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Uncertainty model of electro-optical thrombus growth estimation for early risk detection

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ABSTRACT

It is proposed a device for the study of the real-time aggregation of white thrombi in whole blood. This device allows extrapolating the 3D-shape of platelets clotting within artificial capillaries, by simultaneously monitoring the electrical impedance between a pair of specifically designed gold electrodes and 2D optical image of pixel luminance of the fluorescent labeled platelets. Up to 30 3D images per minute have been obtained, for a process which requires few minutes before the aggregation of large thrombi structures, a noticeable result with respect to other 3D-shape reconstruction methods. The contribution of this paper mainly consists in the study of an uncertainty model which, in our opinion, is of fundamental importance to provide a valuable metrological estimation of thrombus growth under flow conditions.

1. Introduction

Noninvasive and real-time monitoring of the clotting process, in artificial capillaries under *ex vivo* conditions that mimic as close as possible the *in vivo* ones, has received a considerable attention in the recent days medical research. Usual state of the art devices mainly perform a visualization of the thrombogenesis process by means of Epi-fluorescent video microscopy, confocal microscopy, Optical Coherence Tomography (OCT), or light scattering [1–5]. Epi-fluorescent microscopy visualizes a 2D image of thrombotic kinetics in flowing whole blood and reconstructs, through a digital image processing, the three-dimensional thrombus geometry. However, the solid models of the thrombi can only be estimated in an approximate way. The confocal microscope is a bulky and expensive instrument which performs multiple scans on different focus planes. The time needed for a high number of scans on multiple planes can take several seconds (approximately 30 s, see Section 4) because of the exposure time required for each plane scan, so that the instrument is not suitable for the evaluation of the time evolution of the aggregation process and/or the thrombus stability. In fact, the volume estimation can be performed only at the end of the aggregation process. Optical Coherence Tomography and light scattering instruments would allow fast thrombus geometry reconstruction, however, their size and costs allow their usage only in research centers and specialized laboratories. The goal of the proposed device, instead, is the development of a new, small, and low cost Point-Of-Care prevention screening system which allows tracing the thrombogenic profile also outside specialized clinical laboratories or hospitals. This work, which is the extension of [6], focuses on the analysis and metrological characterization of a novel device able to capture the time evolution of white thrombus (consisting mainly of platelets) formation induced on a collagen-coated glass within artificial capillaries during whole blood flow. The novelty content of the proposed

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device lays on the capability of extrapolating the actual three-dimensional structures of the thrombi and to measure the volume of thrombus during its formation, by simultaneously monitoring electrical impedance between a pair of gold electrodes specifically designed and 2D optical image of pixel luminance of an aggregating blood sample. It is known that an accurate information on volume estimation is of paramount importance in the hematology field to study the thrombogenic response [7]. Moreover, the uncertainty model proposed in the present work is of fundamental importance to provide a valuable metrological estimation of thrombus growth under flow conditions in the routine clinical decision-making.

Impedance measurements applied to blood analysis, in particular in the form of Electrical Impedance Spectroscopy (EIS) [8,9], has received a considerable attention because of non-invasive and realtime monitoring of the clotting process. The present paper is the evolution of previous works [6,10–15] and focuses on the uncertainty estimation of volume reconstruction of white thrombus fusing optical and impedance data.

The acquired impedance data are post processed and compared to a corresponding computed impedance obtained from an electromagnetic numerical model of the overall system formed by the microchannel, the thrombus, and the gold electrodes. The geometry of the thrombus is represented by a solid model deduced from the 2D optical image of pixel luminance. The height of the thrombus with respect to surface of the slide, is assumed proportional to the pixel luminance according to a unique scale factor. The scale factor is determined by matching the computed impedance obtained by a 3D electroquasistatic numerical model of the thrombus with the measured impedance, according to a bisection iterative algorithm. The white thrombus volume is then evaluated from the thrombus solid model.

The paper is organized as follows. In Section 2 a system description is performed, focusing on the microfluidic sensor and on the measurement set-up. Section 3 is dedicated to the experimental results. In Section 4 a model is proposed with the aim of evaluating the uncertainty of the thrombus volume estimation and, finally, in Section 5 we discuss the novelty of the proposed approach on the basis of experimental results.

2. System description

The measurement setup is shown in Fig. 1; the microfluidic sensor, described in detail in next Subsection, is posed under an optical microscope with a Charge Coupled Device (CCD) camera and connected to an LCR meter; the camera and the LCR meter are connected to a laptop through USB interfaces and a software control panel simultaneously acquires optical and impedance data. Blood flows inside the microchannel at controlled flow rate in order to obtain a proper shear rate and reproduce the *in vivo* conditions at controlled temperature of 37°C.

Before each experiment, a collagen is spread on the quartz slide which forms the upper wall of the microchannel; the collagen will activate platelets which will adhere

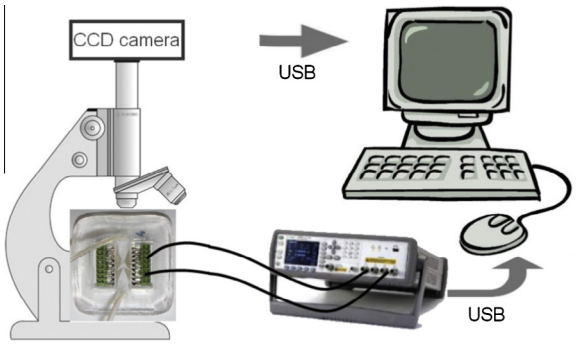

Fig. 1. Measurement setup: the thrombus growth in the microfluidic sensor provides an optical information through a microscope and an electrical information through an LCR meter. Electrical and optical information are both automatically acquired by a computer.

to the slide thus starting the thrombus growth process from the slide surface. After one hour of incubation time, the device is assembled. Platelets are labeled with quinacrine, a fluorescent marker which emits fluorescence in the green zone (522 nm wavelength) when excited with violet (488 nm wavelength) light. With the marker, thrombi of platelets (also known as white thrombi), will provide a fluorescent emission and the light intensity sensed by the CCD camera will change proportionally to the volume of the thrombus.

The sensor is assembled and placed on the microscope; whole blood is pumped in the microchannel for a time interval of three minutes at a flow rate $Q = 750\ \mu l/\min$, in order to obtain the desired shear rate γ

$$\gamma = \frac{6Q}{wh^2} = 3000\ \mathrm{s}^{-1}, \tag{1}$$

where w is the width and h is the height of the rectangular duct. Such a high value of shear rate confines the platelets (which are smaller than red and white blood cells) close to the walls of the channel, where collagen is spread; here platelets are activated by the collagen and start adhering to the slide so as to clot. Moreover, the subsequent collisions between the moving platelets and the aggregate enhance the thrombus formation [18]. At the end of the experiment, all the acquired impedance and optical data are processed with a novel inversion methodology in order to obtain the time evolution reconstruction of the thrombus volume and geometry.

At the same time, a 3D scan on the investigation area is performed with a state-of-the-art confocal microscope, used as a reference, in order to characterize the accuracy of the reconstructed volume with the actual volume of the thrombus at the end of the experiment. Notice that confocal microscope cannot be used for estimating all the aggregation evolution, but only the final state.

2.1. Microfluidic sensor

The developed sensor is composed by a Poly-Dimethyl-Siloxane (PDMS) substrate, where the microchannel with microfluidic connections is molded, and a quartz slide

forming the upper wall of the channel with sputtered gold electrodes. The microchannel measures 400 μm × 25 mm, with 250 μm of depth. The upper wall of the microchannel is the quartz slide with 200 nm thick sputtered gold electrodes, arranged as shown in Fig. 2. The outer electrode is 30 μm wide and extends on a 280 × 280 μm^2 investigation area; the inner electrode is 20 μm. A SiO_2 passivation layer (pink in Fig. 2) is set over the tracks in order to obtain that only the electrodes are electrically active and in contact with blood. The microchannel and electrodes were designed so as to obtain a good optical spatial resolution in a wide investigation area with standard microscope magnification factors. In particular, the optical device in the experiments uses a 10× magnification factor allowing an investigation area of 650 × 650 μm^2 with a pixel area of 1.35 × 1.35 μm^2, while the single platelet diameter is in the order of 3 μm.

2.2. Measurement setup

The microfluidic sensor is connected to the LCR meter and posed under the microscope. The microscope magnification factor is 10× and the CCD camera acquires 512 × 512 pixels images recorded at a frame rate of 10 frames per second; under these conditions, as previously said, the investigation area is 650 × 650 μm^2 with a resolution of 1.35 × 1.35 μm^2 and the time resolution of optical measurements is 0.1 s.

The impedance measurements are performed by using the high precision LCR meter Agilent E4980A in the frequency range 1–300 kHz with eight logarithmic evenly spaced steps with a two-wire configuration. In order to avoid that redox reactions will take place between electrodes and salts dissolved in plasma, a drive voltage of 100 mV has been chosen, since it is by far lower than the standard half cell potential for gold (that is 1.5 V).

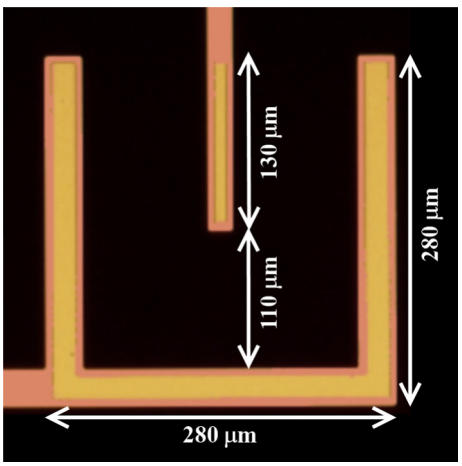

Fig. 2. Detail of electrodes arrangement evidencing gold layer (yellow) and passivation layer (pink). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In this frequency interval, the growing thrombus behaves like an insulator, thus providing an increase of measured impedance between the electrodes. The lipidic membrane surrounding the platelets which form the thrombus, behaves like an insulator at frequencies lower than 300 kHz [11,13]; in this conditions the thrombus impedance is much higher than the blood impedance and platelet aggregation can be measured by evaluating the impedance increase. Before each experiment, open and short compensation were performed in order to remove parasitic effects; in these conditions, the impedance measurement accuracy is in the order of 0.1%. With these configurations, the instrument can perform a measurement sweep every 1.7 s that is the time resolution allowed for the thrombus reconstruction.

The Real – Imaginary plot of the impedance over a time interval of three minutes is shown in Fig. 3; as previously said the time resolution of acquired spectra is 1.7 s, but for graphical reasons in Fig. 3 the plotted curves are 17 s spaced. It is apparent that the interface impedance between electrode and blood (known as double layer) dominates the impedance behavior at low frequency, masking the effects of thrombus formation, since all curves are overlaid. The estimated impedance of the double layer is thus $Z_{DL} = \frac{1}{C_{DL}(j\omega)^{\alpha}}$ with $C_{DL} \approx 5.9$ nF and $\alpha \approx 0.59$. At high frequency the overall impedance behaves more resistively, justifying the decrease of the imaginary part of impedance in the Figure. Since the resistive section of blood between electrodes is partially obstructed by thrombi, in this frequency range the blood resistance increases with a rate proportional to thrombus height and width.

At the end of each experiment, the thrombus geometry and volume were reconstructed by a novel inversion methodology based on optical and impedance data fusion. The geometry of the thrombus is represented by a solid model deduced from the 2D optical image of pixel luminance $L = 0.299R + 0.587G + 0.114B$, being R (Red), G (Green) and B (Blue) the RGB values of the acquired images. In the implemented model we assumed that the thrombus height is linearly proportional to the intensity of emission of marked platelets, as well established in literature [16]; this approximation provides good results

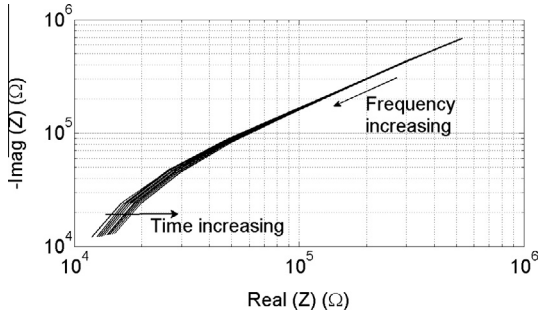

Fig. 3. Impedance spectroscopy during thrombus formation. Test frequencies are log-spaced in the range [1–300] kHz with eight steps. The time evolution is acquired on a 3 min interval with a resolution of 1.7 s; for graphical reasons only impedance data acquired every 17 s are plotted.

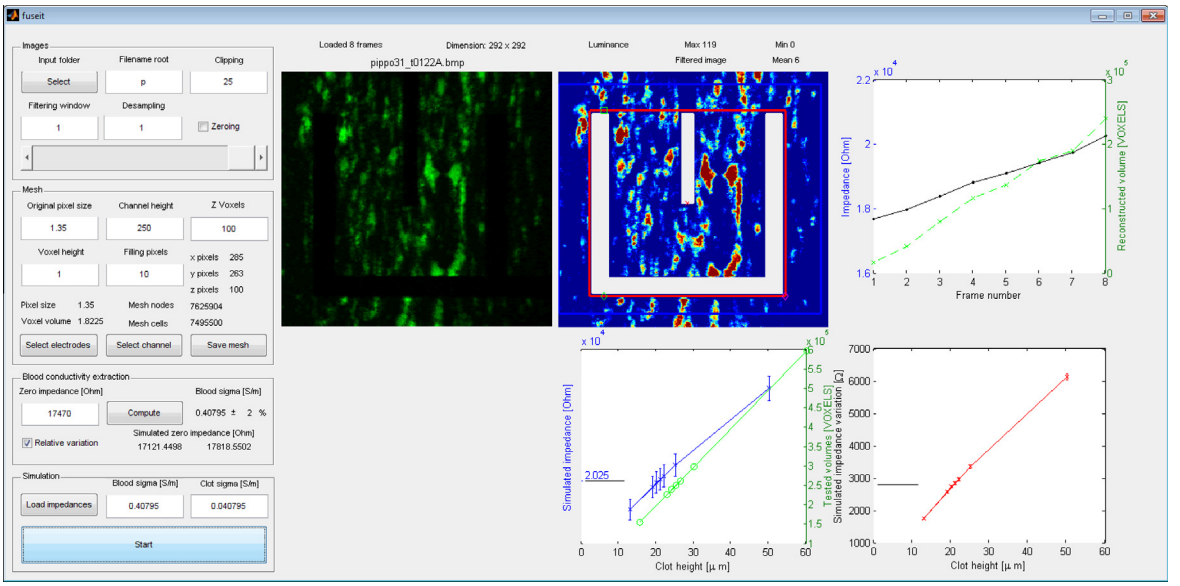

Fig. 4. The developed tool used for the solid model reconstruction allows to automatically recognize electrodes and microchannel, compute blood conductivity and modify the scale factor to match measurements with solid model simulations.

from the experimental point of view, as discussed in Section 4. Accordingly to this assumption, each pixel luminance results proportional to the thrombus height with respect to the slide surface, with a unique scale factor for all the pixels. In order to determine the scale factor, a 3D solid model is derived from luminance data and the pixels heights are multiplied by the scale factor; the resulting geometry is iteratively simulated changing the scale factor until the computed impedance matches the measured one; the thrombus volume is then calculated by the thrombus solid model. The electrical parameters of blood at high shear rates that have been used in the simulations can be found in [11,13] while the thrombus electrical properties are considered as perfect insulator below 300 kHz. The 3D simulation tool expressly developed for this application is based on the Discrete Geometric Approach developed by the authors. The tool, shown in Fig. 4, automatically recognizes the electrodes and the microchannel in the microscope images, solves the direct problem to compute the blood conductivity from the impedance measured at the beginning of the experiment in absence of thrombi, and, for each frame, iteratively modifies the height scale factor to match simulations and measurements. The initial solution of the direct problem is necessary since blood conductivity can vary significantly from a patient to another for many reasons, for example because of the hematocrit concentration and the platelets count.

3. Experimental results

Fig. 5 shows some significant frames acquired from the CCD camera during an experiment. The blood flow direction is from the top to the bottom of each frame; the frames acquired at 40 s, 60 s, 80 s, 100 s, 120 s, 140 s, 160 s and 180 s after the experiment start are shown in Fig. 5(a)–(h), respectively. The growing thrombi appear as green[1] spots inside the investigation area. At the beginning of the process the single platelets are activated and start adhering to the collagen; then the platelets aggregate with each other and the thrombus increases both in base area and volume to form, at the end of the experiment, stripes in the direction of blood flow.

Fig. 6 shows the time evolution of measured impedance magnitude at 300 kHz (blue axis and line) and the reconstruction of the thrombus volume (red axis and markers); error bars on the markers represent the uncertainty on volume reconstruction computed with the method proposed in the next Section.

With available commercial measurement systems (e. g. confocal microscope) the evaluation and measurement of the time evolution of growth shown in Fig. 6 is not possible since the multiple planes scans can be performed only at the end of the experiment. However, the volume of the thrombi can be estimated at the end of process, and this estimation can be used for a comparison with the estimation provided by the proposed method at the same time.

4. Uncertainty evaluation

The main contributions to the uncertainty of the volume reconstruction arise from the camera resolution and from the solid model used in the finite elements simulation. The LCR meter uncertainty can be neglected, since it contributes for less than 0.1%, far lower than the previous terms.

The volume of the reconstructed thrombus is calculated as the sum of each pixel area (the base) multiplied by the

[1] For interpretation of color in Figs. 5, 6, and 9, the reader is referred to the web version of this article.

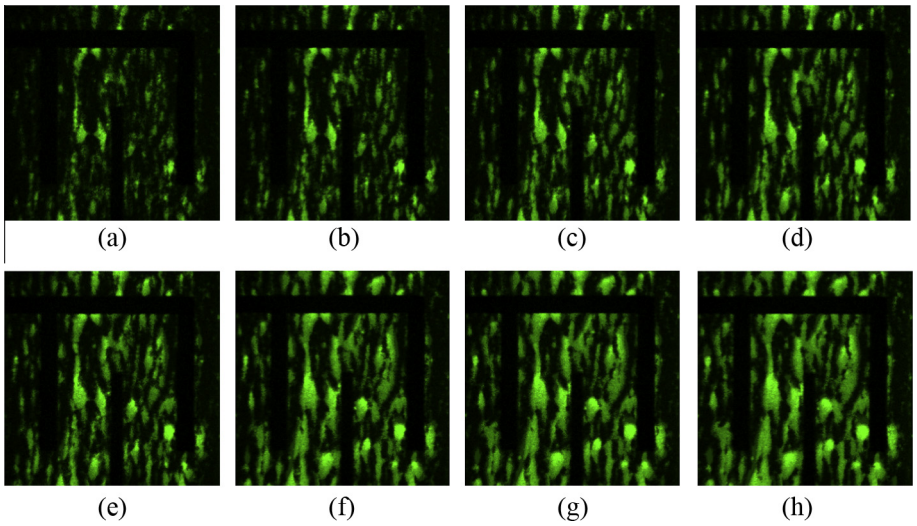

Fig. 5. Acquired frames from microscope during thrombus growth, blood flows from the top to the bottom of each frame. Frames are acquired after the experiment start: (a) after 40 s; (b) after 60 s; (c) after 80 s; (d) after 100 s; (e) after 120 s; (f) after 140 s; (g) after 160 s; (h) after 180 s.

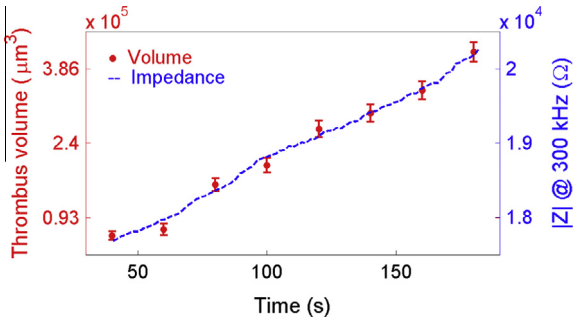

Fig. 6. Impedance change over time measured at 300 kHz (––) and thrombus volume estimation (●).

respective computed height; hence, each pixel with non-zero luminance is a parallelepiped whose volume is V_i and, if $N = N_I + N_F$ is the total number of pixels with non-zero luminance, N_I the internal pixels of the thrombus, N_F the frontier pixel, the total volume is estimated as

$$V = \sum_{i=1}^{N} V_i = \sum_{i=1}^{N_I+N_F} Ah_i \tag{2}$$

where A is the pixel area, and h_i is the computed parallelepiped height.

As reported in the previous Section 2.2, the voxel heights h_i are obtained by multiplying each pixel luminance for a scale factor which has been estimated by the simulator with a resolution of 1 μm for computation time reduction, pointing to an uncertainty $u(h_i) \approx 1/\sqrt{12}$ μm, under the hypothesis of uniform quantization error. Since there is only one scale factor for all pixels, the finite element simulation contributes $NA \times u(h_i)$ to the measurement uncertainty of the volume.

In the matter of the uncertainty of the parallelepiped base area, this is negligible in correspondence to the internal pixel of the thrombus, while it is related to the pixel quantization on the border of the thrombus, i.e. $u(A) = A/\sqrt{6}$, [19]. Finally, the uncertainty of the volume can be estimated as

$$u(V) = \sqrt{\sum_{i=1}^{N_F} h_{Fi}^2 u^2(A) + (NA\,u(h_i))^2} \tag{3}$$

where h_{Fi} is the ith frontier pixel.

Fig. 7 shows the recognition of frontier pixels used for the uncertainty evaluation (3). Fig. 8(a) shows the reconstructed solid model of the experiment shown in Fig. 6 while Fig. 8(b) shows the uncertainty relative to each pixel. It is apparent in Fig. 8(b) that the main source of uncertainty on volume reconstruction, is related to the voxel height quantization, which has been set to 1 μm to reduce the computation time. In these conditions in fact, the time required for inversion is 20 s/frame.

Fig. 9 shows the detail of a single thrombus reconstruction (dark red) with the uncertainty contribution (light red) due to the voxel quantization.

Fig. 10 reports the volume estimation performed on three different experiments by the proposed technique and by the confocal microscope, used as a reference instrument. The confocal microscope settings were: image size 300 × 300 pixel, exposure 165 ms, number of z-scans 200, z-scan step 0.5 μm, z-scan range 100 μm, total scan time 36 s. In laboratory practice, the uncertainty of volume reconstruction with the confocal microscope is not easily quantifiable; at each experiment in fact, the operator manually sets the optimal brightness and contrast of the scanned images in order to remove noise from the z-scans, thus improving the 3D reconstruction. In any case, confocal microscope uncertainty is by far lower than the presented method uncertainty, and, actually, represents the state of the art. For this reason the uncertainty on thrombus geometry reconstruction performed with confocal microscope is assumed to be negligible with respect to the proposed device. Fig. 10 points out that the proposed

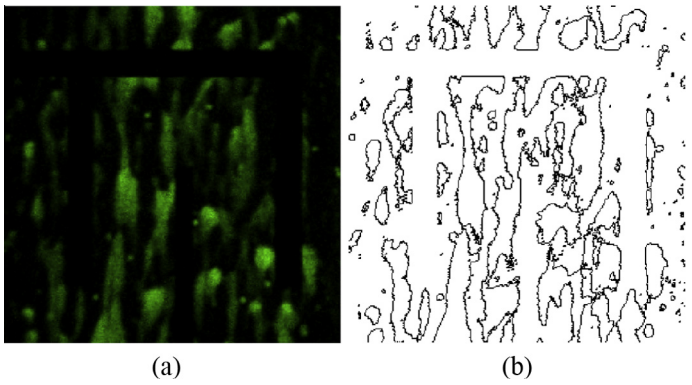

Fig. 7. (a) Camera frame with thrombus luminance and (b) thrombi's frontier pixels.

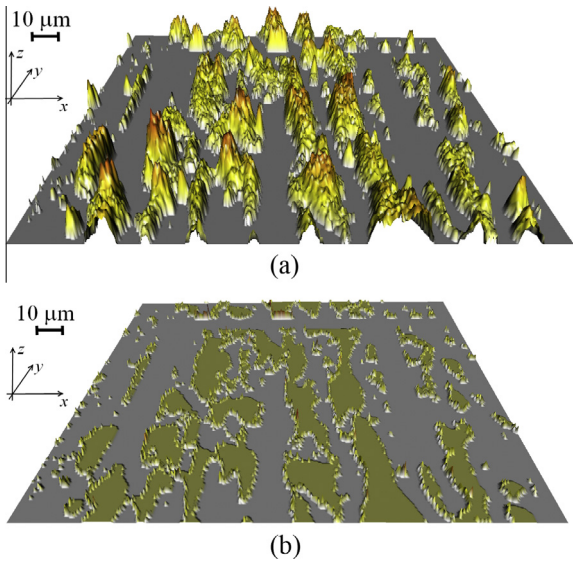

Fig. 8. (a) Reconstructed thrombus geometry, (b) uncertainty estimation on reconstruction.

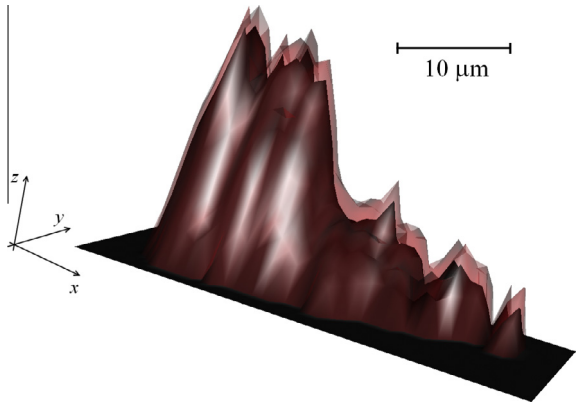

Fig. 9. Detail of thrombus geometry (dark surface) and the uncertainty contribution (light surface).

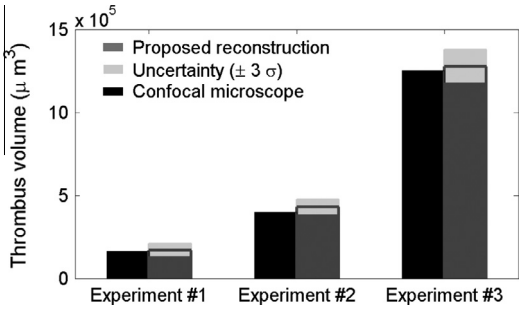

Fig. 10. Thrombus volume estimation provided by the proposed method, along with its uncertainty, compared to confocal microscope results, for three different experiments. The confocal microscope uncertainty is difficult to quantify, but is approximately one order of magnitude lower than the present method.

method provides compatible measurements with the confocal microscope, and that its uncertainty is lower than 10%.

5. Discussion

Monitoring of platelet thrombus aggregation is critical to better understand some pathologies, to understand the mechanism of mural thrombogenesis or to evaluate the thrombogenicity of various biomaterials and, finally, to guide hemostatic therapies, so that it was recognized as an important issue in several studies already in the 1980s [16].

Dynamic, real-time 2D-visualization is now a common technique for studying thrombus generation on a collagen-coated surface by blood from patients with various congenital platelet aggregation disorders perfused under different shear rate [17]. Similarly, 3D-visualization has gained in recent years more interest, being used in specialized laboratory for periodic controls, at the expenses of lower frame rates [18].

In this paper, the results of the conducted experiments showed that our technique provides thrombus reconstruction compatible with state-of-the-art confocal microscope at 1 standard deviation level, with the advantage of allowing the study of the thrombus growth at a frame rate of

approximately 0.5 frames per second for 4D (3D + time) thrombi imaging.

This technique, that in our opinion is of interest to the academic community, is likely to be rapidly translated into new experiments that may ultimately hold great promise in the clinic practice. However, effective diagnosis and treatment of disease requires the medical laboratory to produce reliable, reproducible, and comparable measurement results. According to ISO15189 (Medical Laboratories Requirements for Quality and Competence) and ISO/IEC 17025 (General requirements for the competence of testing and calibration laboratories) standards, laboratorian should be aware of the components that influence measurement uncertainty, so to validate each internally developed methods for both accreditation and certification of the laboratory. This paper represents a necessary step in this direction.

6. Conclusions

The developed device with the proposed measurement methodology provides a good estimate of the volume and shape of thrombi during their formation, by combining the global electrical impedance and the corresponding 2D optical images of a flowing whole blood sample aggregating on the plane of a glass. The uncertainty on volume reconstruction is related to the voxel height quantization, which has been set to 1 μm in order to reduce the computation time needed for the inversion which is about 20 s/frame. Moreover, the values of the volume evaluated on three different experiments have been compared to the relative value provided by a confocal microscope used as a reference instrument; the proposed methodology provides compatible measurements with respect to the reference confocal microscope with an uncertainty lower than 10%. These results demonstrate how the optical and electrical data fusion can provide an accurate and fast information on the individual predisposition to form thrombi.

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