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Abstract. This work presents the development of a protocol based on the dynamic laser speckle designed to monitor the reaction of cancer cells of line MEL-RC08 to the application of the drug Colcemid in two different concentrations: 0.2 and 0.4 μg/mL. The protocol was designed using the forward scattering approach with an He-Ne laser of 632.8 nm illuminating the samples, a control, and two variations of Colcemid, being monitored along 8 h. The data were analyzed numerically in the time and in the frequency domain, and the results presented the ability of the technique to monitor the action of the drug, particularly Colcemid (0.4 μg/mL). © 2014 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: 10.1117/1.JBO.19.5.057008]

1 Introduction

The preclinical models of the action of drugs in medicine is a key point to evaluate the effectiveness of those drugs over tissues or cells, such as tumors, before their adoption in serial treatments. The advanced evaluation allows a thorough knowledge of the cells' response and in addition allows the adjustment of the optimum concentration of the chemicals. In order to achieve the models, tests can be carried out in cells *in vivo* merged in a nutritive medium, which creates an adequate condition for the cells to survive and to introduce different drugs. The monitoring of the drugs and of the cell interactions can be done by means of some routine techniques, such as chemotaxis,[1] DNA synthesis speed,[2] investigation of existing inactive zymogens in living cells, apoptotic caspase becoming activated during apoptosis,[3] and apoptotic DNA fragmentation.[4]

The measure of the activity of the interactions between cells and drugs can also be provided by optical techniques, and dynamic laser speckle would be elected as a potential alternative.[5] Dynamic laser speckle is based on the monitoring of the Doppler beating phenomenon caused by the multiple scattering of the coherent light of a laser returning from or passing by the illuminated sample. The noisy signal observed is, thus, analyzed by means of many techniques, such as those associated with statistical analysis. Therefore, the ability to monitor the activity of the illuminated sample without any contact and the use of a low-power light set the dynamic laser speckle as a potential tool in a broad range of applications in biological material.

Moreover, the ability of that technique to offer objective outputs and at real time makes it an outstanding tool when compared to the traditional methods to test biological features, which are time consuming and demand the subjectivity of human judgment.

Applications of dynamic laser speckle in biological samples have been recounted, for example, in fruits,[6] in seeds analysis,[7] in motility of bovine frozen semen,[8] as well as in blood flow[9,10] and in bacterial chemotaxis.[11] In addition, dynamic laser speckle presents itself as a reliable tool to monitor nonbiological samples, such as in paint drying[12] and in zeta potential in colloids.[13]

Particularly related to the analysis of cancer tissues, one can see some reports of the adoption of dynamic laser speckle in the identification of melanoma in live human beings,[14] or even the signature in frequency of different types of cancer in animal tissues.[15]

Despite the great application of dynamic laser speckle, and despite the reports of many methods to monitor the response of cancer cells,[16] there is no account in the literature about the adoption of dynamic laser speckle associated with preclinical models to monitor the action of drugs in cancer cells.

Therefore, this work evaluated the feasibility of providing reliable outputs of dynamic laser speckle related to the reaction of cancer cells of line MEL-RC08[17] to the introduction of Colcemid (Gibco, Invitrogen, Life Technologies LTD, Paisley, United Kingdom) in different concentrations with time.

2 Materials and Methods

2.1 Specimen

In this study, the cell line MEL-RC08 was used. This cell line was derived from a pericraneal metastasis of a cutaneous melanoma; it is well characterized and has been cultured for >150 passages.[17] Culture medium used was sterile RPMI-1640 (Gibco BRL, Grand Island, New York) with a defined standard composition and a good stability at a pH of 7 to 7.2. The medium was supplemented with 10% bovine fetal serum (Gibco), 1% l-glutamine (Gibco), and an antibiotic mix with penicillin

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(50 U/mL), and streptomycin sulfate (50 μg/mL) (Gibco). The cells grew into 25-cm^2 tissue culture flasks (Nunc, Roskilde, Denmark) in controlled conditions, maintained in a humidified atmosphere at 5% CO_2 and air at 37°C, until use.

The bioactive substance assayed in this work was Colcemid. Colcemid is a microtubule-depolymerizing drug. It has different functions: suppression of both dynamic instability and cell migration, and inhibition of microtubule assembly during cell division.[18] Colcemid inhibits mitosis by suppressing microtubule plus-end dynamics; it leads to cell-cycle arrest in metaphase of cell division, and therefore, it allows to obtain metaphase chromosomes to make cytogenetic studies.[19] The mechanism of action of this drug is similar to that of Colchicine, but toxicity of Colcemid is lower in mammals.

In this experiment, MEL-RC08 cells were seeded in multi-well cell culture plates with clear-bottom polystyrene treated with CellBIND® (Corning Life Sciences, Hazebrouck, France), which enhances the attachment, thus making it easier to grow cells under difficult conditions. Four wells were used: well number 1 had only 1.5 mL of complete medium. In the other wells, 100,000 cells per well were seeded upon 1.5 mL of complete culture medium (wells 2, 3, 4) and were grown for 48 h, until they were semiconfluent (Fig. 1). After this time, in well numbers 3 and 4, Colcemid was added at 0.2 and 0.4 μg/mL, respectively. Well number 2, with cells and without drug, was used as a control.

We checked the standard concentration of Colcemid for metaphase-arresting in human lymphocyte cultures, which is 0.2 μg/mL,[19] because this concentration induces cell cycle arrest without being toxic to cells. A twofold concentration, 0.4 μg/mL, was selected since it shows sufficient differences to assess the effect of the drug in a short-term study, near to a toxic concentration.

2.2 Experimental Setup

Figure 2 presents the experimental setup to acquire the speckle patterns generated in time by the illumination of the samples using a linear polarized He-Ne laser beam (632.8 nm, 30 mW). The beam size of the expanded laser light was 110 mm in diameter, presenting a monitored area of a square edge with 38 mm. The well with a diameter of 16 mm was situated in the center of the illuminated area, which presented a uniform illumination provided by a diffuser.[11,20] In addition, a high contrast of the speckle grains was adjusted, in order to have grains with high definition of their shapes. The distance
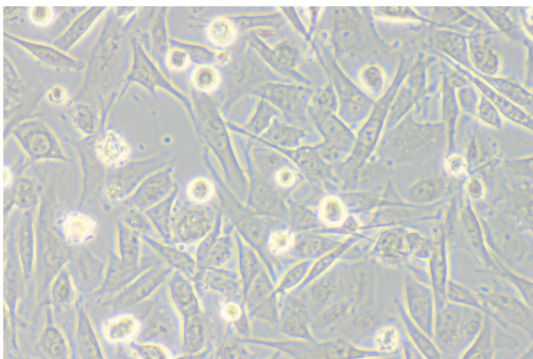

Fig. 1 Human cell line *in vitro* (MEL-RC08) before the treatments with drug Colcemid.
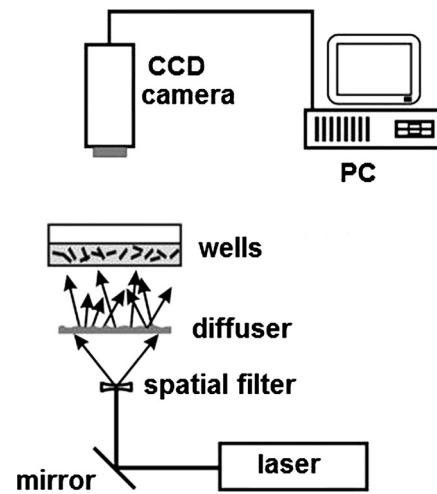

Fig. 2 Scheme of experimental setup used to record the dynamic speckle patterns produced by human cell line activity.

between the diffuser and the well was 7 mm. The well contained a sample with a thickness of 7.5 mm related to a volume of 1.5 mL.

The images were acquired by a macro (SIGMA) with a focal length of 50 mm, numerical aperture of f/16, connected to an Allied Vision Technologies CCD Camera (AVTMarlin F-145B, pixel size of 4.65 μm).

Image quality was tested in order to avoid speckle grains with unworthy information about the phenomena. Therefore, the setup was biased in order to avoid speckle grains smaller than the pixel size and to avoid large speckle grains with blurred appearance. Blurring was avoided using the contrast test.[21] The setup was maintained during all the analysis; thus, the speckle grain sizes remained in the same range. The speckle size in the image plane was 12.35 μm.

At the beginning of each hour, along 8 h, a collection of 128 images (8 bits, 640×480 pixels and shutter speed 1/125 s) was acquired during 8.53 s at a time rate of 15 frames per second.

2.3 Biospeckle Image Processing

The setup was evaluated using the homogeneity test,[22] and the images of each well were cropped in four regions of interest (ROIs) in the homogeneous areas.

Each ROI was analyzed with respect to its behavior in time by means of the absolute values of the differences (AVD) method, after the creation of a time history speckle pattern (THSP) matrix, as can be seen in Fig. 3. The AVD method can be expressed by Eq. (1):

$$\text{AVD} = \Sigma_{ij}\text{COM}_{ij}|i - j|, \tag{1}$$

where COM is the co-occurrence matrix[23] related to the THSP,[24–26] and i and j variables represent the line i and column j of each point of the COM matrix.

The data were also analyzed using the filtering of the signals in different frequency bands. Thus, wavelet transform was used to perform the filtering and the reconstruction of the signal in different bands in order to analyze again by the AVD. The decomposition of a signal using the continuous wavelet transform (CWT) can be summarized in Eq. (2), where CWT (t, j) represents the coefficients as a function of time (t) and scale (j).
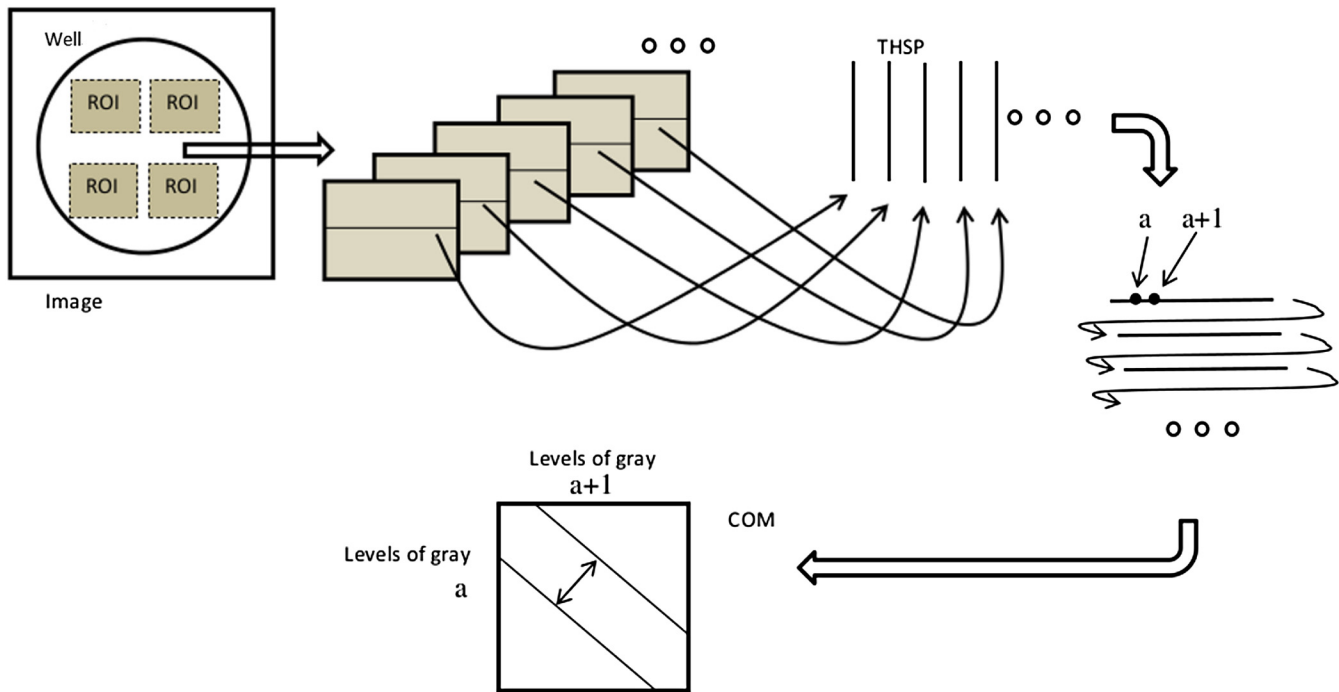
Fig. 3 Biospeckle image processing protocol adopted in each region of interest of the image. The construction of the time history speckle pattern and its co-occurrence matrix are showed.

Therefore, the signal in time $f(t)$ is convolved with $W(j, t)$, which is the mother wave[27]

$$\text{CWT}(t, j) = f(t) * W(j, t). \tag{2}$$

After the decomposition of each pixel from the speckle pattern in time, the reconstruction of the collection of images was carried out at each scale, related to the real value of the frequencies, by means of Eq. (3).[27] The number of frequency ranges varies in accordance with the number of images assembled and with the time rate of image acquisition.

$$f(t) = \Sigma_j \Re\{W(j, t)\}, \tag{3}$$

where the scale (j) represents a range of frequencies.

2.4 Statistical Analysis

IBM Statistical Package for the Social Sciences version 19.0 was used for statistical analysis. An analysis of variance of two factors with repeated measurements was carried out to determine the difference between variables studied. Bonferroni's test was used for multiple comparison and the test was considered significant for $p < 0.05$. The AVD relative error was determined as a percentage relation between standard deviation and mean value, and lies between 15 and 19% (Table 1).

3 Results and Discussion

The AVD results from the four ROIs within each treatment were averaged, and the evolution in time can be seen in Fig. 4. The AVD values for the activity of the culture medium (RPMI-1640) alone maintained the same in time, which means that the culture medium was stable and, therefore, would not interfere in the analysis of the cancer cells and of the cancer cells with the

Colcemid. After the transitions presented in the first 2 h, the treatment of the cancer cells with 0.4 μg/mL of Colcemid can be distinguished statistically ($p \ll 0.001$) from the other two cases [control culture cells and cancer cells with 0.2 μg/mL of Colcemid ($p > 0.2$)]. At time zero, it is possible to see a difference (not significant, $p > 0.07$) among the treatments, which was expected since the solutions had different densities caused by the distinct concentrations of the drug.

Therefore, with time the drug (Colcemid) acts arresting the cell cycle in mitosis and, thus, the activities of the cells are affected, mainly where the concentration of the drug is the highest, 0.4 μg/mL, which was expected. The information of the exact moment of the effect of the chemical is key information and presents the dynamic of the drug, and its effect in the cancer cells helps the election of the best drugs and concentrations.

Variations in activity between the control cultured cells and cells exposed to 0.2 and 0.4 μg/mL of Colcemid shown in Fig. 4 may be explained taking into account the mechanism of action of the drug and its concentration.

- In the case of the control, culture cells proliferate, increasing their activity until they occupy the entire culture surface available, when due to contact inhibition (a characteristic feature of many cell cultures), their activity stabilizes.

Table 1 Relative error of absolute values of the differences (AVD) for temporal and frequency analysis.

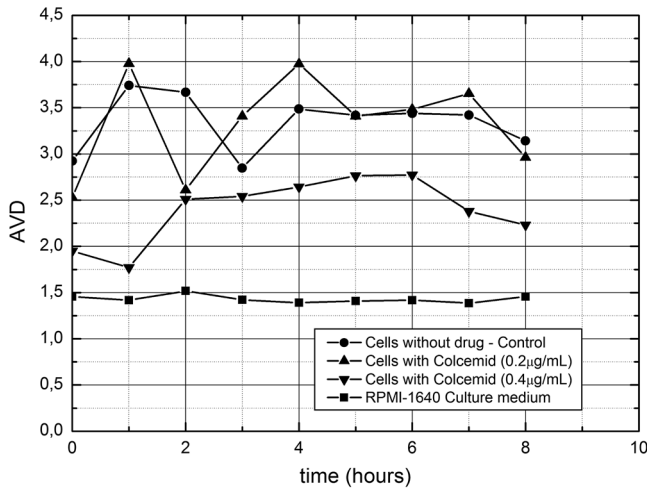|	Mean (%)	Minimum (%)	Maximum (%)
Temporal analysis	15	8	25
Frequency analysis	19	7	27

Fig. 4 Evolution temporal of the biological activity, quantified by absolute values of the differences (AVD) for control cells, 0.2 and 0.4 μg/mL Colcemid-treated cells, and culture medium (RPMI-1640) during 8 h.

- Cells exposed to 0.2 μg/mL of Colcemid behave differently from the control cells at 2 h of study; at this point, the cell cycle of cells that had started mitosis stopped at metaphase,[17,19] and thereby, a decrease of biological activity is observed. Then, cells that were in interphase proceed within the cell cycle, so an increase of activity is observed, until they are arrested in metaphase. From this point, activity remains constant and similar to the control.

- Cells exposed to 0.4 μg/mL of Colcemid show at 2 h an activity similar to those exposed to Colcemid 0.2 μg/mL. Afterward their activity stabilizes. It must be taken into account that 0.4 μg/mL is close to the toxic effects, since the cells that are in interphase are unable to proceed within the cell cycle, and this justifies their stabilization after 2 h. This is a key point in the biospeckle sensitivity, which is relevant to further works.

Further analysis of the data was conducted using the frequency domain. The relations of biological activity of
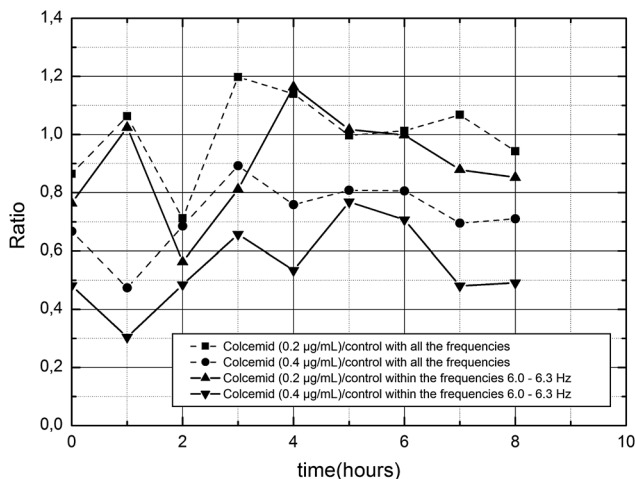
0.2 μg/mL Colcemid and 0.4 μg/mL Colcemid respectively, with the control were analyzed at all frequencies (whole signal) and at all frequency ranges possible regarding the sampling theory. The range of 6.0 to 6.3 Hz was empirically found to be the best region to monitor the phenomena, due to the largest difference between the drug and the control (Fig. 5).

Therefore, this signature in frequency can be used to compare the signals from the biospeckle of a cancer cell with the action of the drug. The differences were shown to be greater than the other intervals after 3 h. Using this signature, it was possible to separate even more the response of the cells with the Colcemid (0.4 μg/mL) and the control cells if compared to the signal with all the frequencies. This suggests the use of the monitoring under frequency analysis, and particularly in the range of 6.0 to 6.3 Hz in this particular case.

4 Conclusions

One can conclude that the dynamic laser speckle, in the experimental configuration proposed and associated with the analysis in the frequency domain, could monitor the interaction of the drug in different concentrations with the cancer cells. In addition, it was possible to identify when the action of the drug, particularly of Colcemid (0.4 μg/mL), provides changes in the biological activity of the cells with melanoma line MEL-RC08 as was expected, since in the literature it is possible to see some works[28–30] addressing the effects of Colcemid in cancer cells by means of the traditional chemical methods.

The ability of the dynamic laser speckle to provide numerical evaluations, avoiding human judgments and without contact with the biological material, offers analysts a safe tool to monitor the different reactions of cancer cells to drug delivery.

Fig. 5 Ratio of biological activity of Colcemid-treated cells with control cells during 8 h. The whole signal is compared with the signal in the range of 6.0 to 6.3 Hz.

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