TITLE PAGE

Citation Format:

Paiè, Petra, Federico Sala, Roberto Memeo, Andrea Bassi, Roberto Osellame, and Francesca Bragheri. "Three-dimensional and dual-color fluorescence microscopy on a chip." In *Optical Methods for Inspection, Characterization, and Imaging of Biomaterials V*, vol. 11786, pp. 206-211. SPIE, 2021.

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DOI abstract link:

https://doi.org/10.1117/12.2592982

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Event: SPIE Optical Metrology, 2021, Online Only

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Three-dimensional and dual-color fluorescence microscopy on a chip

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ABSTRACT

In this work we present two microscopes on chip based on Light Sheet Fluorescence Microscopy, capable to automatically perform 3D and dual-color imaging of specimens diluted in a liquid suspension. A microfluidic channel is used for automatic sample delivery, while integrated optical components such as optical waveguides and lenses are used to illuminate the sample flowing in the channel. The devices are fabricated by femtosecond laser micromachining in a glass substrate. Benefiting from the versatility of the fabrication technique we present two prototypes that have been optimized for different samples such as single cells and Drosophila embryos.

Keywords: 3D microscopy, integrated optics, optofluidics, imaging, laser micromachining

1. INTRODUCTION

Among different fluorescent-based techniques Light Sheet Fluorescence Microscopy (LSFM) stands out for its significant advantages [1]. This technique is based on a non-invasive optical sectioning of the sample through a light sheet. The signal coming from the illuminated plane is orthogonally collected by a microscope objective and focused on the acquisition camera. The subsequent sample translation permits to collect a stack of images that contains the information of the whole sample, allowing a subsequent 3D reconstruction. This approach allows the acquisition of high signal to noise ratio images, fast sample scanning and low photobleaching. A major drawback is the limited throughput that prevents its use for large sample populations. To overcome this limitation, a possible solution consists in performing automatic sample delivery through microfluidics channels [2-4]. Nevertheless, this requires a precise and stable alignment between the components, and it often introduces aberrations in the imaging process. In this work we present integrated microscopes on chip that perform an automatic sample delivery and illumination, combining both optical and fluidic integrated components. These devices permit to automatically investigate all the samples diluted in a liquid suspension with no need of any manual sample alignment. The fabrication technique used is femtosecond laser micromachining [5]. This is a powerful and enabling technology that permits a straightforward fabrication of both optical and fluidic components in the same substrate depending on the irradiation parameters used. Advantaging of the versatility of the fabrication technique we present different microscopes on chip that address the specificity of the different sample under investigation, such as single cells and *Drosophila* embryos. These two samples present distinct dimensions with a difference of about an order of magnitude, as single cells have a diameter of about 15-20 μm, while the embryos are ellipsoid 200 x 500 μm^2. Therefore, the light sheet dimensions must be properly optimized to achieve a uniform sample illumination without affecting the sectioning capabilities. This was particularly demanding for single cells, where a light sheet waist of about 1 μm is required and the impact of spherical aberrations can be detrimental on the minimum waist achievable value. Lens profile optimization has been necessary to achieve our target. On the other side, larger samples introduce different kind of problematics. The light scattering induced by the sample itself is not negligible anymore, affecting the quality of the light sheet and of the acquired images. To overcome the impact of this problematic we have performed both optical and fluidic optimization, designing a microscope on chip capable to perform both dual side illumination and automatic sample rotation. 3D images of the acquired specimens will be presented.

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Optical Methods for Inspection, Characterization, and Imaging of Biomaterials V, edited by Pietro Ferraro, Simonetta Grilli, Monika Ritsch-Marte, Christoph K. Hitzenberger, Proc. of SPIE Vol. 11786, 117861Q · © 2021 SPIE · CCC code: 0277-786X/21/$21 · doi: 10.1117/12.2592982

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2. MATERIALS AND METHODS

2.1. Device fabrication

The fabrication technique used is femtosecond laser micromachining (FLM), which allows the fabrication in glass substrates of both microfluidic and integrated optical elements. Indeed, using the correct irradiation parameters it is possible to induce a localized increase of refractive index with respect to the pristine material, which leads to the fabrication of integrated optical waveguides. On the other side, using different irradiation parameters it is possible to obtain an increased etching selectivity of the irradiated substrate with respect to the unirradiated one. The subsequent exposure of the substrate to an aqueous solution of hydrofluoric acid allows microchannel formation [6-8].
We have used two different laser sources for the two prototypes. Regarding the microscope on a chip for *Drosophila* embryos imaging the laser source was a commercial femtosecond laser system (Pharos, LIGHT CONVERSION), with an emission wavelength of 1028 nm and 1 MHz repetition rate. The sample was mounted on a three-dimensional translation stage (FIBERglide3D, Aerotech, Pittsburgh, PA, USA). The laser beam was focused in a 3 mm thick fused silica substrate with a 50x, 0.65 NA microscope objective with correction ring (LCPLN 50XIR, Olympus). We used different fabrication parameters for the microfluidic channels and the optical waveguides. Optical waveguides were irradiated 750 µm deep from the substrate surface with a sample translation speed and a pulse energy of 0.5 mm/s and 50 nJ, respectively and a repetition rate of 50 kHz. A multi-scan approach has been implemented, irradiating 7 parallel lines with a shift of 0.4 µm. This permitted to obtain a square cross section of the modified region with final dimensions of 3.5 µm x 3 µm and a uniform refractive index modification. Waveguides were characterized using both blue and green light. We have estimated propagation losses equal to 0.26 dB/cm and a mode diameter equal to 6 x 6.5 μm^2 at 561 nm. Whereas, we have estimated 0.08 dB/cm and a mode diameter of 4.2 x 4 μm^2 at 488 nm. Regarding the microfluidic channel fabrication, we have used 2 mm/s as translation speed and 760 nJ as laser pulse energy, while the repetition rate was set to 500 kHz. We have irradiated several parallel lines to define the microchannel profile with a line-to-line separation of 5 µm for vertical planes and of 1 µm for horizontal surfaces. The microchannel spans through the whole height of the fabricated device (3 mm) and has a maximum cross section of 965 x 500 μm^2. Due to the large dimensions of the microchannel, we also irradiated multiple lines inside the volume of the structure (with a separation of 5 µm) to facilitate the chemical etching process. After the irradiation step, the glass substrates were exposed to an aqueous solution of hydrofluoric acid (HF at 20%) in a sonic bath at 35°C. The irradiation and the etching processes lasted approximately 10 and 7 hours, respectively. Subsequently, the substrates were polished to optical quality and the waveguides were fiber pigtailed.
Regarding single cell imaging we have used a different commercial femtosecond laser system (femtoREGEN, HighQLaser, Rankweil, Austria), with an emission wavelength of 1040 nm and 1 MHz repetition rate. The second harmonic of this laser is focused through a 50x, 0.6 NA microscope objective in the fused silica substrate. In this case the energy per pulse and the sample translation speed were set to 300 nJ and to 1 mm/s, respectively.

2.2 Sample preparation

Drosophila flies were maintained in dedicated incubators at 25°C. Embryos collection was performed using air-permeable cages with removable bottom plates for egg collection. Due to the continuous egg deposition, plates were changed every day, and embryos were collected and processed within 24 hours from deposition. Collected embryos were subsequently fixed for 20 minutes using a solution of 4% Paraformaldehyde (PFA) in PEM (0.1M PIPES, 1mM MgCl2, 1mM EGTA, pH 6.9). In this work, we have processed two different embryos populations encoding fluorescent proteins. The first one expressing cytoplasmic GFP ubiquitously (Act5C-GAL4, UAS-GFP courtesy of D. Grifoni, Università di Bologna) and the second expressing RFP in nuclei of all cells (w-; His2av-mRFP1; BDSC stock 23651). During device validation experiments, the embryos were diluted in a liquid suspension of water and 0.1% agar, to prevent them from freely sinking in the liquid solution: they can thus be approximated as neutrally buoyant.

As cellular line, we have used a hTERT-immortalized human mammary epithelial cells (WT IMEC). These cells are transduced with PGK-H2B mCherry lentiviral vector and immunolabeled with Alexa488 targeted to alpha-tubulin. For the immunofluorescence procedure, 1 million cells were fixed in 4%PFA for 10 minutes and then were processed in suspension as follow: permeabilization and blocking with PBS/1% BSA/5% goat serum/0.5% Triton X-100 (blocking solution-BS) for 1 hour at room temperature- RT, incubation with primary antibody diluted in the BS for 2 hours at RT, 3 washes in PBS, incubation with secondary antibodies (diluted in the BS) and other 3 washes in PBS. The sample concentration was set to 1 million cell/ml in PBS buffer solution.

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2.3 LSFM setup

Our devices can be mounted in standard fluorescence microscopes, allowing the acquisition of 3D images. In this work we have used a custom-made inverted microscope, as in Figure 1. The collection objective used depends on the specimen under investigation. We have used a 20x, 0.45NA microscope objective with a correction ring for up to 2 mm of glass (CFI S Plan Fluor ELWD 20X, Nikon) for *Drosophila* imaging, while we have used a 60x, 1.1 NA water immersion objective (Olympus LUMFLN60XW) with correction ring. The fluorescence signal collected by the objective was filtered with a dual band filter (Semrock FF01-512/630-25) and focused through a tube lens (Olympus U-TV1XC) on a high speed CMOS camera (Hamamatsu Orca Flash 4.0 V3), which acquires images with maximum frame rate of 400 Hz. We used two Coherent OBIS lasers with 561 nm and 488 nm emission wavelengths as excitation light sources.

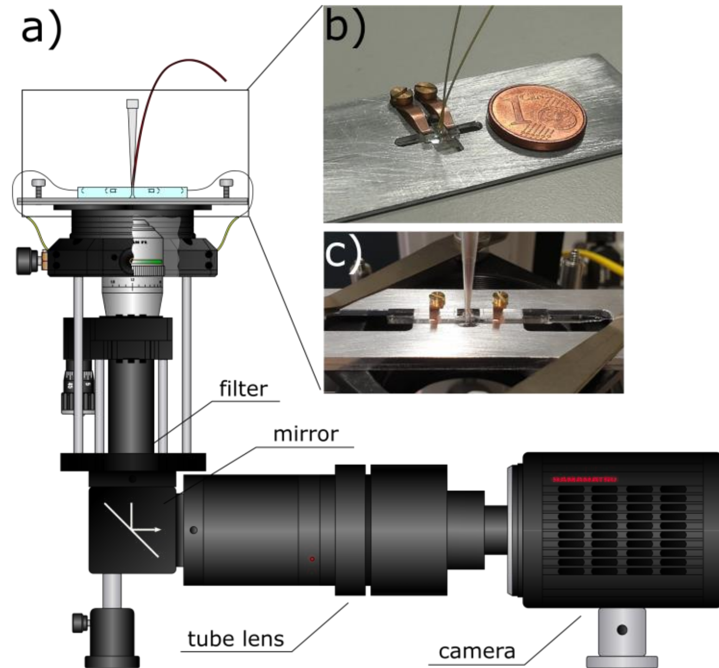

Figure 1. a) schematic of the custm-made invesrted microscope. b) and c) pictures of the microscope on chip for single cell and *Drosophila* embryos imaging, respectively

3. RESULTS & DISCUSSION

3.1 Microscope on a chip for single cell imaging

The objective of this activity is the realization of a microscope on chip for automated imaging of single cells, that in addition allows dual color fluorescence excitation. Considering that cells have an average size of about 15 µm, this implicates that a thin light sheet should be used to have a sufficiently good optical sectioning. We have set as targets, to obtain a waist of about 0.8 µm and to have the focused beam centered in the middle of the sample channel, for both green and blue light. In this regard, we have performed the optimization of the integrated lens profile through simulations in OpticStudio (Zemax). This has allowed us to retrieve an aspherical lens profile, that permits to target the desired beam waists, with a focal shift of only 3.1 µm between the two light-sheets generated at different wavelength, which is negligible if compared to the detection channel size (about 50 µm). To obtain integrated lenses that present the optimized profile after the etching process, we have irradiated a profile that compensate for the non-perfect acid selectivity (Figure 2.a). To retrieve the optical properties of the fabricated lenses, we have collected and analyzed the fluorescence signal from the sample channel (Figure 2.b and c). We have measured a beam waist of about 1.05 µm, which still guarantees a good optical sectioning for single cells. The discrepancy between the theoretical and experimental value could be due to the residual surface roughness of the lens that affect the optical properties of the lens.

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We have performed dual color imaging of single cells, by alternating the two excitation sources. To synchronize the acquisition with the excitation, both the lasers and the camera were triggered with two external signals generated by a DAQ board (National Instrument NI USB-6212) combined with a custom logical port. Results are illustrated in Figure 2.d, were the MIP of a dual color cell is illustrated. To guarantee the acquisition of a sufficient number of frame the cells are acquired flowing in the microchannel at 15 µm/s. To further increase the throughput, we have successfully processed cells also at 150 µm/s. An automated image analysis protocol has been developed to recognize the cell presence and to record the frames above a predefine threshold.

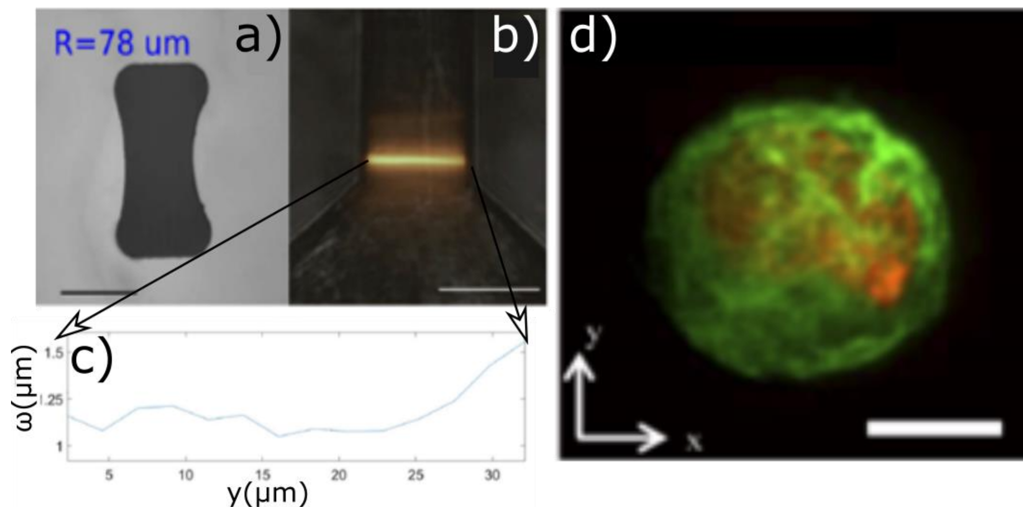

Figure 2. a) Microscope image of the integrated lens, with aspherical profile. b) and c) microscope image of the fluorescence excitation in the sample channel and corresponding analysis. Scale bars are 50 µm. d) MIP of a single cell flown in the device. Scale bar is 5 µm.

3.2 Microscope on a chip for *Drosophila* embryos imaging

Drosophila Melanogaster is widely used by biologist, as it constitutes a valuable animal model in biomedical research. The embryos are thick and elliptical in shape being 500 x 200 μm^2. These dimensions introduce several problematics during the imaging. i) the specimens scatter light and this affects the light sheet quality, degrading the optical sectioning capabilities. ii) The images are acquired from the collection objectives positioned below the chip, through the embryos themselves. Thus, at the beginning of the sample sectioning the images are neat. Then, they become more and more blurred during the acquisition, as the images are acquired deep into the specimens. This is particularly accentuated when embryos are oriented vertically (with the major axis parallel to the flow direction). To reduce these problematics, we have performed both an optical and a fluidic device optimization. Indeed, we have introduced a dual side illumination to guarantee a more uniform sample illumination as well as a passive approach for an automatic sample rotation in order to have them aligned horizontally when flowing through the light sheet.

To perform a dual side illumination, it is important to guarantee a precise alignment between the two-light sheets, otherwise the two planes do not overlap in the sample channel. This might increase the effective light sheets thickness and the device optical sectioning capabilities. Thus, we have decided to take advantage of the versality of the fabrication technique, by fabricating during the irradiation step two waveguides that face the sample channel from the two opposite sides. This approach guarantees a precise and stable alignment between the integrated components. We have first characterized the properties of a light sheet obtained focusing the light coming from a single waveguide with an integrated lens. We have measured a beam waist of about 5 and 6 µm, for blue and green light, respectively. Which is in good agreement with previous lens optimization. Subsequently we have investigated the overlapping of the two opposite light sheets, and we have measured a shift 0.7 µm, which is widely acceptable if we consider the thickness of the two light sheets.

Regarding the automatic sample rotation, we have optimized the layout of the microfluidic channel as well as the flow rate value by simulations with COMSOL Multiphysics. We have introduced an expansion chamber asymmetrically located with respect to the microfluidic channel and we have retrieved the correct dimensions of the device that allows

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the embryos to be correctly rotated when flowing through the light sheet. We have validated our simulations experimentally, and we have measured a rotation efficiency of 85%.

We have subsequently fabricated the microscope on chip, combining the previously described optical and fluidic optimization. We have validated this chip using two different populations of embryos. Images are reported in Figure 3, where it is possible to observe both the uniform illumination as well as the optical sectioning capabilities, as we can detect volumetric information from the specimens.

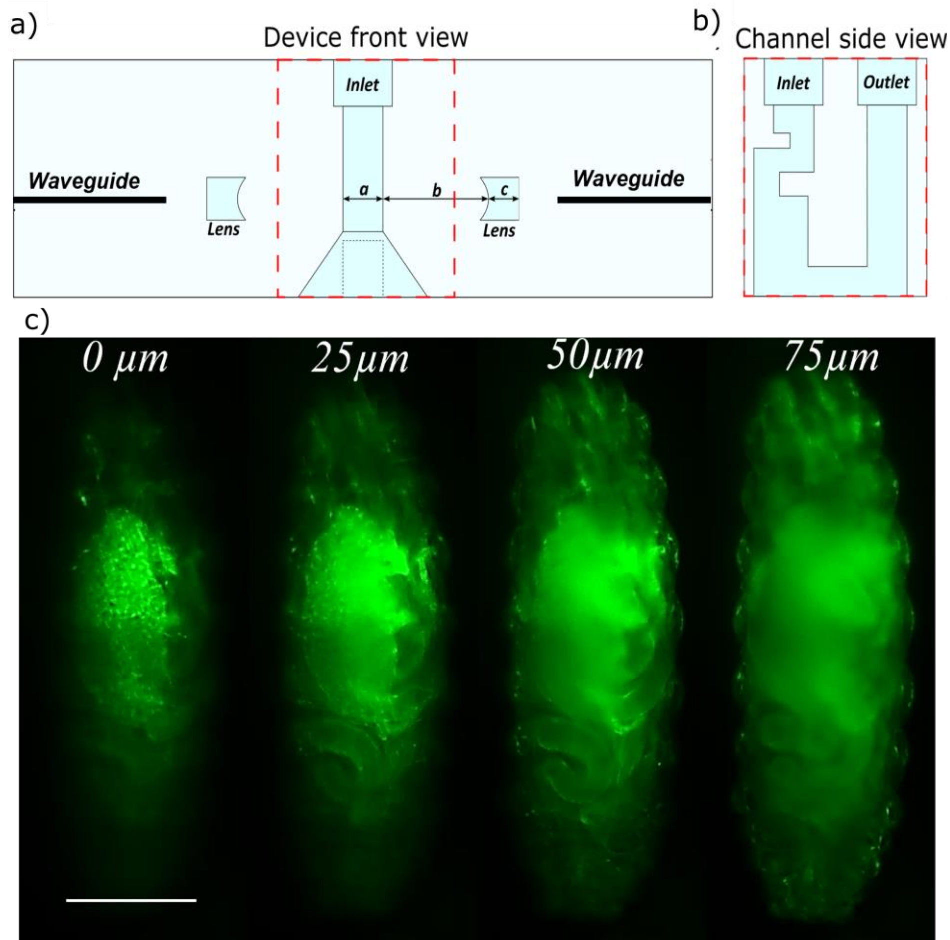

Figure 3. a) and b) front and side views, respectively of the microscope on chip for Drosophila embryos imaging. c) frame acquired at different depths into the specimens. Scale bar is 200 μm.

4. CONCLUSIONS

In this work we have presented two prototypes of microfluidic-based microscopes on a chip, capable to address the specific requirement for the investigation of samples characterized by different sizes and shapes. These devices allow obtaining three-dimensional images and dual color analysis. In addition, the measurements are entirely automated, and all the specimens diluted in a liquid suspension can be processed with no need of manual alignment.

ACKNOWLEDGEMENTS

This work was supported by PROCHIP, an H2020-EU -FET Open project (Grant agreement ID: 801336).

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