Holographic microscopy for biomedical imaging.

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Abstract

Blood flow imaging techniques are widely used in biomedical research and clinical diagnostics, since they can assess physiological processes or can be used for early detection of disease. However, the majority of existing techniques are invasive and require, for imaging purposes, the use of a contrast agent. Recent developments in Digital Holography and Laser Doppler Holography techniques can be considered to alleviate this issue. In addition, the possibility to reconstruct the microcirculation in 3D and in real-time is not an issue.

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Introduction

Blood flow imaging (BFI) is nowadays widely used in many biomedical applications, like ophthalmology [1,2] and cardiology [3], since its skill to assess physiological processes or for early detection of disease. However, many BFI techniques require the use of a contrast agent for imaging purposes, making blood flow characterization invasive [4,5]. Scanning laser-Doppler imaging can be considered to alleviate this issue by losing in acquisition speed due to the scanning process [6]. The imaging of blood flow dynamics in real time can be achieved by analyzing the spatial statistics of the dynamic speckle with the Laser Speckle Contrast Analysis/ Imaging (LASCA/LSCI) technique [7-9]. Improvement of the acquired contrast image has been achieved through exposure time optimization [10], or intensity fluctuation analysis [11] resulting in high quality perfusion images. However, these techniques are limited to perfusion monitoring in 2D and unable to reconstruct the structure of the blood flow in 3D or 4D. Recent developments in Digital Holography and Laser Doppler Holography techniques can be considered to alleviate this issue [12].

Holography invention and developement

Proposed in 1948 by Gabor [13] as an optimization of electron microscopy, classical holography aims to record, on a photosensitive material, the interference between a reference field and a diffracted object field. However, within the proposed common-path interferometric configuration, holographic imaging suffers from the so-called twin-image noise. Leith and Upatnieks [14] improve this technique by introducing an off- axis reference field, which leads to a separation of the object and its twin-image contribution in spatial frequencies domain. It is then possible to extract, from this interference pattern, the scattered field that reaches the holographic detector. This is particularly easy in digital holography, by using a camera detector. Development and democratization of high-resolution photodiode sensors played a major role in holographic imaging diffusion, making possible both digital recording and processing of holographic images [15]. Further development conduct to the introduction of heterodyne digital holography [16], in which the reference field is dynamically phase-shifted with respect to the object field. Therefore, the recorded hologram is time modulated, enabling phase-shifted interferometric measurements [17]. Temporal modulation feature of digital heterodyne holography can also be used to investigate dynamic phenomena, and being considered as a laser-Doppler imaging technique [18]. The ability of heterodyne digital holography to perform Doppler imaging has been demonstrated really valuable to investigate *in vivo* vasculature assessment, without contrast agent [19-21]. So, thanks to these developments in recent time, laser-Doppler holography and transmission microscopy can be coupled to investigate blood flow microcirculation by adapting a laser-Doppler holographic setup to a standard bio-microscope [22,23].

Challenges in 3D imaging

Since the Maxwell equations can be used to back propagate the field from the camera to any point of the 3D space [15], holography is an intrinsically 3D technique: the acquired hologram allows to localize and track a point-like object that scatters light, since its position coincides with the maximum of intensity of the reconstructed field [24]. The localization accuracy along the z-axis depends strongly on the experimental conditions, in particular on the detection numerical aperture (NA). Nanometric accuracy can be reached in holographic microscopy with a high numerical aperture (NA $\geq$ 1) objective [25,26].

By now, digital holography has been confirmed as a useful technique to image and qualitatively analyze blood flows in 2D [27,28]. Moving to the next step of 3D imaging is challenging for many of reasons. The biologically relevant size of the microcirculation makes it impossible to use a high NA objective, since the field of view would be too small. Blood cells are much larger than the wavelength, and their refractive index is close to the plasma one. The light is then scattered within a small angle in the forward direction. In transmission geometry, this corresponds to a small NA giving a poor accuracy along the z-axis. In reflection geometry, this leads to a poor signal, which competes with light scattered by the surrounding living tissues, whose refractive index is not

homogeneous in time and space. These challenges has been an improvement path that is still wide open and each improvement in this direction can open other views in several applications.

Real-time 3D holography

Some examples of real time 3D holography are the in-line holographic setup for plankton *in-situ* observation proposed by Pfitsch et al. [29] and a parallel phase-shifting digital holography setup developed by Awatsuji et al. [30] that has been applied to the 3D reconstruction of transparent objects. Due to the complexity of using two different setup and the long acquisition time, both the above methods lack 3D reconstruction in real time.

Recently, moving red blood cells in a preclinical model have been imaged in real-time in 3D [12] with the help of a technique that combines digital holography, illumination of the sample and reconstruction along several axis [31], and calculations that involve both standard holographic propagation and 3D reconstruction by a cleaning algorithm [32-34]. More specifically, Kim et al. [35] use interferometric microscopy on mice with a method to filter out static scattering signals. The results show 3D refractive index tomograms of individual red blood cells flowing through micro capillaries. However, in this work, the part of a thin mesentery tissue should be placed on the sample holder.

Another application for zebrafish embryos is presented by Donnarumma et al. [23] and consists in a holographic microscopy working in transmission. Two illumination beams oriented in two different directions have been used to enhance the slicing along the z-axis; the angle between these two beams can variate between 30° and 90° [23,36]. Thus, each illumination beam signal has been selected with the aim to obtain two holograms from which two 3D intensity maps have been calculated. The highest values of correlation of these intensity maps have been selected by using a greedy algorithm to obtain a 3D reconstructed image. This procedure has been repeated over time to obtain a 4D reconstruction of the moving objects. In comparison with other *in vivo* imaging techniques dedicated to blood flow monitoring, including laser-Doppler and laser-speckle, the proposed approach has the major advantage of evaluating complex 3D aspects of the blood cells movements, specifically due to the recording of holograms. The proposed method can be still improved by using a more efficient cleaning algorithm for the 3D reconstruction and a setup allowing a larger angle of separation of the two illuminations beams [36,37]. These improvements should yield faster calculations and higher volume resolution.

Conclusion

In conclusion, multimodal techniques that combines digital holography, dual illumination of the sample and reconstruction with a greedy algorithm are rapidly developing, allowing 3D imaging of microcirculation over time with the possibility to evaluate physiologic parameters and investigate pathologies and anomalies. By now, these techniques have been validated on preclinical models and *in vitro* cultures. Other quantitative applications of this techniques like the evaluation of the individual blood cell motion with possible coincidence of images can be also studied from sequences of reconstructed images. In the next few years, these techniques can turn out in a label-free *in vivo* imaging as a robust alternative to other techniques for medical imaging. Meanwhile, the image treatment developed to achieve this result consists in an improvement of general application for other medical imaging techniques as computed tomography, magnetic resonance imaging, and ultrasound scans [38].

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