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Diffuse reflectance measurements using lensless CMOS imaging chip

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Abstract. To assess superficial epithelial microcirculation, a diagnostic tool should be able to detect the heterogeneity of microvasculature, and to monitor qualitative derangement of perfusion in a diseased condition. Employing a lensless CMOS imaging chip with an RGB Bayer filter, experiments were conducted with a microfluidic platform to obtain diffuse reflectance maps. Haemoglobin (Hb) solution (160 g/l) was injected in the periodic channels (grooves) of the microfluidic phantom which were covered with ~250 µm thick layer of intralipid to obtain a diffusive environment. Image processing was performed on data acquired on the surface of the phantom to evaluate the diffuse reflectance from the subsurface periodic pattern. Thickness of the microfluidic grooves, the wavelength dependent contrast between Hb and the background, and effective periodicity of the grooves were evaluated. Results demonstrate that a lens-less CMOS camera is capable of capturing images of subsurface structures with large field of view.

1. Introduction
Functional capillary density (FCD) and heterogeneity of microvascular pattern (MVP) are widely used clinical parameters associated with a number of medical conditions such as inflammation, benign lesions, early mucosal cancers, pathophysiology of sepsis, and quality of the tissue perfusion [1]–[4]. It is due to the complex relationships between microvascular evolution and an early detection of cancer, assessment of its grade and prediction of treatment outcome still remain a formidable challenge of modern medicine [4].

The normal mucosa is composed of an avascular epithelium and a subjacent vascularized dermis. Dense arborescent vessels nourish the epithelial layer via a network of thin vessels, which rise to the surface of the mucosa and form intrapapillary capillary loops (IPCLs). Individual IPCLs are periodically distributed in the dermal papilla space with an average interval of about 100-180µm, which corresponds to the maximum distance that oxygen diffuses from a vessel [5], [6]. In mucosal layers, this capillary network is located, on average, 350 µm beneath the surface [7], [8].
With the onset of mucosal cancer, the progressive neovascularization leads to an increase in microvessel density. This process results in gradual transformation of the organized microcirculation network, inducing structural changes of the local capillary structure, and disturbing the spatial pattern of the initially periodically distributed haemoglobin [6].

Diffuse reflectance spectroscopy is widely used to evaluate tissue averaged haemoglobin changes in a volume sampled by the light traveling from the source to the detector. The spectroscopic technique offers a potential to determine optical properties of a sample, and/or quantify relative concentrations of the chromophores of interest integrated over the interrogated volume. A spatially resolved diffuse reflectance spectroscopy (SRDR spectroscopy) based on a fibre bundle probe can potentially provide a sufficient resolution to capture the structural distribution of the capillary loops but it lacks proper visualization of the tissue [9].

Current article is focused on the results of the development of a novel miniature, spatially resolved diffuse reflectance technology based on a lensless CMOS imaging chip to monitor spatial parameters of subsurface microfluidic pattern. These experiments are designed to test the ability of the method to evaluate the width of the haemoglobin grooves in the microfluidic phantom, to estimate the difference in haemoglobin contrast as reordered by the RGB channels of the conventional CMOS chip, and to determine the periodicity of the embedded structure.

2. Method

2.1 Equipment

A Motic AE31 Inverted Microscope, on-board RGB CMOS chip (Motic, China) was used. The CMOS matrix had physical dimensions of 6.8cm x 5.3cm (the active dimensions are less but this information was not provided by the manufacture), and a maximum resolution of 3 mega pixels (MP) (2048 x 1536) which corresponds to a pixel pitch of ≈ 3.2µm, neglecting packaging area losses at the chip boundary. The CMOS read-out offered variable resolutions ranging from 0.2 MP, 0.8MP, 3MP, corresponding to an effective pixel pitch of 12.5µm, 6.3µm, and 3.2µm respectively. Spectral resolution of the CMOS was determined by the spectral width of the Red-Green-Blue channels (≈ 100 nm). The analog to digital conversion had an 8-bit resolution. Software Motic Images Plus 2.0 for PC software package was used for image acquisition. The visualization of images and the image processing was performed by an algorithm created in MATLAB (MathWorks, USA).

Illumination of the sample was achieved with two 0.400 mm core optical fibers (Silica fibers, NA 0.22, Thorlabs Inc., USA) coupled to a broadband white light source, HPLS-30-02 (Thorlabs Inc, US). Placing the fibres at the extreme edges of the CCD (as shown in Fig. 1) results in a non-uniform illumination of the sample. For analysis, a region of interest was chosen manually which portrayed the lowest variation in illumination and a background compensation was performed as described in the “Image processing” section. Although non-uniform illumination can be compensated with background subtraction techniques, a diffusive illumination setup is planned to be designed for future experiments to provide uniform illumination.

Microvasculature-simulating microfluidic phantoms with narrow periodic grooves filled with haemoglobin extracted from red blood cells were used in the experiments. The microfluidic structure was produced in collaboration with the Microfabrication Laboratory at University Health Network, Techna Institute (Toronto, Canada). The device was designed to reproduce capillary spatial pattern with close to realistic physical dimensions: on average, 34µm wide groove, separated by 82µm spacing, the groove’s height - 30µm.
Figure 1. Design of the experimental setup for the diffuse reflectance measurements: an axonometric view and a side view. The magnified schematic of the microfluidic channels shows an average value of the groove’s width, 34µm, a separation of approximately 82 µm. In Z-direction (depth), the groove is 30µm. The total area covered by the microfluidic channels was 5mm x 5.75mm.

The phantom was manufactured from a clear PDMS (silicone) material, n=1.56 in the visible spectrum without addition of scattering particles. The microfluidic channels were covered by a layer of intralipid to mimic the biotissue light diffusive properties. Original solution of intralipid (20%) (I141, Sigma-Aldrich Inc, USA) was diluted in deionized water to obtain 1% and 2% intralipid concentrations, which were used within 10 minutes of the preparation time. Based on the scattering properties of the intralipid reported in the literature, [10],[11],[12] 1% and 2% concentrations were selected to match the scattering properties (µs’) of the epithelium [13].

The thickness of the intralipid layer on the top of the grooves was ≈ 250µm. The solution of the haemoglobin (160 g/l, Sigma Aldrich, USA) was injected in the reservoir A (2mm x 2mm) by means of an 25 gauge needle and a syringe (Fig.1). Another needle was placed in reservoir B (2mm x 2mm) to facilitate the flow of the haemoglobin from reservoir A to reservoir B, and to obtain a uniform distribution of haemoglobin in the microfluidic channels.

2.2 Image Processing
Denoising of the images was performed by a 2D Wiener filter implemented in MATLAB, with a pixel neighbourhood of the dimensions 5 x 5 pixels. The Wiener filter is designed as an adaptive filter which performs little smoothing where the variance is large, and more smoothing where the variance is small. This helps to preserve edges and the high frequency components of the image. Images were rotated for the alignment of channels vertically to facilitate the calculation of microfluidic channel widths. Line scanning was performed on the images and due to the nature of illumination in our experiments, a low pass filter was implemented in the form of a moving average with a window size of 30 samples per scan line to estimate the background compensation required. It was estimated that the spatial frequency of the channels was much higher than the frequency pertaining to the variance of the background illumination across the illumination field. Hence, illumination variance compensation was achieved by the subtraction of a low-passed output from the raw image data line by line. The resultant data was mapped as a binary image [-1, 1] and the zero crossings in each line were obtained to calculate the channel widths. The zoomed section of the Fig.2 represents a sample of processed output.
3. Results

In measurements of the diffuse reflectance signal with on-surface CMOS device, two optical fibres irradiate the surface of a sample while the RGB CMOS camera captures an image. Some part of unpolarised light returning from the subsurface layers of the microfluidic phantom, passes through a set of red, green, and blue filters integrated in the CMOS chip array. The corresponding image is stored digitally as three matrices representing the red (600–700 nm), green (500–600 nm), and blue (400–500 nm) colour planes, respectively [14].

Evaluation of the thickness of the grooves in three different experimental conditions are shown in Fig. 3. Left column contains two images (original and binarized) and a histogram of the condition when no diffusive media (intralipid) was added on top of the capillary layer, whereas the middle, and the right column show the results for a low and high scattering layers over the grooves of the microfluidic. The intralipid layer on the top of the microfluidic phantom produces an intensity gradient at the boundaries of the haemoglobin channels. As can be seen in the histograms (bottom plots), the thickness of the channels’ images acquired through the intralipid layer demonstrates broadening of their width due to the longer paths of light (Fig. 3, central, and right bottom charts).

Results of the comparative analysis of the RGB channel intensities for the grooves and background are displayed in Fig. 4. At first, the intensity values in the grooves and the background were normalized to the 8-bit integer maximum (255) to obtain values in the range [0, 1]. The distribution of the normalized intensities (X-axis) in the grooves (solid lines) and background (dashed lines) are colour-coded, and plotted (Fig 4). The distributions were normalized (Y-axis) by the respective maximum frequency to account for variations in the field of view as well as for the better representation of the peak separations. Percent difference representing the peak shifts in intensities for each colour channel was calculated to observe the contrast for each spectral band.
Periodicity of the haemoglobin grooves in three experimental conditions (no intralipid, 1% and 2% intralipid on top of the microfluidic channels) was evaluated by obtaining the prominent spatial frequency contained within the line scans performed on the field of view depicted in Fig. 3. Fast Fourier Transform (FFT), corresponding to a sampling interval of 0.0063mm (effective pixel pitch), was performed line-by-line from top to bottom, and a mean spectrum was obtained to represent the most prominent frequency as shown in Fig. 5. The peak in the spectrum was observed at an 8 mm\(^{-1}\) mark, corresponding to a spatial separation of 125µm. This value closely resembles the actual pitch of the haemoglobin carrying grooves (34µm + 82µm = 116µm).

![Figure 3. Top panel: Original images of the microfluidic phantoms: no intralipid (left), intralipid 1%, intralipid 2% (right). Binarized images of the region of interest for three experimental conditions are in the centre row of the figure. Histograms (bottom panel) of the recovered widths of the grooves, corresponding to the binary images above.](image)

4. **Discussion**

A novel method of the application of a lensless CMOS imaging chip for the on-surface measurements of the diffuse reflectance signal has a potential for further development into a clinical technique, which can provide both, structural and metabolic information about functional heterogeneity of microvascular pattern (MVP) and capillary density (FCD). Since application of the lensless CMOS chip for the on-surface measurements of the diffuse reflectance data is a novel approach, the most important research question to be answered is how the camera can capture subsurface images of embedded structures. The main objective of these experiments is to evaluate the image quality of the periodic channels under various scattering conditions, using intralipid as a diffusive media topping the surface of the haemoglobin grooves.

The experimental results have confirmed that the lensless CMOS imaging set-up can be used to acquire the diffuse reflectance signal from the subsurface of a microfluidic phantom with the embedded periodic structure. The collected signal contains both components of the reflected light, i.e., surface reflectance and subsurface reflectance [14]. To separate these two components a set of polarizers will be included in the future system, so the surface and subsurface information could be de-coupled by the polarization gating techniques.
Noise reduction in the images was achieved by using an adaptive 2D Wiener filter which was chosen for its ability to preserve the dimensions of the original spatial pattern. The noise in the images was assumed to be Gaussian, and performance of the image processing was manually verified. This approach allowed us to recover the spatial frequency of the grooves which was found sufficiently close to the original dimensions.

Although the 100nm bandwidth of the RGB channels is too broad to be considered for spectroscopic applications, the absorption contrast of the Red, Green and Blue channels have been qualitatively compared. In tissue spectroscopy, wavelengths dependent absorption is linked to the quantitation of the relative concentrations of tissue chromophores. For hemoglobin, there are several absorption peaks present in the green and blue regions of the spectrum. Hence, it was expected that, despite the breadth of the spectral bands, we should observe an enhanced contrast for the Green-Blue channels of the RGB image as compared to the Red channel values. Indeed, the largest peak difference (contrast) between the grooves and background intensities was found for the Blue and Green spectral bands, while the red component demonstrated relatively insignificant shift of the curve (Fig.4). Virtually no significant difference between the Blue and Green contrasts was observed when the scattering properties of the phantoms were increased. That is the condition when the narrow band imaging would have greater advantage. In future modification of the system, a monochrome CMOS imaging chip would provide more clinically valuable information as it enables the experiment to be fine-tuned for the absorption by a specific chromophore using a selected spectral band. Switching multiple spectral bands opens up advantages of the hyperspectral imaging.

The periodicity of the pattern was evaluated by observing the FFT spectrum. All the images were rotated before processing to align the microfluidic channels parallel to the direction of line scans (Y-axis in Fig. 1). The microfluidic phantom was placed on a flat surface, and the channels were parallel to the CMOS chip plane. The difference in the measured spatial frequency and the actual spatial frequency was $\approx 7.2\%$. We believe this difference is attributed to the uneven orientation of the layers to the
direction of line scans, non-uniform illumination, and the scattering top layer (in case of the intralipid layers). Since our set-up contained a linear pattern of grooves, 1D Fourier transform was sufficient to provide an estimate of the periodicity of the pattern. Also, the variability in the thickness estimation can be used to qualitatively evaluate the periodicity of the pattern. This shows that we can design application-based computational techniques to evaluate the periodicity of the microvascular patterns. In future, correlation matrix based method can be implemented to assess the periodicity, and the quantitative analysis (e.g. covariance measures on thickness estimation) can be performed to obtain a measure of periodicity as a clinical parameter.

There are several advantages of the usage of the CMOS imaging chip for the diffuse reflectance measurements of the subsurface space. The larger coverage area facilitates collection of the optical signal from a large field of view, whereas the small sampling interval increases the method’s spatial resolution. Its pixel size enables “continuity” of the signal, and satisfies sampling criteria for robust 2D resolution of the microvasculature in epithelial tissue. The interrogated depth and blood contrast can be improved by exploiting the Optical Clearing technique [15].

Future modifications of the device will target improvements of the design, and reproducibility of the measurements. The uniform illumination of the sample is an important factor in prevention of the saturations spots, which cause losses of imaging area [16]. Overall, the technique has potential for subsurface imaging.

5. Conclusion
The main application of the technique considered in this paper is a targeted imaging of the spatial parameters of subsurface microfluidic pattern. The experiments with a lensless, on-surface CMOS chip imaging on the intralipid microfluidic tissue phantoms have demonstrated a potential of such 2D spatially resolved method. It offers the advantage of the real time, non-invasive imaging of the structural architecture of subsurface patterns.

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7. References


