

Restoration of phase contrast microscopy images for the analysis of lung epithelial scratch wound repair assays

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Abstract

In phase contrast microscopy living biological specimens can be studied without the need for staining or fluorescent tagging. This technique works by converting small differences in the optical path length of light passing through a transparent specimen into changes in image brightness. Objects appear typically dark surrounded by bright halos. The halo artefacts make automatic segmentation non-trivial. This paper develops a simplified model of image formation and its inversion implemented as a deconvolution with a Difference of Gaussians filter. In the restored images, values are proportional to the cell thickness and simple thresholding provides effective segmentation. This method is applied to segmentation and analysis of epithelial scratch wound repair assays used in basic bioscience research into treatment of acute lung injury. The computational method shows excellent agreement with the biosciences gold standard (correlation coefficient 0.92) whilst being objective and much faster.

1 Introduction

In phase contrast microscopy (PCM) small differences in the optical path length of light passing through a transparent specimen are converted into changes in image brightness. This makes it possible to image living biological specimens without the need for staining or fluorescent tagging. In PCM images background takes on mid-grey-level values (corresponding to little or no difference) whereas objects typically show as dark shapes surrounded by bright halos. The contrast between the dark interior and the bright halo is

related to *local* differences in specimen thickness. For this reason any global segmentation techniques such as thresholding cannot be straightforwardly applied.

Acute lung injury is a clinical condition that reduces the efficiency of the lungs to transfer gases in and out of the blood, causing hypoxia. There are no pharmacological approaches to promote the recovery and the current research aims to identify proteins and signalling pathways involved in the repair process to identify or develop pharmaceuticals that could help reduce the mortality rate. There are several models of repair of epithelial lung cells. The most common one is conceived as a two-phase process: of cell spreading and migration to cover the affected area, followed by cell proliferation to increase the cell density. An *in vitro* experimental procedure that is relatively straightforward to use is the wound repair assay. This method mimics the repair of cells. It comprises of growing a monolayer of cells that is then scratched to simulate wounding and loss of cells in the alveolus. Images are taken at predefined time points to calculate the percentage cell coverage of the wound, the rate of cell migration and the cell density [1]. As the epithelial cells are transparent, phase contrast microscopy is a method of choice.

Current gold standard protocol for processing the scratch wound images is to draw around the wound by hand. However, this method is time consuming, subjective, and can lead to bias [1]. Several computer image analysis methods have been applied to this problem. Holistic methods focus on textural or frequency differences between the scratch region and the regions populated by cells (e.g. [2]). Such methods work well for assessing cell migration but are not suitable for computing cell density. Methods, which are capable of this, rely on segmentation of individual cells. A simple approach is to apply thresholding followed by post-processing [3] which can often be quite considerable, for instance by evolving a geometric active contour around each cell candidate [4]. Watershed-based methods exploit the fact that the cell interior forms a “basin” whose edges define the boundaries of the cell. Over-segmentation is a common problem here although it can be overcome by careful pre-processing [5][6]. In a recently published method image restoration is carried out prior to segmentation. By modelling the optical pathway of a phase contrast microscope and its inversion the estimate of the optical path length is computed at every pixel [7]. In the resulting images the cells show as bright objects on a dark background and the halo effect is completely eliminated making segmentation easy.

This paper presents a method for assessing both the cell migration and the changes in cell density. Individual cells are segmented by applying a simplified inverse model of image formation in PCM. Individual segmented cells are then grouped on the basis of their proximity, subdividing the image into three regions: the central scratch area and the two confluent groups of cells on both sides of the scratch. The size of the scratch is computed for the central region and cell density is computed from the size of the areas occupied by cell and from the number of cells occupying it.

2 Methods

2.1 Simplified model of image formation

The two key processes responsible for the PCM image appearance are (1) the optical “computation” of local differences in optical path length and (2) the blur introduced by the microscope’s optics. In a gross simplification these processes combined can be represented by a difference of Gaussians (DoG). Figure 1 shows the profile through a PCM cell image and a profile of a wedge convolved with a DoG filter. A hypothesis is that by de-

convolving a PCM image with a DoG filter with suitably chosen parameters it is possible to compute relative estimates the optical path length for each point in the image. The parameters sought are a point spread function (PSF) for a given microscope and the magnitude of the difference between the two Gaussians, which is related to a phase shift.

2.2 Parameterisation of the Difference of Gaussians filter

This is a semi-manual process which needs to be performed only once for a given microscope and an imaging protocol. It requires the user to choose a profile though a single individual cell chosen to have a typical appearance. The profile must include a length of background. Three quantities are then computed from the profile (see Fig. 1): the difference between the maximum and minimum of the cell (D_{obj}), the difference between the maximum of the cell and the background (D_{BgMax}) and the difference between the minimum of the cell and the background (D_{BgMin}).

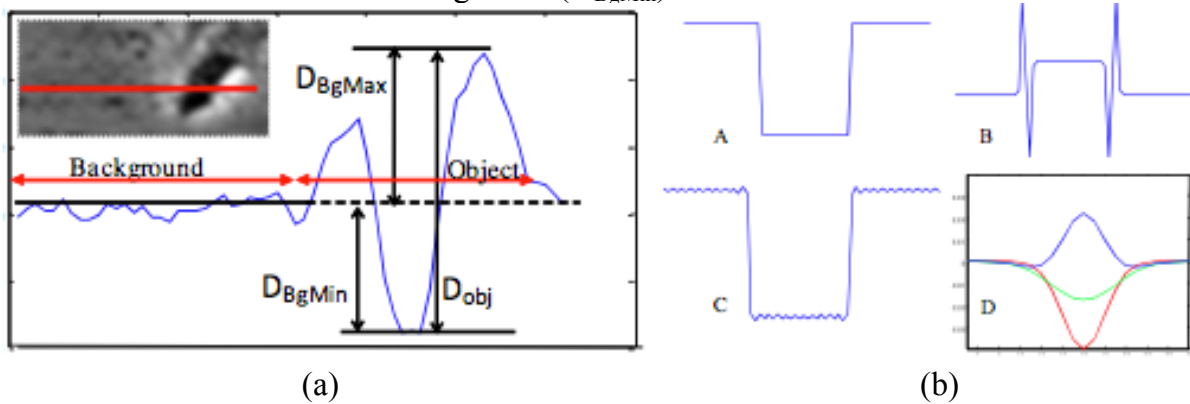


Figure 1: (a) A single cell in a PCM image (inset) and its image profile; (b) (A) A wedge, (B) the wedge convolved with DoG filter, (C) restored edge: (B) deconvolved with the same DoG, (D) DoG filter (blue) generated by subtracting Gaussian (σ_1) (red) from Gaussian (σ_2) (green).

The parameters sought are the spread of Gaussian1, σ_1 , the spread of Gaussian2, σ_2 , and the difference $\Delta\sigma = \sigma_1 - \sigma_2$. To obtain values for σ_1 and σ_2 a 2D grid search was implemented. The axes of the grid are the two variables of the DoG, σ_1 and σ_2 . The values on the grid are derived from eq. 1 when the sample image profile is deconvolved with the DoG parametrised by σ_1 and σ_2 . The key problem was to formulate an objective function that uses only PCM image data. Initial research used synthetic objects where the DoG parameters were known and so the search could be directed (Fig. 2). By using parameters D_{obj} , D_{BgMax} and D_{BgMin} – all of which can be obtained from the PCM single cell image – it was observed that there was a distinct minimum in the value by using a specific combination (Eq. 1). The correct DoG occurs when the search is directed from different grid directions (vertical, horizontal and diagonal). The slope both before and after the minimum is the same so a gradient based method would be ineffective. Limits in the grid search were set for the ranges of both Gaussians and then every DoG was calculated and measured by the parameters in the equation:

$$Combination = D_{obj} \times \left(\frac{1}{D_{BgMin}} \right) \times L_{BgMax} \quad (\text{Equation 1})$$

The D_{BgMax} is not used directly within this calculation. Instead it is used as an indicator as to whether the DoG is too big or too small, which can cause the deconvolved image to be blurred; L_{BgMax} is set to 0 or 1 to ensure that D_{BgMax} does not become too large. Large DoG also has smoothing effects and so D_{obj} is small which mimics an ideal situation.

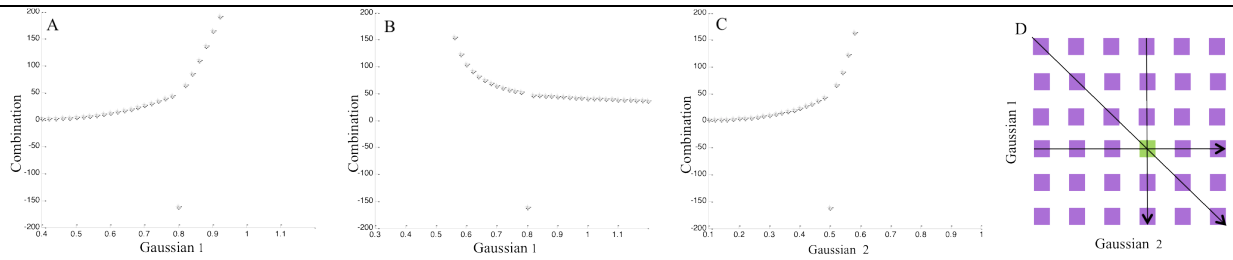


Figure 2: Directed search for the optimal difference of Gaussian. (A) Vertical search, (B) Diagonal search and (C) horizontal search. (D) shows how the directed search was made to overlap the correct combination of Gaussians (green square).

To evaluate the robustness of the recovery of parameters σ_1 , σ_2 , and $\Delta\sigma$ the process was repeated many times for different cells in the same image and for different images from the same batch of experiments. As these parameters characterise the imaging system they should show little difference across the experiments. The variation between the recovered parameters σ_1 and σ_2 was 0.073 and 0.054 respectively with a mean of 1.40 (σ_1) and 0.4 (σ_2) demonstrating the robustness of the process.

2.3 Deconvolution and post-processing

The phase contrast images were deconvolved with the DoG filter, $\text{DoG} = G(\sigma_1) - G(\sigma_2)$ yielding a relative estimate of cell optical pathlength which is proportional to cell thickness. The deconvolved images were observed to have large background variations (Fig. 3B). To remove them the original PCM image was strongly smoothed, deconvolved with the same DoG filter and subtracted from the restored original image (Figs. 3C and D). After the application of a top hat filter [8] (Fig. 3E) a single threshold value produced clear segmentation of the cells. To remove the artefacts the cell size and cell distance to neighbouring cells (obtained from distance transform) were computed and those segmented objects whose properties were outside one standard deviation from the respective mean were removed (Fig. 3F).

2.4 Derivation of quantitative parameters

By applying a sequence of morphological operations (dilation, erosion and hole filling with the structuring element of the size equal to half-diameter of a mean-size cell) to the binary segmented image three regions were obtained, corresponding respectively to the scratch wound and to the groups of cells on either side of the wound. The outline of the scratch wound is shown in red in (Fig. 3A). The manually drawn outline is shown in blue for comparison in Figure 3F. The cell count on each side of the wound divided by the segmented area provided an estimate of the cell density. The size of the central region provided an estimate of the scratch wound area.

3 Results and evaluation

Wound repair images are analysed in pairs, at zero and at an end time points (typically 18 or 24 hours). The ratios of the wound areas at the two time points are then computed. 22 pairs of images have been analysed both manually and by the method described. As the computer analysis was carried out retrospectively the actual outlines of the wound areas were not available in all the cases, only the wound area ratios for image pairs. For this data

there was good agreement between the manually and automatically derived ratios (correlation coefficient of 0.92, the slope of the trend line of 0.88). Reproducibility of the manually derived ratio is not known for the data sets used for these experiments. The latter could suggest that the method is more prone to error with pairs of images where the wound has closed to the greatest extent. In such samples at the end-time point the wound has partially closed and therefore the wound area is substantially more intricate. Overall trend between the two data sets shows that the gold standard produces a larger wound coverage value than the method described here. This could be due to errors in the boundary of the wound produced by this method or errors in bias in the gold standard.

The analysis has been carried out on two further experimental data sets of similar size and all the results showed similar correlation with the gold standard. In all the cases the same DoG filter was used, demonstrating the robustness of the imaging model and its derivation.

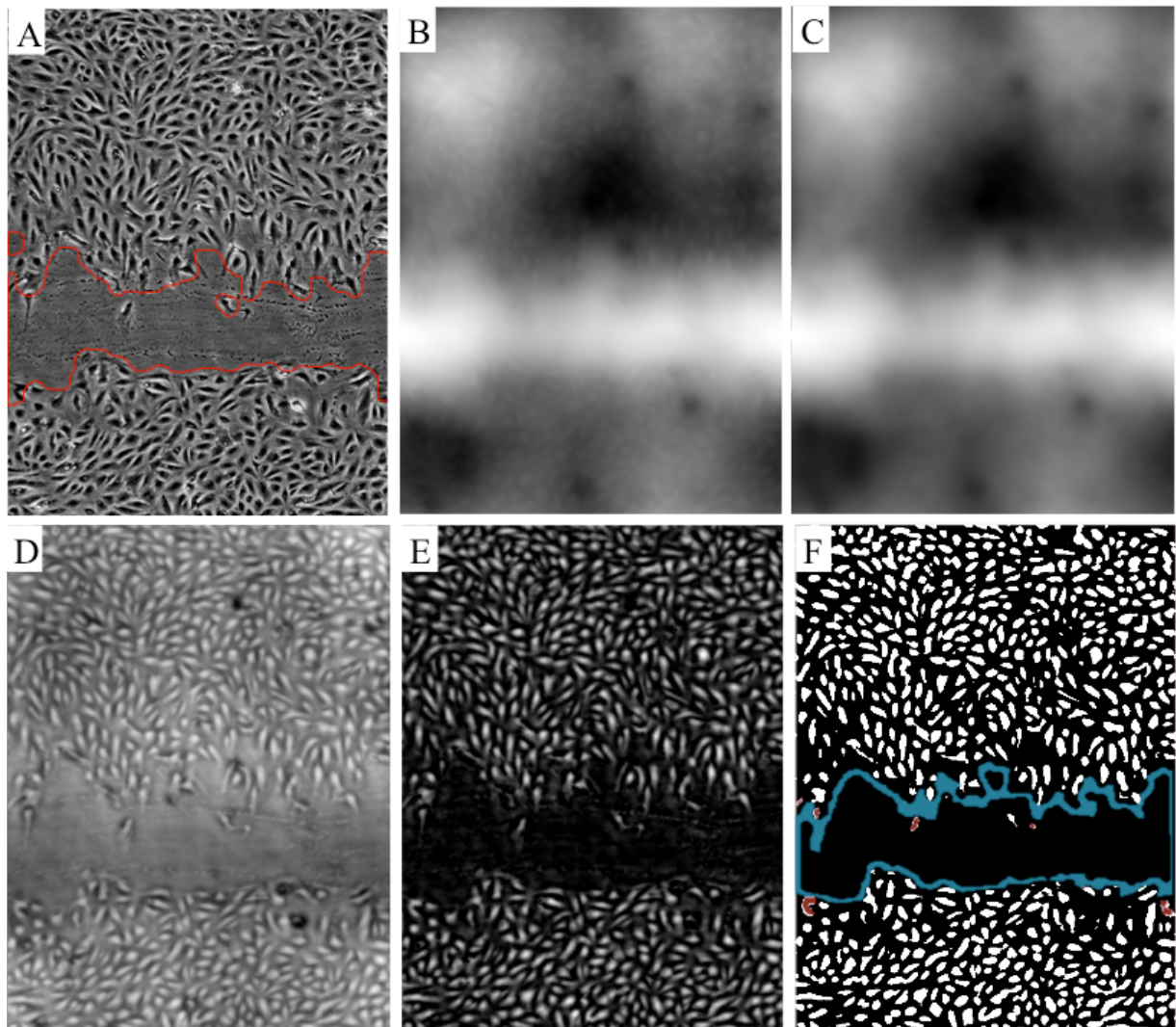


Figure 3: The Steps of the Method. (A) Initial phase contrast image with outline of automated segmentation produced by method described in this paper. (B) Image after deconvolution with DoG filter; (C) Background generated by deconvolving smoothed original image; (D) Image after background subtraction; (E) Top-hat algorithm applied to (D); (F) Binary image of (D) after thresholding; superimposed are cells that have been considered outliers and the outline of the wound when analysed by hand.

4 Discussion and Conclusion

The novel aspect of this work is the development, parameterisation and use of a model of image formation in the analysis of PCM images. The model itself is a very simple linear filter, DoG, lacking the nuance and quantitative aspects of more complex models (e.g. [7]). The key to its successful performance is its parameterisation, which has been computed via optimisation using a small image sample. Once the parameters have been established there is no regularisation involved in the process of image restoration as it is the case in [7]. The model inversion is implemented as a deconvolution. In the restored images the values are proportional to cell thicknesses, making cells easy to segment by simple global thresholding. Artefacts characteristic of the PCM, such as halos, are absent.

In application to the analysis of lung epithelial scratch wound repair assays this semi-automatic method has a number of advantages over the manual gold standard method. First is that by using this method the results are unbiased. Secondly, the method is significantly faster than the gold standard. Once a single cell profile has been determined (see 2.2) the method takes only 7 seconds to run on a standard PC using an uncompiled Matlab script. With the cell profile analysis time is increased to approximately 3 minutes, but this needs to be carried out only once per batch. In contrast, manual analysis takes approximately 10 minutes per image. The presented method can compute parameters related both to cell spreading and to cell proliferation. As the epithelial scratch wound repair assays are used in many areas of biosciences, and are applicable to the cell lines that can form monolayers [9], the authors plan to develop a practical tool for use by bioscientists.

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