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An Enhanced Pre-Processing Technique for Evaluation of Cells, *in vitro* through Image Analysis

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Abstract: ***This paper proposes a method for denoising and enhancement in vitro images using various filters. It deals with enhancing the contrast of suspicious areas in the image and also involves the removal of background noise from the image. Lipoproteins (LDL+VLDL) were isolated, oxidized in vitro and used to differentiate monocyte into macrophage. Images were captured at the end of experimental period for image analyzing. This proposed technique can extract cells, in vitro information which is not apparent by visual inspection. The objective of preprocessing is to improve the quality of the image and make it ready for further processing by removing the irrelevant noise and unwanted parts in the background of cells, in vitro. The proposed method enhances the image into maximum level of transformation and produces good SNR ratio with minimum mean square error. Evaluate the cells; in vitro before proceed to the gene expression studies.***

Keywords: Image enhancement, Cells, *in vitro*, Monocytes

1. Introduction

Monocyte to macrophage differentiation plays an important role in atherosclerosis. Inhibiting this differentiation will be a first line of prevention and treatment of inflammatory diseases. Monocytes are the largest corpuscles in the blood. Monocytes replenish resident macrophages under normal state and in response to inflammation signals. Monocytes undergo leukocyte extravasation process and differentiate into macrophages and dendritic cells to evoke an immune response. Macrophages are highly heterogeneous cells that can rapidly change their function in response to local micro environmental signals[1]. Macrophages are tissue-resident professional phagocytes and antigen-presenting cells (APC), which differentiate from circulating peripheral blood monocytes. Macrophages appear as adherent cells with typical morphology: prominent nucleus with flatly outspread cytoplasm and multiple pseudopodia. The monocyte/macrophage is a critical cell in the pathogenesis of atherosclerosis []2–[4] capable of secreting many factors, such as chemokines, cytokines, growth factors, and reactive oxygen species, which contribute to lesion development. The *in vitro* differentiation of human blood monocytes might serve as a model for the *in vivo* maturation process of emigrating monocytes. Upon differentiation, the cell loses its ability to replicate and its antibacterial properties are markedly enhanced, allowing it to participate in the inflammatory and immune responses [5].

Numerous pro- and anti-atherogenic properties have been attributed to Ox-LDL[1]-[3],[6]-[8]. Monocytes undergo leukocyte extravasation process and express several receptors for ox-LDL, known as scavenger receptors. Uptake of ox-LDL by invading macrophages but also vascular smooth muscle cells contributes to foam cell and fatty streak formation. Ehara et al [9] positively correlated levels of Ox-LDL with the severity of acute coronary syndromes and proposed that more severe lesions contain a significantly higher percentage of Ox-LDL positive macrophages. Diosgenin is a precursor of steroid hormones, which can be found in several plant species. Diosgenin has been shown to have a variety of biological activities including anti-inflammatory activity, but through a mechanism that is unclear. Especially, the effect of this agent on macrophage function has not been characterized in detail [10]. Diosgenin exhibits anti-inflammatory properties in the interaction of adipocytes and macrophages by inhibiting the inflammatory signals in macrophages [11].

The rapid growth in digital imaging techniques associated with light microscopy allows researchers from the fields of biology, medicine etc. to produce large amounts of image data in a variety of experiments. This overwhelming amount of image data sometimes needs to be handled carefully to allow the extraction of the required information in a resourceful manner. Cells and the internal structures of the cells can be observed using many different forms of light microscopy ranging from the normal bright field microscopy to advanced systems like the Stimulated Emission Depletion microscopy (STED)[12]. The *in vitro* modeling of the angiogenesis related phenomena of cell growth and differentiation into tubular structures was feasible after background correction [13].

In this study, we used Image processing technique (MATLAB language) to analyze the 2D image of monocyte differentiation into macrophage by Oxy-LDL induction and the treatment by Diosgenin.

2. Materials and Methods

Human serum was collected from VHS (Voluntary Health Service) hospital, Taramani, Chennai, India. U937 cells were obtained from NCCS (National Centre for Cell Science), Pune, India.

2.1 Isolation of Lipoproteins (LDL+VLDL)

LDL+VLDL (Low density lipoprotein and Very low density lipoprotein) were isolated by the method of Burstein [14] with slight modifications. 100 g of sucrose was dissolved in 100 ml of human serum (1 g/ml of serum) expanding the volume to 160 ml. 1.6 ml of 5% heparin and 8ml of 2 M $MgCl_2$ were then added. Opacification occurs immediately. After 15 min at room temperature, the mixture was centrifuged in stoppered tubes for 30 min at 6000 g. Due to the high density (sucrose), the precipitated lipoproteins float to the top and form a pellicle on the surface of the clear subnatant. The subnatant solution was completely removed by aspiration with a syringe and needle. The tubes were then centrifuged again for 1 min in order to sediment the pellicles. The precipitates were combined and dissolved in 2 ml of 5% NaCl by incubation for 30 min at 37°C. To remove contaminating serum proteins, the lipoproteins were precipitated by adding 100 ml of the Tris-HCl buffer and 2.5 ml of 2 M $MgCI_2$. Since the ionic strength is low (0.1% NaCl), the complete precipitation of the lipoproteins occurs in the absence of sucrose. The precipitate sediments on centrifugation (10 min at 6000 g) and was dissolved again in 2 ml of 5% NaCl, the lipoproteins were reprecipitated as above to eliminate the last traces of impurities. The washed precipitate was dissolved in 0.5 ml of 10% sodium citrate. The solution was dialyzed for 24 hr against 400 ml of the Tris-HCl-NaCl buffer to remove the citrate and the Mg++ ions. In order to remove the heparin, the dialysis bag was transferred to another flask containing 5% $BaCl_2$. After 24 hr, the insoluble heparin-barium salt was removed by centrifugation at 4°C. The supernatant was dialyzed against Tris HCl -NaCl buffer in order to remove the $BaCl_2$. This results in a clear yellow solution of concentrated lipoproteins (LDL+VLDL). Such a highly concentrated solution of isolated lipoproteins remains clear even after prolonged dialysis.

2.2 Protein estimation and *in vitro* oxidation

The protein content was quantified by the method of Bradford [15] using the Biorad Protein assay kit. The estimated lipoproteins (LDL+VLDL) were subjected to *in vitro* oxidation. Lipoprotein was oxidized by incubating protein (200 µg of protein/ml) with 5µM $CuSO_4$ in phosphate-buffered saline for 20 h at 37°C. Oxidation was arrested by refrigeration and addition of 100µM EDTA and 20µM butylated hydroxytoluene. The above method, oxidation of lipoprotein was reported by Maggi E, et al [16]. Electrophoresis was used to determine the electrophoretic mobility of oxy-LDL + VLDL).

2.3 Metiltetrazolium reduction assay for cytotoxicity (MTT)

U937 cells were cultivated for 24hand 48 h in 96-well plates, 1.5 to $3x10^5$ cells/well in 200 µL RPMI 1640 supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) antibiotic mixture, under 5% CO2 atmosphere, 37°C and 70% humidity. Diosgenin was dissolved in ethanol (100 µM) and mixed with the fresh medium to achieve the desired concentration. The final ethanol concentration in all cultures was 0.5%, which did not alter cell growth and cell cycle measurements when compared with the vehicle-free medium [17]. Then, medium was replaced and cells were exposed to the different concentrations of Diosgenin (0, 2, 4, 6,8,10,12,14,16, 20µM) in the same volume of RPMI 1640 pH 7.4 supplemented with 1% (v/v) antibiotic mixtures for 24 and 48hrs. Blank reaction controls were run without diosgenin. Next day, the medium was carefully removed and 20 µL of a 5 mg/ml MTT solution was added to each well and the plate was horizontally shaken for 4h, 37°C. After addition of 100 µL of DMSO to each well, the plate was delicately shaken and the absorbance at 570nm was measured in an ELISA reader, within 10 min. The cell viability was calculated by using the formula:

Cell viability % = $OD_{Test}/OD_{Control}$) X 100

The concentration of Diosgenin at which the cell viability is maintained at more than 90 % was taken as the ideal concentration for further experiments.

2.4 Grouping of cells

The cells were categorized into three types (Control, Induced and Treated). The “control” which contains U937 cells alone, “induced” in which the cells were induced with Oxy LDL + VLDL at 10µg/ml and “treated” in which the cells were induced with Oxy LDL + VLDL at 10µg/ml + with 12 µM Diosgenin. Monocytes which were cultured for 7 days in the presence of human serum differentiate into macrophages [18],[19]. All the experimentally grouped cells were maintained for 7 days.

2.5 Image capture

At the end of experimental period, colored photomicrographs of the cell cultures were captured with a Samsung ES80 camera, in 4000 x 3000 pixels resolution using an inverted microscope equipped with a plan achromatic 10x objective.

2.6 Image processing

Digital image processing can be thought of as a sequence of steps: image acquisition, pre-processing, segmentation, feature extraction, analysis and evaluation. These steps can also be expanded into more intermediate steps depending on the application. For example, the sample preparation can also be thought of as an initial step prior to image acquisition. There can also be steps that can be categorized as post-processing [20].

2.7 Digital image data

A digital image is generally represented with a square grid consisting of picture elements (pixels) in 2D. Each element has a value that describes the content of the position of the imaged object that it represents. In a binary image the value is either 1 (part of an object) or zero (part of the non-object or background regions). In a gray valued or gray scale image the range of values change depending on how the structure that stores the information is defined. If an 8-bit representation is used 2^8 is the upper limit and each element can have a value ranging between 0 and 255. This is the most common form of representation but it is not so uncommon to use other representations such as the 16-bit representation that gives a value between 0 and 65535. The appropriate representation can be decided based on the amount of information needed to represent the object of interest and also the amount of storage available [20].

2.8 Pre-processing

Pre-processing, as the name suggests, alters the content of the image and makes it more suitable for the following image analysis steps. The aim of this step should however not be to make the images "look nice" since by doing so, one might risk throwing away useful information, but rather to make them more suitable for the next steps to be applied. One common example of a pre-processing step is noise reduction. Filtering in spatial domain, performed in a controlled manner will decrease the effect of noise on images [21].

2.9 Image Enhancement

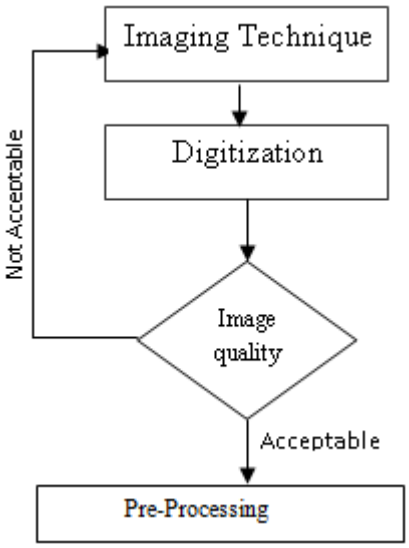
Image Enhancement is one of the most important and difficult techniques in image research. The aim of image enhancement is to improve the visual appearance of an image, or to provide a “better transform representation for future automated image processing. Many images like medical images, satellite images, aerial images and even real life photographs suffer from poor contrast and noise. It is necessary to enhance the contrast and remove the noise to increase image quality. One of the most important stages in medical images detection and analysis is Image Enhancement techniques which improves the quality (clarity) of images for human viewing, removing blurring and noise, increasing contrast, and revealing details are examples of enhancement operations. The enhancement technique differs from one field to another according to its objective. The existing techniques of image enhancement can be classified into two categories: Spatial Domain and Frequency domain enhancement. In the present work, image enhancement processing techniques in spatial domain has applied [21].

2.10 Denoising and Enhancement on Cells, *in Vitro* Images

Image enhancement is a technique which reduces image noise, removes artefacts, and preserves details. Its purpose is to amplify certain image features for analysis, diagnosis and display. The technique can be performed by either suppressing the noise or increasing the image contrast. Regions that do not contain cells in the coarsest (lowest resolution in the decomposition process) image will be removed.

2.11 Histogram Modification

Many images contain unevenly distributed gray values. It is common to find images in which all intensity values lie within a small range, such *as* the image with poor contrast shown in Figure. Histogram equalization is a method for stretching the contrast of such images by uniformly redistributing the gray values. This step may make threshold selection approaches more effective. In general, histogram modification enhances the subjective quality of an image and is useful when the image is intended for viewing by a human observer.



2.12 Linear Filters

As mentioned earlier, images are often corrupted by random variations in intensity values, called *noise*. Some common types of noise are *salt and pepper* noise, *impulse* noise, and *Gaussian* noise. Salt and pepper noise contains random occurrences of both black and white intensity values. However, impulse noise contains only random occurrences of white intensity values. Unlike these, Gaussian noise contain variations in intensity that are drawn from a Gaussian or normal distribution and is a very good model for many kinds of sensor noise, such as the noise due to camera electronics. Linear smoothing filters are good filters for removing Gaussian noise and in most cases, the other types of noise as well. A linear filter is implemented using the weighted sum of the pixels in successive windows. Typically, the same pattern of weights is used in each window, which means that the linear filter is spatially invariant and can be implemented using a convolution mask. If different filter weights are used for different parts of the image, but the filter is still implemented as a weighted sum, then the linear filter is spatially varying. Any filter that is not a weighted sum of pixels is a nonlinear filter. Nonlinear filters can be spatially invariant, meaning that the same calculation is performed regardless of the position in the image, or spatially varying.

2.13 Mean Filter

One of the simplest linear filters is implemented by a local averaging operation where the value of each pixel is replaced by the average of all the values in the local neighborhood.

$$h[i,j] = \frac{1}{M} \sum_{(k,l) \in N} f[k,l] \quad \rightarrow (1)$$

where *M* is the total number of pixels in the neighborhood *N*. For example,
taking a 3 x 3 neighborhood about [i,j] yields:

$$h[i,j] = \frac{1}{9} \sum_{k=i-1}^{i+1} \sum_{l=j-1}^{j+1} f(k,l) \quad \rightarrow (2)$$

Now if h[i,j] = 1/9 for every [i,j] in the convolution mask, the convolution operation in Equation (2) reduces to the local averaging operation shown above.

The size of the neighborhood *N* controls the amount of filtering. A larger neighborhood, corresponding to a larger convolution mask, will result in a greater degree of filtering. As a trade-off for greater amounts of noise reduction, larger filters also result in a loss of image detail.

When designing linear smoothing filters, the filter weights should be chosen so that the filter has a single peak, called the main lobe, and symmetry in the vertical and horizontal directions. Linear smoothing filters remove high-frequency components, and the sharp detail in the image is lost. For example, step changes will be blurred into gradual changes, and the ability to accurately localize a change will be sacrificed. A spatially varying filter can adjust the weights so that more smoothing is done in a relatively uniform area of the image, and little smoothing is done across sharp changes in the image.

2.14 Median Filter

The main problem with local averaging operations is that they tend to blur sharp discontinuities in intensity values in an image [21]. An alternative approach is to replace each pixel value with the median of the gray values in the local neighborhood. Filters using this technique are called *median filters*. Median filters are very effective in removing salt and pepper and impulse noise while retaining image details because they do not depend on values which are significantly different from typical values in the neighborhood. Median filters work in successive image windows in a fashion similar to linear filters. However, the process is no longer a weighted sum. For example, take a 3 x 3 window and compute the median of the pixels in each window centered around *[i, j]*.

In general, an odd-size neighborhood is used for calculating the median. However, if the number of pixels is even, the median is taken as the average of the middle two pixels after sorting.

3. Results and Discussion

The result of electrophoresis in this study shows that the oxidized LDL + VLDL which showed more electrophoretic mobility than native LDL + VLDL. This may be due to the fact that oxidant copper cation has conferred new physicochemical properties on LDL + VLDL by binding to it and increases its negative surface charge and consequently enhanced its electrophoretic mobility (Figure 1).

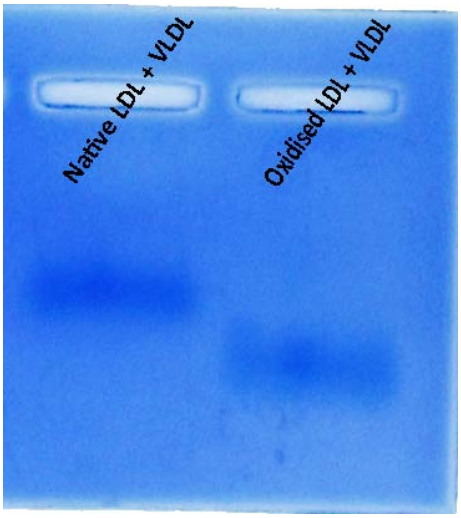


Figure 1: Electrophoretic mobility of Native and Oxidized lipoproteins

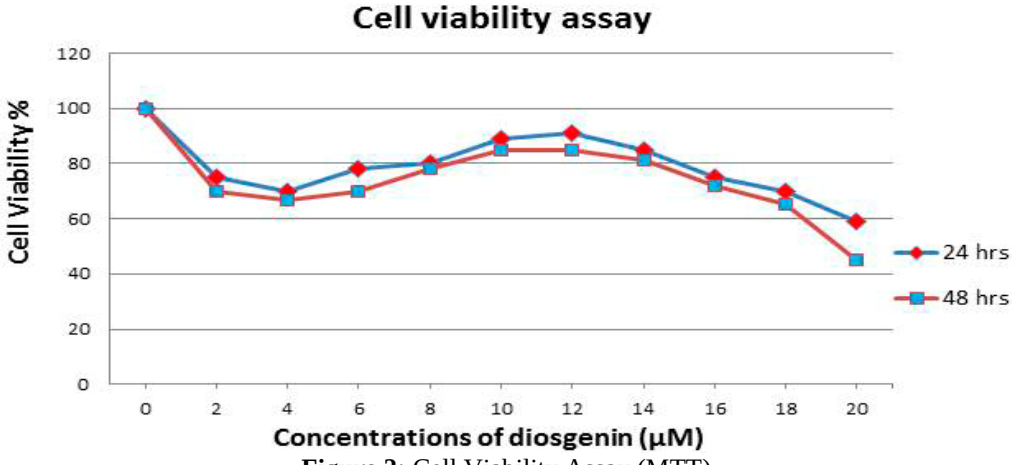


Figure 2: Cell Viability Assay (MTT)

Effect of diosgenin on cell viability is represented by the results of MTT assay (Figure 2). It is suggested that 12 µM of diosgenin was an ideal concentration at which the cell viability was more than 90%. This dosage was used throughout the time period of study. Our findings also coincide with Esfandiarei M, et al [22, 23]. They suggested that over the concentration range of 10-15 µM, diosgenin may provide overall beneficial effects on diseased vascular smooth muscle cells, by blocking migration and contraction without any significant cytopathic effects, implying a potential therapeutic value for diosgenin in vascular disorders. Diosgenin≥25 µM) induces apoptosis as measured by the number of annexin V-positive cells and caspase – 3 cleavages, while decreasing cell viability as indicated by protein kinase B/Akt phosphorylation [24].

Filtering is a very promising algorithm for removing the noise such as speckle noise from cells, cell image. Two filtering techniques namely Averaging filter and Median Filter have been applied for image denoising and enhancement in cells, in vitro image. It is noted from the above figure, salt and pepper noise is added to the gray scale image, the median filter gives a better image quality than averaging filter.

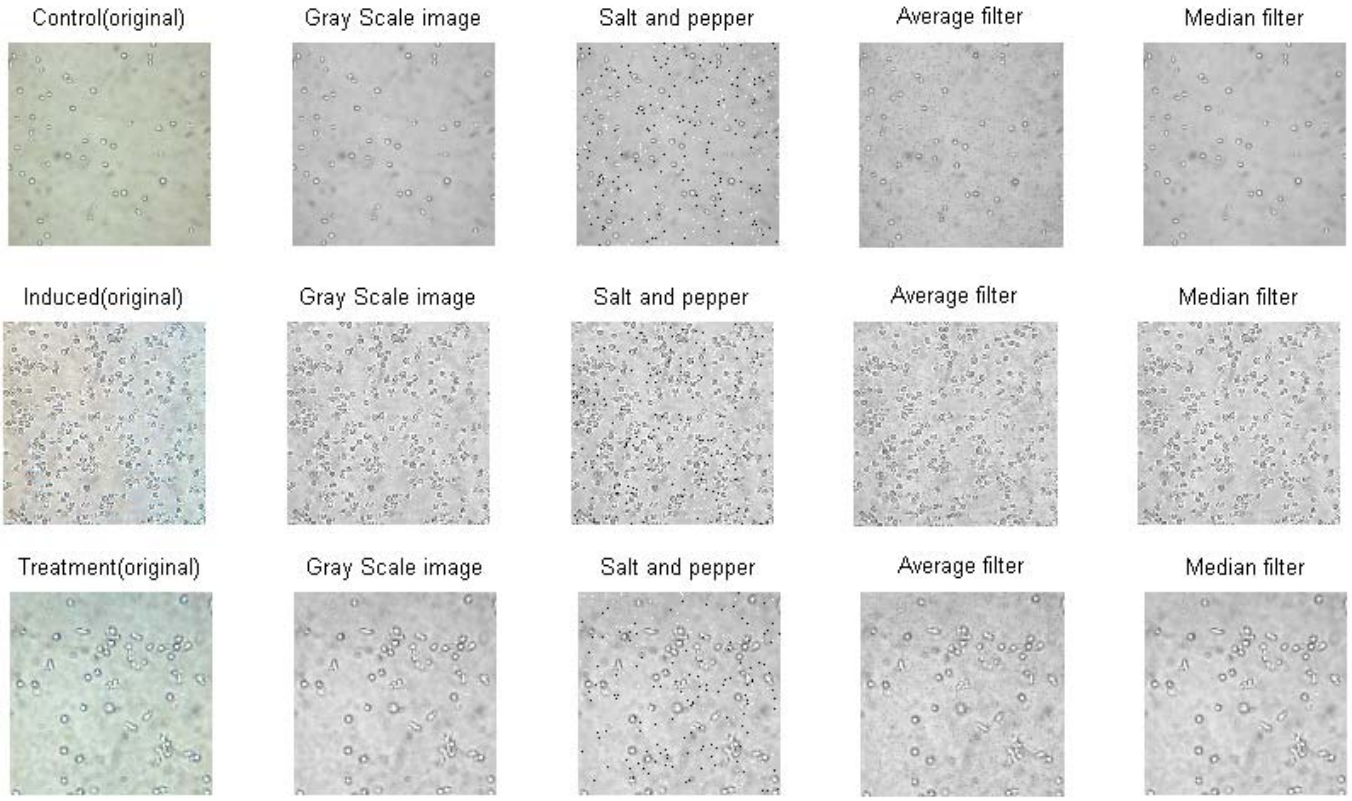


Figure 3: (a) Original control image with filtering output (b) Original induced image with filtering output (c) Original treatment image with filtering output

Two filtering techniques namely Averaging filter and Median Filter have been applied for image denoising and enhancement in cells, *in vitro* as shown in figure 3. To measure the performance of the noise removal techniques several parameters were used for comparison. The common parameters used for analyzing the present work were signal noise ratio (SNR) and mean squared error (MSE).

The comparison results of averaging and median filters are summarized in Table 1

Table 1

S.No	Filtering Technique	SNR (dB)	MSE
1.	Original image	16.695	811.012
2.	Average Filtering	17.396	799.563
3.	Median Filter	22.120	680.054

Higher the value of SNR and lower the value of MSE of denoised and original image implies that the performance of the denoising filter method and visual quality of the denoised image is good. From Table 1, it is clear that the Median Filter method shows a better result for noise reduction from cells, in vitro image than averaging filter.

4. Conclusion

In this paper, different filtering techniques for removing noises in cells, *in vitro* images have discussed. Furthermore, it has been presented and compared results for the various filtering techniques. The proposed algorithm is tested with various sample images. The results on cells, *in vitro* images indicate that the proposed method is able to enhance the image without noise of background cells. Future work will concentrate on developing an appropriate algorithm to validate the segmentation and image analysis.

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