing the signal under consideration on a 2-dimensional plane.

One important application of the technique capable of measuring quantitative changes of subcellular structure *in situ* is early diagnosis of cancer or precancerous lesions [7]. The various forms of epithelial dysphasia exhibit some common morphological changes on microscopic examination, the most prominent of which relate to the nuclear morphology. The Light Scattering Spectroscopy (LSS) is capable of providing quantitative information to characterize these features and, as a non-invasive optical technique; it is well suited for use in dysphasia surveillance [8]. The LSS studies have shown that light scattered by cell nuclei can be modelled using Mie theory [9-11].

Now is known that Mie scattering could be used to obtain a diameter distribution of cell nuclei and correlate the findings with pathological aspects of the sample [12-14] from backward light scattering spectroscopy data in the visible spectral range.

The light beam entering the cell holder surface and the outgoing beam are brought close together in a fibre optic attachment mounted below the microscope sample holder.

The aim of the present research was to design an improved method for measuring, under microscope control of the investigated field, the backward Mie LSS energies due to nuclei of cells cultured on transparent surfaces of various cell holders, in order to correlate the fractal dimensions due to diameter distribution changes of these nuclei with pathological processes.

2. Materials and methods

To estimate changes in size distribution of cellular nuclei using fractal analysis method it was implemented an experimental model for *in vitro* infection with Virus Herpes Simplex (HSV) in human cervix epithelial carcinoma cell line (HeLa CLL#2). This line is used currently in virology studies due to her susceptibility in HSV virus infection.

The experimental device used was registered patent [15] and described (design and construction) in [16]. Here it was presented analysis of complexity of

spectra collected with the LSS device. The block diagram of data processing is shown in Fig. 1.

The outgoing beam spectrum was recorded with a personal computer system Intel, Pentium III assisted diode array MCS 420 (C. Zeiss – Jena) simultaneous spectrophotometer, and the corresponding ASPECT v.2.02 software.

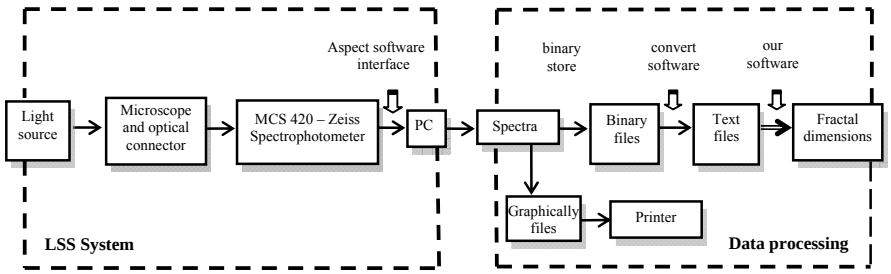


Fig. 1. Schematic diagram of data processing

In order to verify that only in the presence of cells the Mie LSS spectra recorded in the visible domain show the characteristic pattern of several equidistant peaks [7], a non-infected HeLa cell monolayer on a culture flask and a HSV infected HeLa cell monolayer on a culture flask were used as samples, and a cell free part of the flask tissue as reference. The cells were cultivated in 25 cm^2 flask (Falcon, Becton Dickinson). As immersion liquid between the optical fibre attachment tip and the cell holder wall Merck Silicon oil 7742 was used. The method is based on light energy output measurements of a sample versus those of a reference.

For non-infected HeLa cells it was registered spectra from 5 different areas from culture flask, microscopic chooses. But for HSV infected HeLa cells, the culture flask was scanned each 5 mm length and each 4 mm width, so it was collected spectra from the whole flask area. In this way it was obtained spectra for 60 different areas from the flask's HSV infected HeLa cells, because of a high level of heterogeneity due to cellular nuclei diameter variation, function of infection degree.

The optical parameters of the measurements are described, where Mie LSS energy outputs recorded from a monolayer of HeLa cultured cells in terms of the input light energy are used in a theoretical approach of the optical phenomena involved. Five individual spectral curves were recorded for each reference and ten individual spectral curves were recorded for each sample. Diffusion spectra were registered in the same experimental conditions.

The spectra were accounted for using a set of recording parameters chosen from the list offered by the ASPECT software. They are: 84 ms integration time, 10 accumulations for each spectrum, ordinate expressed in relative energy units,

350 and 619 nm initial and final wavelengths of the spectra, automatic dark correction, and manual file saving and print command. Also, spectra were registered in a 'cyclic scan' way by setting time parameters: 2 s delay, 20 s total scan time for each area, 5 s scan interval) and number of scan 5. Because at 350 nm energy values of all spectra were higher than zero, an empirical apparatus correction was necessary to bring all ordinates at 350 nm at the origin and difference spectra were calculated also with the ASPECT software set of routines.

In many applications based on spectral tools, data are registered using a conventional way and then processed using a transformation algorithm. So, Mie diffusion spectra registered using computer – spectrophotometer system needed extra processing to remove unexpected effects (noise, perturbations).

Spectral data processing algorithm consist in the following steps:

1. Compute averaged Mie diffusion spectra;
2. Bring all ordinates at the origin and make needed corrections;
3. Smoothed spectra;
4. Subtract sample spectra from the reference ones;
5. Normalised spectra;
6. Subtract normalised spectra from mathematically simulated smoothed spectra.

Regarded signal analysis a variety of methods is available to compute the fractal dimension, *Df* representing the measure of the shape irregularity.

To determine the fractal dimension, below denote *Df* value, data were treated using two of the most prominent methods: the Katz's method and Hurst exponent methods. The data raw were then analysed using several methods to obtain information about HeLa cells nuclei. A variety of algorithms are available for the computation of fractal dimension. Many algorithms for estimating the fractal dimension of biomedical signals was analyzed and compared [17].

In the following we describe algorithms of extraction of fractal dimensions for signal analysis.

2.1 Fractal dimension algorithms analyzed

Hurst's R/S - analysis

The rescaled range R/S analysis has been introduced by Hurst [18,19]. The R/S statistic for a series ξ_t in the discrete integer valued time is conventionally defined as:

$$X(t,\tau) = \sum_{u=1}^{t} \left(\xi_u - \langle \xi \rangle_s \right), \tag{1}$$

Viewing the ξ_t as spatial increments in a one-dimensional random walk $\sum_{t=1}^{s}\xi_t$ is the distance of the walker from the starting point at time *s*.

$$\langle\xi\rangle_s = \frac{1}{s}\sum_{t=1}^{s}\xi_t \,, \tag{2}$$

The range *R(s)* is the distance between minimum and the maximum value of *X*, and is rescaled by dividing it by the standard deviation *S(s)*:

$$R(s) = \max_{1\le t\le s} X(t,s) - \min_{1\le t\le s} X(t,s), \tag{3}$$

$$S(s) = \left[\frac{1}{s}\sum_{t=1}^{s}\left(\xi_t - \langle\xi\rangle_s\right)^2\right]^{\frac{1}{2}}, \tag{4}$$

On the other hand, Hurst had found empirically that many time series of natural phenomena are described by the scaling relation:

$$R(s)/S(s) \sim s^H \,, \tag{5}$$

where *H* differs significantly from 1/2. In the context of fractional Brownian motion a Hurst exponent of *H*=1/2 corresponds to the vanishing of correlations between past and future spatial increments in the record. For *H*>1/2 one has persistent behaviour, which means a positive increment for some time in the past lead to a positive increment in the future (if the increments are distributed symmetrically around zero). Correspondingly, the case of *H*<1/2 denotes antipersistent behaviour. If the time series is long enough, the relationship between the fractal dimension and the Hurst exponent *H* is:

$$Df = 2 - H \,, \tag{6}$$

HH exponent, "Height-height correlation" algorithm

Method known as Height-height correlation (HH) is very close to Hurst exponent method, the difference consist only in the type of the computed dimension [20].

So, for given *X(t)* data string, we can compute for a given window τ, the dimension:

$$R(\tau) = \sqrt{\left\langle [X(t) - X(t+\tau)]^2 \right\rangle}, \tag{7}$$

Repeating the operation for different τ values we check if *R(τ)* comply with a power low regarding τ.

$$R(\tau) \sim \tau^{HH}, \tag{8}$$

HH exponent determined, if the power low is validated by experimental data, became the wanted fractal estimator:

$$Df = 2 - HH. \tag{9}$$

2.2 Cell culture

The culture conditions have been described elsewhere [21]. Briefly, HeLa cells were grown in DMEM (Sigma) supplemented with 10% fetal calf serum (Sigma) at 37^{o}C, 5% CO_2, in a humidified atmosphere. Exactly 5x10^5 cells were seeded onto flask. After 24h, cells grown on flask were fixed in methanol: water (8:2) for 5 min.

The digitised image of the microscopic picture was captured by a digital camera (CCD; Creative PC-CAM 300) attached to optical microscope. The magnification used was x200. The nuclei and some subnuclear structure of these cells, such as nucleoli and the heterochromatin clumps, are visible.

3. Results and discussion

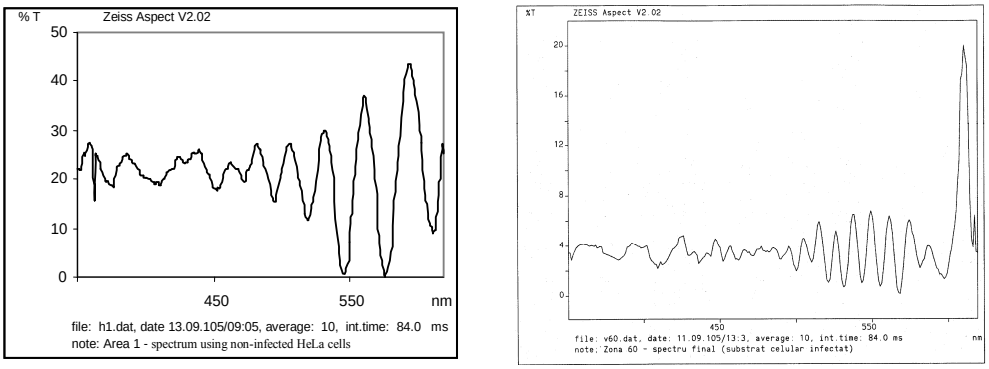
Fig. 1 shows two Mie light scattering spectra examples in visible range, after completing the processing algorithm: (a) investigated aria 1 spectrum using non-infected HeLa cells flask and (b) investigated aria 60 spectrum using HSV infected HeLa cells flask.

Mie light scattering spectra data obtained from HSV infected and non-infected HeLa cells were stored in separate files. Using fractal dimension algorithms and corresponded computing programs they were processed all spectra data files for each area involved.

The algorithms to compute the fractal dimension have been proposed and applied to experimental data derived from HeLa cells light scattering spectrum.

Fractal dimension values were calculated in the λ = 400 - 600 nm spectral range, hence by 201 points. Each of the algorithms described above was implemented in Microsoft Visual C++ 6.0 software.

Fractal dimension, representing the aim of data processing program obtained from signal analysis, is a parameter which characterise the complexity of investigated cellular nuclei size distribution.



(a) (b)

Fig.1 Processed Mie light scattering spectra

Based on experimental data were computed average and standard deviation for fractal dimensions related to non-infected HeLa cellular nuclei size distribution, for a group of 5 investigated areas using both methods. The results computed using each method is: Hurst - Df = 1.357 ± 0.02; HH - Df = 1.317 ± 0.02.

So, fractal dimensions for HSV infected HeLa cellular nuclei size distribution computing using both methods needed first of all an ascendant average for values, followed by 5 value groups dividing. After that they were computed the average and standard deviation for each group. For non-infected HeLa cells it was had only one group with 5 values.

Table 1 presents the average and standard deviation for fractal dimensions by groups for infected and non-infected HeLa cells.

Because the cellular distribution in culture flask is randomly, part of sample area has no cell zones leading to smaller values for Mie light diffusion spectra, respectively to erasing of corresponding structures. On other areas we noticed cells agglomeration so the light beam meet only meet only a small part of the wall/air reflection beam [3], in the way Mie diffusion spectra have higher values and better defined structures.

Based on data presented in Table 1 it comes out that average values for computing fractal dimensions in case of infected HeLa cellular nuclei size distribution are well higher compared to non-infected HeLa cellular nuclei. That

expected because of more complex and heterogeneous structure of infected HeLa nuclei. So, both methods evidenced aspects regarded cellular nuclei size distribution discrimination.

Table 1

The average and standard deviation for fractal dimensions by groups

Cells type	Groups	Fractal dimension methods	
		Hurst	HH
Non-infected HeLa cells	1	1.357 ± 0.02	1.317 ± 0.02
HSV infected HeLa cells	1	1.422 ± 0.004	1.376 ± 0.007
	2	1.429 ± 0.004	1.387 ± 0.004
	3	1.442 ± 0.003	1.399 ± 0.003
	4	1.451 ± 0.002	1.405 ± 0.001
	5	1.457 ± 0.004	1.414 ± 0.004
	6	1.469 ± 0.001	1.425 ± 0.001
	7	1.474 ± 0.002	1.430 ± 0.002
	8	1.481 ± 0.002	1.437 ± 0.003
	9	1.486 ± 0.003	1.441 ± 0.002
	10	1.493 ± 0.002	1.448 ± 0.002
	11	1.498 ± 0.001	1.454 ± 0.003
	12	1.523 ± 0.01	1.488 ± 0.02

Analysing this data it was concluded that ascendant arranged averages for computed fractal dimensions are not normal statistics distributions.

Based on computed averages for fractal dimension groups, they were plotted graphics for non-infected and HSV infected cells using both methods (Fig. 2, Fig. 3) to identify the distribution way of obtained values.

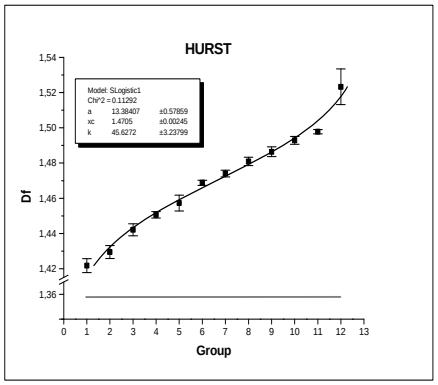


Fig. 2. Representation of Hurst method computed fractal dimension groups for HSV infected and non-infected HeLa cellular nuclei size distribution

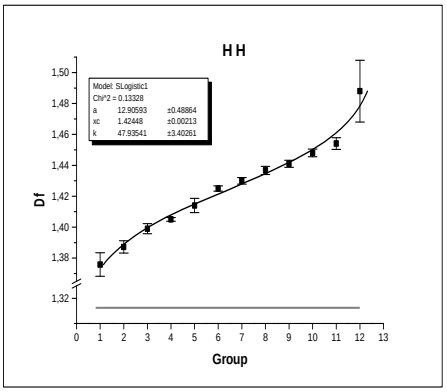


Fig. 3. Representation of HH method computed fractal dimension groups for HSV infected and non-infected HeLa cellular nuclei size distribution

It comes out that fractal dimension averages for HSV infected cells have a type of the following logistic equation:

$$y = \frac{a}{1 + e^{-k(x - x_c)}}, \tag{10}$$

where a, x_c and k are real parameters.

Using software product MicroCalc Origin 5.0 (Data Analysis and Technical Graphics) there were represented graphics for fractal dimension averages distributions by groups for above equation. The equation has non-linear type and has a different parameter variation for the two methods involved, as they were noticed in Table 2.

Table 2

Logistic equation parameters for both methods

Parameters of the logistic equation	Fractal dimension methods	
	Hurst	HH
a	13.384 ± 0.578	12.909 ± 0.489
x_c	1.47 ± 0.002	1.424 ± 0.002
k	45.627 ± 3.238	47.935 ± 3.403
χ^2	0.113	0.134

To evidence the correlation between the two methods using HSV infected cells it was plotted the graphic from Fig. 4 using linear regression for fractal dimension by groups.

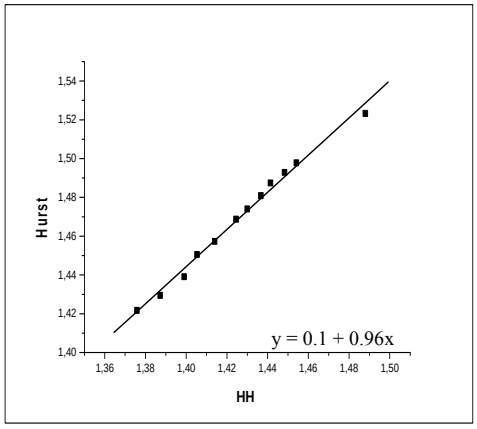


Fig. 4. Correlation between fractal dimension averages calculated using Hurst and HH methods

It comes out that exists a positive correlation between the two methods involved, related to those 12 groups of fractal dimensions averages values and the correlation coefficient $r = 0.996$. The standard deviation was $s = 0.003$ and significance $p < 0.0001$.

By comparing my data with the mentioned ones, the nuclei diameter distribution maximum of the analyzed HeLa cells seems to be at about 15 μm, in a quite good agreement with other microscopic findings [22].

From the above discussed experimental results versus the theoretic approach it is obvious that they are in a satisfactory compliance, so that the finally obtained Mie scattering spectra of the samples used to verify our methodology were representative for the phenomenon described. In addition, the results provide the possibility for quickly investigate the dimension cell nuclei distribution in culture.

4. Conclusions

The study indicated the fact that although they were used different evaluation methods for fractal dimensions, values obtained for non-infected HeLa cellular nuclei size distribution, computed by Hurst and HH methods, are close to value presented in literature applied on microscopic images for malignant hepatocytes [23], and that is due to similarity between the existent way to compute Hurst exponent and fractal dimension using box-counting method.

In Hurst method case comes out that all non-infected HeLa cells has fractal dimension averages by equal 1.357. All HeLa cells groups having fractal dimension averages higher 1.422 were HSV infected. In HH method case comes

out that all non-infected HeLa cells has fractal dimension averages by equal 1.317. All HeLa cells groups having fractal dimension averages higher 1.376 were HSV infected.

It comes out that fractal dimensions values presents much higher variations in case of infected cultures related to non-infected ones, who have nuclei of constant, reduced size.

New facts offered by the fractal dimension itself, as quantitative feature of cell morphology, could add and offer more relevant parameters and better insight into the mode of nuclear behaviour in specific situations, too. Fractal dimension may be a helpful adjunctive technique to discriminate accurately between non-infected and virus infected nuclei.

R E F E R E N C E S

[1] *W. Klonowski*, Signal and image analysis using chaos theory and fractal geometry, Machine Graphics & Vision, **9**, 2000, pp. 403-431.

[2] *L. Ichim*, Caracterizarea prin analiză fractală a structurilor biologice, Stud. Cercet. Virusol., **34**, 2004, pp. 45-52.

[3] *L. Ichim, C. Bleotu, C. C. Diaconu*, Fractal dimensions of cell substructures. An experimental approach, Bull. St. U.P.B., Series C, **66**, 2004, pp. 99-108.

[4] *A. Wax, C. Yang, V. Backman, K. Badizadegan, C. W. Boone, R. R. Dasari, M. S. Feld*, Cellular organization and structure measured using angle-resolved low-coherence interferometry, Biophys. J., **82**, 2002, pp. 2256-2264.

[5] *G. A. Losa, D. Merlini, T. F. Nonnenmacher, E. R Weibel*, (eds.) Fractals in Biology and Medicine, Vol. II, Birkhäuser, Basel, Boston, Berlin, 1997.

[6] *B. B. Mandelbrot*, The fractal geometry of nature, New York: Freeman, 1983, pp. 1-84.

[7] *V. Backman, R. Gurjar, K. Badizadegan, I. Itzkan, R. R. Dasari, L. T. Perelman, M. S. Feld*, Polarized light scattering spectroscopy for quantitative measurement of epithelial cellular structures in situ, IEEE J. Sel. Topics Quantum Electron, **5**, 1999, pp. 1019-1027.

[8] *J. R. Mourant, T. M. Johnson, S. Carpenter, A. Guerra, T. Aida, J. P. Freyer,* Polarized angular dependent spectroscopy of epithelial cells and epithelial cell nuclei to determine the size scale of scattering structures, J. Biol. Opt., **7**, 2002, pp. 378-387.

[9] *M. Gustav*, Optics of turbid Media, Ann. Physik., **25**, 1908, pp. 377-445.

[10] *L. T. Perelman, V. Backman, M. B. Wallace, G. Zonios, R. Manoharan, A. Nusrat, S. Shields, M. Seiler, C. Lima, T. Hamano, I. Itzkan, J. Van Dam, J. M. Crawford, M. S. Feld*, Observation of periodic fine structure in reflectance from biological tissue: A new tehnique for measuring nuclear size distribution, Phis. Rev. Lett., **80,** 1998, pp. 627-630.

[11] *S. S. Cross*, Fractals in pathology, J. Pathol., **182,** 1997, pp. 1-8.

[12] *V. Backman, M. B. Wallace, L. T. Perelman, J. T. Arendt, R. Gurjar, M. G. Müler, Q. Zhang, G. Zonios, E. Kline, T. McGhillican, S. Sphapshay, T. Valdez, K. Badizadegan, J. M. Crawford, M. Fitzmaurice, S. Kabani, H. S. Levin, M. Seiler, R. R. Dasari, J. Van Dam, M. S. Feld*, Detection of preinvasive cancer cells, Nature, **406**, 2000, pp. 35-36.

[13] *V. Backman, V. Gopal, M. Kalashnikov, K. Badizadegan, R. Gurjar, A. Wax, I. Georgakoudi, M. Mueller, C. W. Boone, R. R. Dasari, M. S. Feld*, Measuring cellular structure at submicron scale with light scattering spectroscopy, IEEE J. Sel. Top. Quantum Electron., **7**, 2001, pp. 887-893.

[14] *L. Ichim*, Metodă spectrodifuziometrică de evaluare in situ a distribuţiei nucleelor celulare după mărime, Stud. Cercet. Virusol., **32**, 2002, pp. 46-54.
[15] *L. Ichim*, Dispozitiv spectrofotometric pentru microscop, Brevet Invenţie nr. 120590/27.02.2006.
[16] *L. Ichim, C. Bleotu, C. C. Diaconu*, Light scattering spectrophotometric device for cellular structure characterization, Proc. Ro. Acad., Series A, **5**, 2004, pp. 251-256.
[17] *R. Esteller, G. Vachtsevanos, J. Echaz, B. Litt*, A comparison of fractal dimension algorithms, IEEE Transactions on Circuits and Systems, **48**, 2001, pp. 177-183.
[18] http://www1.physik.tu-muenchen.de/~gamme1/matpack/html/Mathematics/ RSAnalysis.html.
[19] *H. E. Hurst*, Long-term storage capacity of reservoirs, Trans.Am.Soc.Civ.Eng., **116**, 1951, pp. 770-808.
[20] *C. Ioana, F. Munteanu, C. Şuţeanu*, Smoothing dimensions analysis, new effective tools in fractal signal investigation, in: M. M. Novak, R. Dewey (eds.), Fractal Frontiers, Singapore, World Scientific, 1997, pp. 142-154.
[21] *A. Muţiu, I. Alexiu, M. Chivu, M. Petica, G. Anton, C. Bleotu, C. Diaconu, C. Popescu, V. Jucu, C. Cernescu*, Detection of human papillomavirus gene sequences in cell lines derived from laryngeal tumors, J. Cell. Mol. Med., **5**, 2001, pp. 49.
[22] *K. Sokolov, R. Drezek, K. Gossage, R. Richards-Kortum*, Reflectance spectroscopy with polarized light: is it sensitive to cellular and nuclear morphology, Optics Express, **5**, 1999, pp. 302-317.
[23] *A. S. Kerenji, Z. L. Božović, M. M. Tasić, Y. M. Budimlija, I. A. Klem, A. F. Polzović*, Fractal dimension of hepatocytes' nuclei in normal liver vs hepatocellular carcinoma (HHC) in human subjects – preliminary results, Archive of Oncology, **8**, 2000, pp. 47-50.