Topotecan Penetration Assessment in Retinoblastoma Cells using Shannon Entropy and Coefficient of Variation

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***Abstract*—Retinoblastoma is a common intraocular tumor of childhood. One of the medications used as an antineoplastic agent for retinoblastoma treatment is topotecan. Its penetration into living tumorspheres is quantified using confocal microscopy. Topotecan is a fluorescent drug and it dyes the living tissue. Then, it is recorded in a sequence of images over a period of time. The effective penetration of the drug depends on culture characteristics and requires a very specific timing which is calculated empirically by an expert. The purpose of this work is to offer a model to automatically estimate and evaluate the penetration time of topotecan in a cell, based on the information obtained from a sequence of tumorsphere images and using Shannon entropy and coefficient of variation.**

***Index Terms*—Sequence of Confocal Microscopy Image, Statistical Model, Tumorspheres, Retinoblastoma, Shannon Entropy**

I. Introduction

Retinoblastoma is the most frequent ocular tumor in childhood. It is characterized by the appearance of malignant cells in the retina. In Argentina, 45 new cases are detected per year, and 80% are referred for care at Prof. Dr. Juan Pedro Garrahan Hospital, Buenos Aires, where the current cure rate is over 98%. Worldwide, approximately 60% of cases of retinoblastoma occur unilaterally (only one eye) while the remaining 40% compromises both eyes. In the unilateral disease, the average age of onset in children is 2 years of age, while in the bilateral case, it is 1 year of age. For its treatment, Garraham Hospital currently uses a small number of drugs that are administered by different routes according to the criteria of the caring ophthalmologist and oncologist, including: systemic (intravenous infusion), intravitreal and intra-arterial. Each administration route has its advantages and disadvantages and the delivery of treatment or the other depends on medical criterion. Local administration routes, such as intravitreal and intra-arterial routes have advantages over systemic chemoreduction, because they reduce the occurrence of serious adverse events such as severe myelosuppression [6]. One of the drugs used in retinoblastoma treatment is topotecan [9]. The main advantage of having chosen topotecan is that its penetration can be quantified through its fluorescence emission by laser excitation [8]. The purpose of this work is to evaluate topotecan penetration in a cell, when the topotecan is administered directly, using its fluorescence property and image analysis techniques.

Several authors have studied the problem of retinal diseases using different types of medical images and statistical modeling. For example, in [7] a study for tumor classification using a probabilistic framework is presented. In [10] a method for vessel segmentation using active contours is introduced. In [4] a work focused on retinoblastoma detection is presented and several processing techniques are reviewed, all of them based on different types of medical image processing. In [8], the authors contribute to retinoblastoma understanding by studying drug penetration and chemotherapy response in order to optimize patient treatment. However, in these previous studies, the level of penetration could not be quantified nor statistically evaluated. In addition, to the extent of our knowledge, the evaluation of topotecan penetration into retinoblastoma cells has never been performed using automatic image interpretation techniques. One of the ways to measure the penetration of topotecan within the tumorsphere is to measure the homogeneity of gray levels of the pixels within it. There are numerous techniques to perform this measurement. In this work we use Shannon entropy and the coefficient of variation.

Shannon entropy has been widely used as a measure to quantify the disorder of a data set, as well as to detect the presence of distribution patterns. In the case of image analysis it has been used to measure the homogeneity of gray levels of the pixels with several applications [1], [2].

On the other hand, the coefficient of variation is a measure of the relative dispersion of data points around the mean and it can be used as a homogeneity measure in image analysis [3].

In this paper, we focus on analysis and processing of image sequences techniques combined with statistical analysis, in order to evaluate the penetration of topotecan in tumor clusters (i.e. tumor cells agglomerations) using Shannon entropy and coefficient of variation.

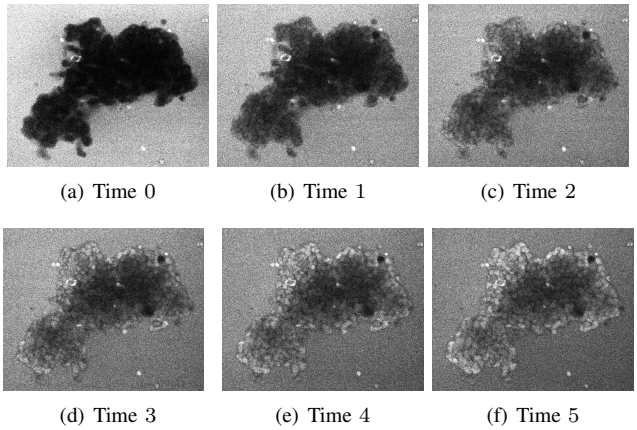
The process for obtaining tumor cell primary cultures is descripted in [8]. The patient models are unique in Argentina and there is a limited number of available models. The experiment consists of the following steps: two primary cell cultures are obtained from the intraocular tumor of two patients, which were enucleated given the advance of the disease. After signing the informed consent (Protocol # 904, Hospital de Pediatria J. P. Garraham), once the enucleation is completed by the ophthalmologist, he takes tumor samples, which are placed in the middle of defined cultures for primary cell cultures. Once the cultures are established and characterized according to the morphology and cellular markers of the retina, they are used to study the penetration of the drug, see [8] for more details.

The microscope used is Olympus Fluoview FV1000 confocal laser scanning microscope (Olympus, Tokyio, Japan) with imaging software (Olympus Fluoview FV10-ASW v1.7c) and equipped with a UPlanSApo 20X / 0.75 NA objective.

The cell cultures studied belong to two patients:

- Patient 1: the patient was enucleated in the first line of treatment without having previously received chemotherapy.
- Patient 2: the patient had received chemotherapy prior to the enucleation of the affected eye.

These cell cultures, to which we refer as tumorspheres, are classified, according to their visualization, into two sizes: medium and large. Then, 1 microliter of a 10 μg/ml solution of topotecan is injected to the cell culture and a sequence of microscopy images are taken. It is desired to evaluate the penetration of the drug in the tumorsphere, according to the fluorescence evolution over time. Figure 1 shows an example of a sequence of microscopy images corresponding to a large size tumorsphere from Patient 1. It can be observed that gray levels of pixels becomes brighter as time increase. As topotecan penetrates the cell, it takes on brighter gray levels. Due to space constraints, we do not show the complete sequence.



(a) Time 0 (b) Time 1 (c) Time 2

(d) Time 3 (e) Time 4 (f) Time 5

Fig. 1. Sequence of microscopy images taken at 30 seconds intervals, corresponding to a large size tumorsphere from Patient 1.

We analyze two image sequences, named $N1$ and $N2$, from each patient. Table I shows a description of their characteristics.

The main purpose of this article is to provide a preliminary assessment of topotecan penetration, considering the previous treatment condition and the culture size, in order to design future experiments.

TABLE I
CHARACTERISTICS OF IMAGE SEQUENCES.

	Patient 1		Patient 2	
	N1	N2	N1	N2
Large	14	8	20	14
Medium	9	7	6	11

The objectives of this work are:

1) Evaluate topotecan penetration within the tumorsphere by means of the analysis of sequences of microscopy images. The tumorsphere is considered to have totally penetrated when it has a homogeneous color in the central region.
2) Compare the penetration rate of topotecan between types of tumorspheres from Patient 1 and Patient 2.
3) Compare the penetration rate of topotecan between different sizes of tumorspheres, derived from the same tumor.

The main idea of this work is to measure the homogeneity of gray levels of the images, in order to evaluate the topotecan penetration. The hypothesis on which this analysis is based is that as the topotecan penetrates the cell, the image should have a more homogeneous appearance, especially in the central area, then the entropy and the coefficient of variation decrease their values.

This article unfolds as follows. In Section II the Shannon entropy and the coefficient of variation are explained. Section III shows the results of applying the methods. Finally, in Section IV final remarks and future works are presented.

II. Entropy and Coefficient of Variation

In this section, two original methodological approaches to assess the penetration time of the topotecan throughout the tumorsphere are described. These methodologies are based on: analysis of Shannon entropy along image sequence and evolution of the coefficient of variation in the image sequence.

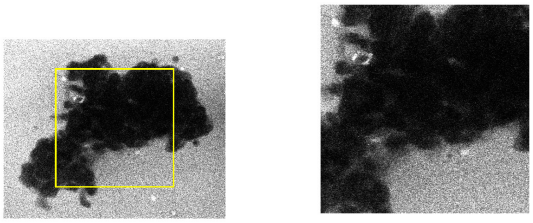
A. Entropy analysis

The Shannon entropy [5] of a continuous probability distribution $f_X(x,\theta)$, where θ is a parameter vector, is given by:

$$\mathrm{H}_S(f_X) = -\int_{-\infty}^{\infty} f_X(x,\theta)\ln(f_X(x,\theta))dx.$$

In the discrete case, the Shannon entropy of an image region I is given by:

$$H_S(I) = -\sum_{i=1}^{256} \hat{p}_i \ln(\hat{p}_i)$$



(a) Original Image (b) Cropped Image

Fig. 2. Square cropped image.

where $\hat{p}_i,\ i = 0, \ldots, 255$ is the relative frequency of gray level i in I, and it is an estimation of the gray level probability. If the value of entropy tends to 0, it implies that the image reaches a greater degree of homogeneity.

B. Coefficient of Variation

The concept of homogeneity of data is associated with the coefficient of variation represented by Cv which measures the percentage of the standard deviation with respect to the mean value. A dataset whose $Cv < 0.2$ is considered homogeneous. Its expression is given by:

$$Cv = \frac{\hat{s}}{|\bar{x}|}$$

where $\bar{x}$ and $\hat{s}$ are estimates of the mean and the standard deviation of the data, respectively.

Therefore analyzing the coefficient of variation over time, a reduction in the value of Cv is expected.

III. Results

In this section the results of applying the methods detailed in Section II to the sequences of images $N1$ and $N2$ corresponding to two cultured tumorspheres for each patient, are presented. In order to take the center of the tumorsphere, the images are cropped to analyze only the center of the image as shown in Figure 2. The tumorspheres are classified in:

- Large
- Medium

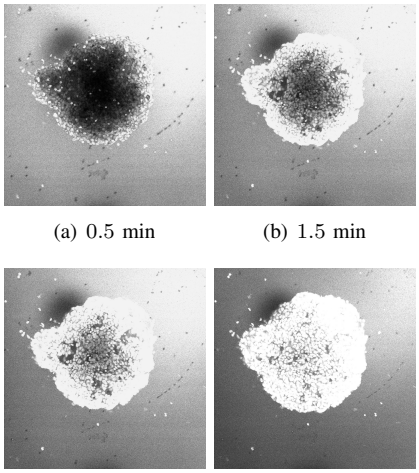
A. Patient 1

The first tumorsphere for analysis corresponds to the sequence of images in Figure 1.

The second large tumorsphere of Patient 1 corresponds to the image sequence observed in Figure 3. Due to space constraints, for each sequence only four images are shown.

Figure 4 shows the result of the entropy analysis for the image sequence of the two large tumorspheres $N1$ and $N2$, corresponding to Patient 1. It can be observed that in the $N2$ tumorsphere, 2 minutes after the topotecan is injected, the entropy is below a threshold value of 75%.

The coefficient of variation analysis, applied to the large size tumorspheres of Patient 1 yields the results shown in Figure 5. It can be observed that the curve decreases in both cases.



(a) 0.5 min (b) 1.5 min

(c) 3.5 min (d) 6 min

Fig. 3. Image sequence corresponding to large size tumorsphere $N2$, Patient 1.

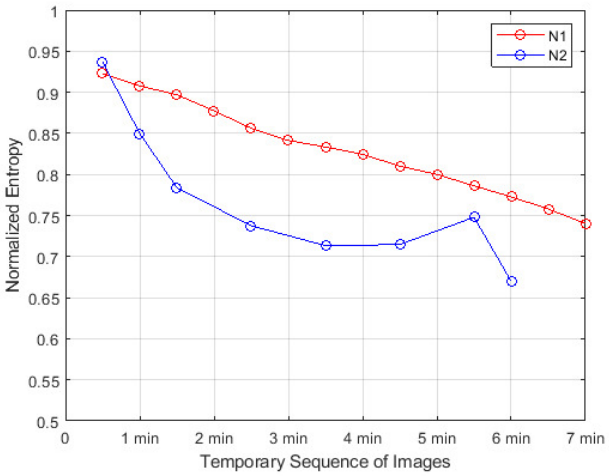


Fig. 4. Entropy analysis corresponding to the image sequences of large size tumorspheres of Patient 1.

The series of medium size tumorspheres of Patient 1 are analized below. Figures 6 and 7 show the image sequences to be analyzed.

Performing the entropy analysis, the results presented in Figure 8 are obtained. It can be seen that at 2 minutes the entropy curves of both tumorspheres are crossing the 80% threshold, and after 4 and a half minutes it drops below 75%. It can be noted that the minimum value of entropy is achieved in the last image.

In Figure 9 the analysis by Cv of the sequence corresponding to the medium tumorspheres of Patient 1 is shown.

A decrease in the values of Cv is observed. The increase in the last image is due to the increase in brightness of pixels on the tumorsphere.

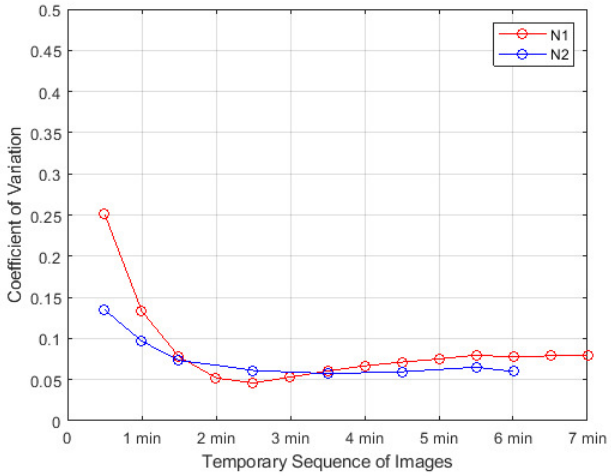
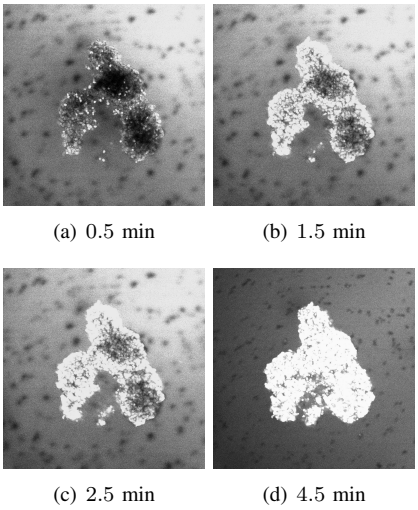


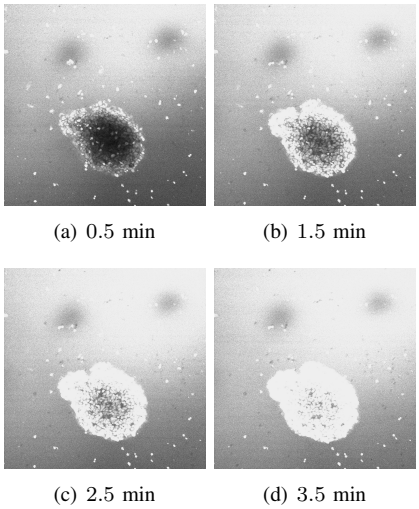
Fig. 5. Coefficients of variation computed using the image sequences of large size tumorspheres of Patient 1.



(a) 0.5 min (b) 1.5 min

(c) 2.5 min (d) 4.5 min

Fig. 6. Image sequence corresponding to medium size tumorsphere $N1$, Patient 1.



(a) 0.5 min (b) 1.5 min

(c) 2.5 min (d) 3.5 min

Fig. 7. Image sequence corresponding to medium size tumorsphere $N2$, Patient 1.

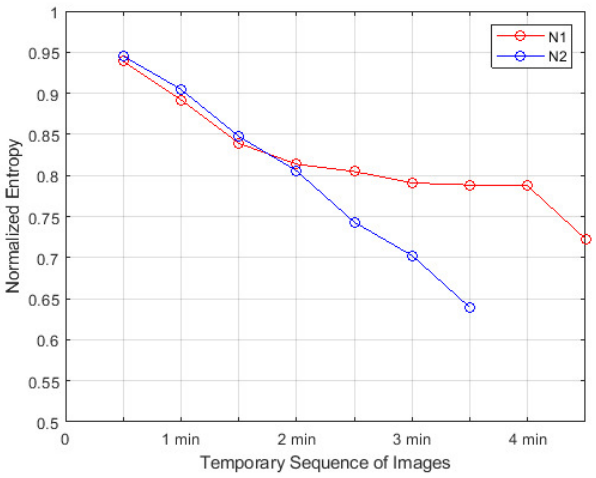


Fig. 8. Entropy analysis of image sequences corresponding to the cultures of medium size tumorspheres, Patient 1.

B. Patient 2

Figures 10 and 11 show two image sequences of cultures of large size tumorspheres of the Patient 2.

The entropy analysis can be seen in the Figure 12.

In Figure 13, the analysis using the coefficient of variation of the sequence, corresponding to the cultures of large tumorsphere of the Patient 2 is observed.

Figures 14 and 15 show two image sequences corresponding to cultures of medium size tumorspheres of the Patient 2.

The entropy analysis is presented in Figure 16. It can be observed that due to the quality of the first images, the entropy value is small and only the third image shows the expected pattern of entropy decrease.

In Figure 17, the analysis by Cv of the image sequences corresponding to the medium size tumorspheres of the Patient 2 is observed.

IV. Conclusions

The aim of this work is to evaluate the degree of penetration of topotecan into groups of Retinoblastoma cells using image sequences analysis by means of the Shannon entropy and the coefficient of variation. Sequences of images from two types of patients and two tumorsphere sizes are compared. In the case of Patient 1, results are conclusive and show that it is possible to use the entropy or the coefficient of variation to evaluate the topotecan penetration into the cell. With respect to the size of the tumorspheres, we can affirm that topotecan penetrates in both of them, but in smaller size tumorspheres the time penetration is lower.

In the case of Patient 2, the results are not conclusive, entropy remains constant and therefore it can not be confirmed that the topotecan penetrates the cell. The coefficient of variation, on the other hand, shows ambiguities.

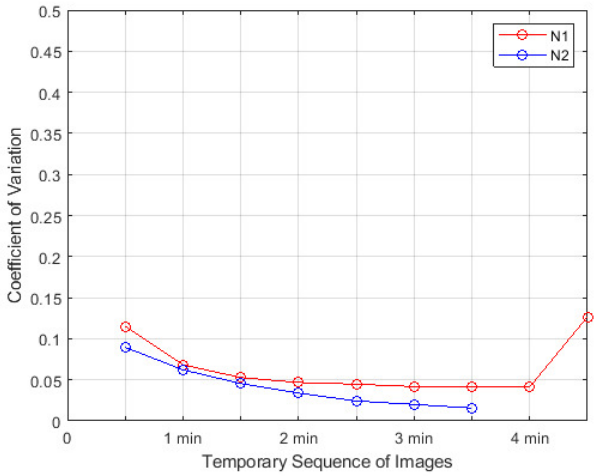


Fig. 9. Coefficients of variation of image sequences corresponding to medium size tumorspheres, Patient 1.

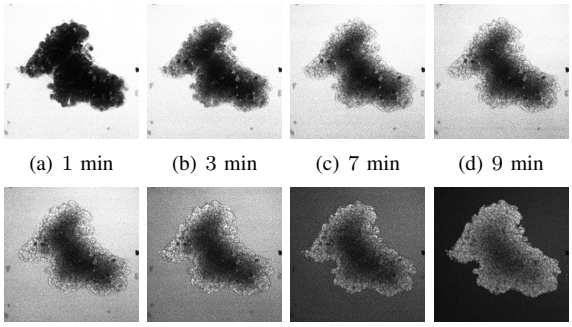


Fig. 10. Image sequence corresponding to large size tumorsphere $N1$, Patient 2.

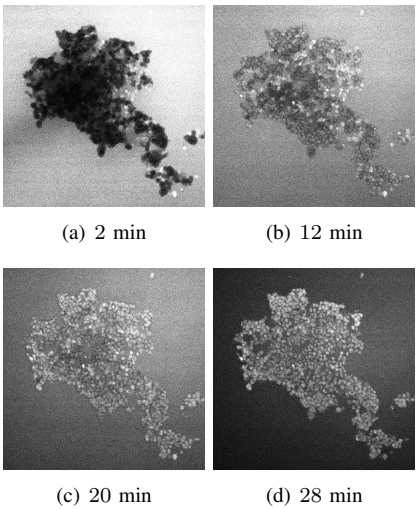


Fig. 11. Image sequence corresponding to large size tumorsphere $N2$, Patient 2.

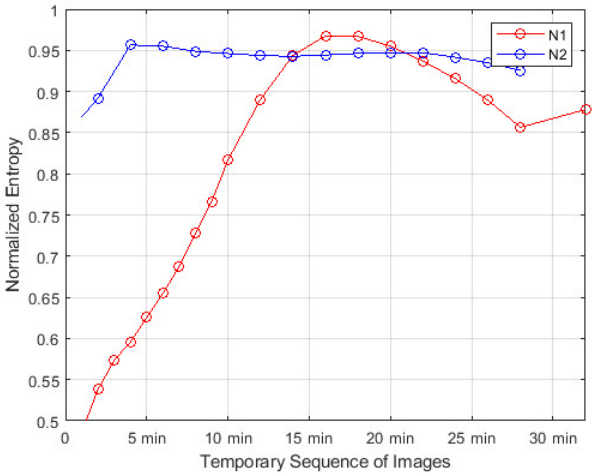


Fig. 12. Entropy analysis for the image sequences corresponding to large size tumorspheres of Patient 2.

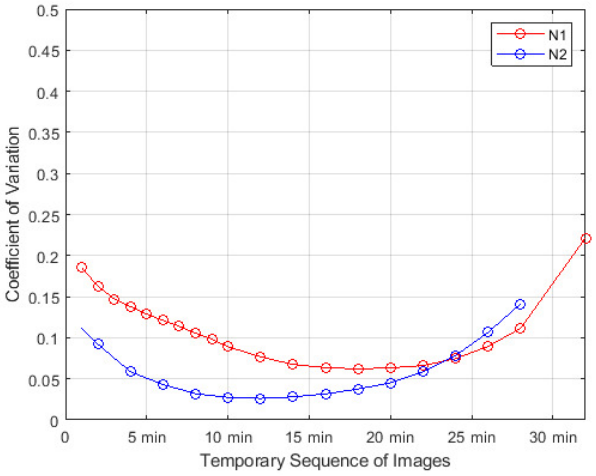


Fig. 13. Coefficients of variation for the image sequences corresponding to large size tumorspheres of Patient 2.

As future works, we want to detect the contour of tumorsphere and evaluate topotecan penetration using all pixels of interest, alternative statistical distances and automatic cropping. As we do not have more tumorspheres of new patients, we want to use permutation methods to establish hypothesis tests.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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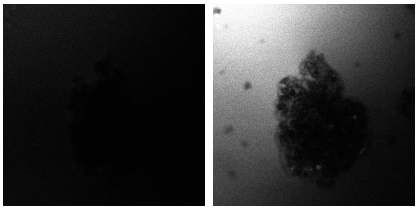
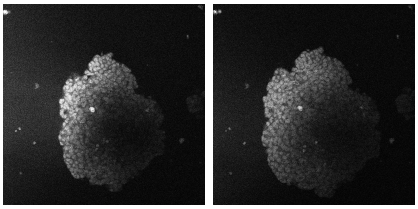
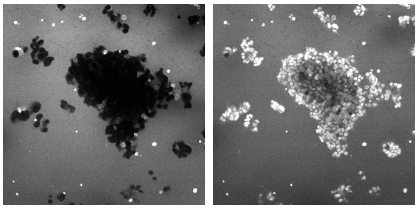


(a) 2 min (b) 6 min

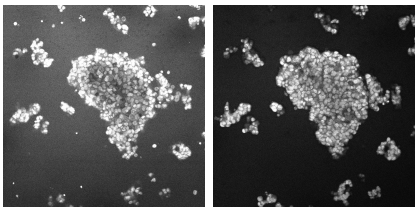


(c) 10 min (d) 12 min

Fig. 14. Image sequence corresponding to medium size tumorsphere $N1$ of Patient 2.



(a) 1 min (b) 5 min



(c) 8 min (d) 11 min

Fig. 15. Image sequence corresponding to medium size tumorsphere $N2$ of Patient 2.

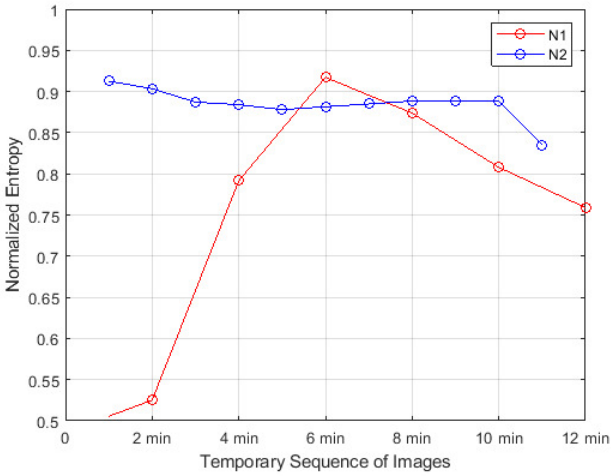


Fig. 16. Entropy analysis of image sequences corresponding to the cultures of medium size tumorspheres of the Patient 2.

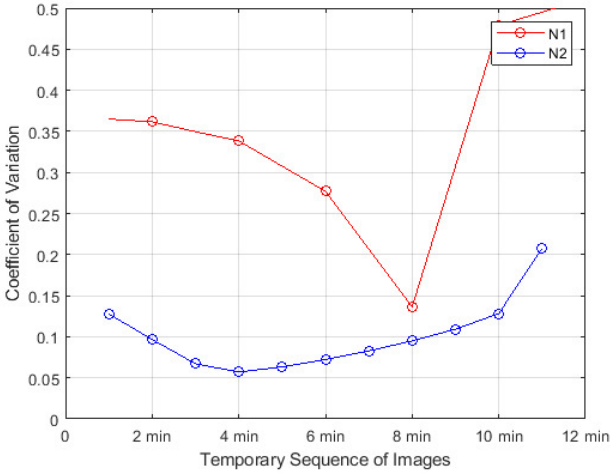


Fig. 17. Coefficients of variation of image sequences corresponding to the cultures of medium size tumorspheres of the Patient 2.

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