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Morpho-molecular characterization of a tattooed skin biopsy using co-localized line-field confocal optical coherence tomography (LC-OCT) and confocal Raman microspectroscopy

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ABSTRACT

Line-field confocal optical coherence tomography (LC-OCT) is an optical technique generating three-dimensional (3D) images of the skin at cellular resolution. Confocal Raman microspectroscopy (CRM) is an optical modality that provides a point-wise molecular fingerprint of samples. We have developed a method to co-localize data acquired by separate LC-OCT and CRM systems. LC-OCT allows for recording 3D morphological overview images in which points of interest (POIs) can be localized for molecular analysis using CRM. A biopsy of skin with a red-colored tattoo with unknown ink composition was analyzed using co-localized LC-OCT and CRM. After acquisition of a 3D LC-OCT image, specific POIs were targeted inside the biopsy, based on their morphological features: POIs located in the epidermis where no ink was expected to be found as well as POIs located in bright areas of the dermis, down to 275 μm in depth, likely indicating the presence of tattoo ink. Analysis of the spectra at these specific POIs confirmed the absence of tattoo ink in the epidermis and its presence in the bright areas in the dermis. A stronger molecular signal of ink in the brightest areas of the dermis identified with LC-OCT was also revealed, suggesting a higher amount of ink. The combination and co-localization of LC-OCT and CRM brings a new level of characterization of the skin, enabling molecular analysis of POIs based on their morphological aspect at cellular-resolution within a volume, which could be of great interest in dermatology and dermo-cosmetics.

Keywords: line-field confocal optical coherence tomography, optical coherence tomography, confocal Raman microspectroscopy, Raman spectroscopy, dermatology, tattoo

1. INTRODUCTION

Tattoos are obtained by intradermal injection of tattoo inks. Tattoo ink contains pigments that give the ink its color and that are dispersed in a solution usually composed of water, alcohol and glycerin. Pigments can be made of mineral, metal oxides or synthetic molecules,¹ and can cause multiple cutaneous reactions such as allergic reactions and non-allergic inflammatory reactions.^{2,3} Residual metal particles including heavy metals were also identified in several inks as potential allergens and possibly toxic elements for human health.^{1,4}

Line-field confocal optical coherence tomography (LC-OCT) is a non-invasive optical technique for imaging the skin *in vivo* at high resolution ($\sim 1 \mu\text{m}$).⁵⁻⁸ LC-OCT provides two-dimensional (2D) and three-dimensional (3D) images of skin tissues with a penetration depth down to 500 μm . LC-OCT has been used for examination and diagnosis of various skin pathologies, including skin cancers.⁹⁻¹¹ LC-OCT was also applied to characterize a case of tattoo reaction.¹² The examined patient had experienced a delayed reaction from red dye of a multicolored tattoo and was examined using dermoscopy, LC-OCT, high-frequency ultrasound (HFUS) and histology. Markers of inflammation were highlighted by LC-OCT and HFUS images and presence of B and T lymphocytes were

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pointed out by immunohistochemical examination of a biopsy taken within the red dye area of the tattoo. In the case of tattoo reactions, it would be interesting to obtain information on the chemical composition of the tattoo ink used in order to identify its potential components causing the reaction.

Confocal Raman microspectroscopy (CRM) is an optical modality that provides point-wise measurements of the chemical content of a sample by probing its molecular vibrations. CRM has been applied to skin analysis for multiple applications: skin cancer diagnosis,^{13,14} skin cancer research,¹⁵⁻¹⁷ dermo-cosmetic applications.¹⁸⁻²⁰ CRM has also been applied to the characterization²¹ and identification of tattoo pigments causing skin reactions³ and has been used as a tool for ink quality control.^{22,23} CRM would therefore allow to enrich the characterization of the tattoo reaction with molecular information at cellular level.

In this paper, we report on a combination of LC-OCT and CRM for *ex vivo* applications, using co-localized acquisitions obtained from two separate systems. The technique allows acquisition of Raman spectra at specific locations within a sample identified in a 3D LC-OCT image, based on their microscopic morphological features. We applied the co-localization method to the characterization of a red-colored tattoo biopsy with previously reported adverse reaction.¹²

2. LC-OCT/CRM CO-LOCALIZATION SET-UP

The LC-OCT system is a custom system developed for *ex vivo* applications. Its architecture is similar to the LC-OCT system used for *in vivo* skin imaging.⁶⁻⁸ In the *ex vivo* prototype, the sample is introduced into a sample holder placed under the microscope objective of the LC-OCT sample arm. The top of the sample holder is covered with a Raman-grade CaF₂ slide. The sample is gently pressed against the CaF₂ slide using a second glass slide underneath that is moved upward with a retaining ring. This allows the sample to be held still for the duration of the experiments. The use of a Raman-grade CaF₂ slide, which shows a single peak at 521 cm⁻¹ in its Raman spectrum, limits background signal added to the Raman spectrum of the skin. A very thin layer of paraffin oil is added on top of the skin sample to minimize reflections at the sample/CaF₂ slide interface.

Raman spectra are acquired using a home-built CRM system. For Raman excitation, a 785 nm continuous-wave laser is focused into the sample using a 40x 0.8-NA water-immersion microscope objective. The Raman signal is collected by the same objective and guided by an optical fiber to a spectrometer with spectral resolution of 11 cm⁻¹. The volume probed by the CRM system is $\sim 1 \times 1 \times 30 \mu\text{m}$ ($x \times y \times z$), which allows both targeting of cell-scale structures in a 3D LC-OCT image and acquisition of the Raman spectrum in reasonable times. The integration time for the CRM measurements was set to 10s with a 6-fold accumulation and the laser power sent on the sample was set to 50 mW. Raman spectra were subtracted from background autofluorescence by fitting a fifth order polynomial function on the acquired spectra.

To perform co-localization experiments, identical XYZ translation stages are placed both under the LC-OCT sample arm microscope objective and the CRM objective. The sample holder is successively placed on the LC-OCT stage and on the CRM stage. Points of interest (POIs) in the sample are identified from 3D LC-OCT images. A calibration of the XYZ coordinates of the stages under each system, performed prior to the experiments, allows to target these same POIs using the CRM system and acquire their Raman spectra.

3. APPLICATION TO TATTOOED SKIN BIOPSY WITH ADVERSE REACTION

We applied the co-localization method to the characterization of a biopsy taken from the red-stained area of a multicolored tattoo already studied by other techniques.¹² 3D images of the biopsy were acquired (see Fig 1). LC-OCT provides images of the superficial layers of the skin, including the epidermis and the superficial dermis, in which diffuse bright areas can be visualized. At the dermal-epidermal junction, hyper-reflective structures with irregular shape can be noticed, as shown by white arrows on the LC-OCT horizontal image. Five POIs to be analyzed with CRM were chosen at different locations within the biopsy: POI 1 was chosen in the epidermis. POIs 2, 4 and 5 were chosen in the dermis at 275 μm , 180 and 250 μm depth respectively, with POI 4 being located in a region appearing darker in the image than POIs 2 and 5. POI 3 targets a hyper-reflective structure near the dermal-epidermal junction (150 μm depth). Raman spectra of the five POIs were acquired, using calibrated XYZ stages under both devices. As expected, since ink is usually injected in the dermis, the spectrum acquired at POI 1 exhibits the Raman signature of the epidermis alone, depicted by blue arrows on the

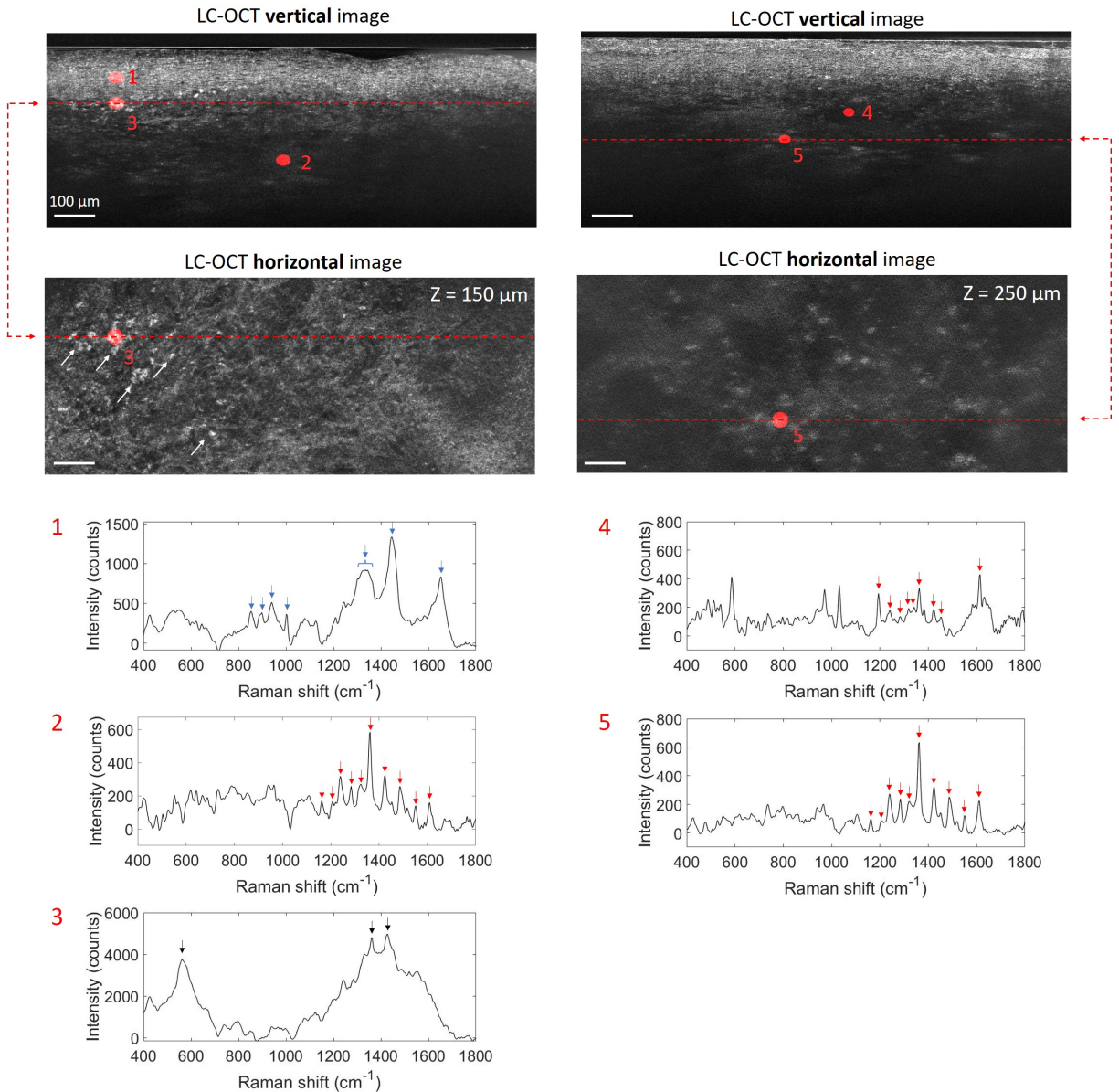


Figure 1. LC-OCT images (vertical and horizontal views obtained from 3D images) of a human biopsy taken within the red dye area of a multicolored tattoo, with associated Raman spectra obtained from CRM for five POIs identified in the images and materialized by red ellipsoids. The dotted line on the horizontal image shows the position of the vertical image above regarding the horizontal plane of the 3D image, and vice versa. The depth Z of the horizontal image is indicated with respect to the skin surface. Blue arrows on Raman spectra depict Raman bands corresponding to the epidermis and red arrows show Raman peaks assigned to red ink. Black arrows represent the main Raman bands associated to bright particles observed in the superficial dermis and pointed out by white arrows on the horizontal image.

spectrum. The spectrum acquired at POI 2, located in a diffuse bright region of the dermis, shows the presence of red tattoo ink, with well-defined peaks depicted by red arrows. According to previous work, this ink shows the molecular signature of an azo-type red pigment,¹ a type of pigment that has previously been reported to be implicated in adverse tattoo reactions.^{24,25} POIs 4 and 5 also show the Raman spectrum associated with the tattoo ink (red arrows). The higher intensity of the Raman peaks associated to the red pigment in the spectrum of POIs 2 and 5, located in brighter regions of the dermis compared to POI 4, suggests that the amount of ink

is higher in the brighter areas of the dermis. The spectrum of POI 3 exhibits a broad and intense peak at 567 cm^{-1} as well as a large band centered at 1400 cm^{-1} with two sharp peaks superimposed at 1360 and 1422 cm^{-1} (black arrows), which are also present in the spectrum of the tattoo ink. Multiple hyperreflective particles were targeted (pointed out by the white arrows in Fig 1), and always showed this same spectrum shape. According to previous work, morphological and molecular information both suggest that the targeted bright particles could be potential inflammatory cells, and more particularly macrophages. Macrophages are white blood cells involved in the destruction of harmful organisms. They were previously observed within tattooed skin²⁶ and are able to engulf tattoo pigments. Studies using RCM showed that macrophages appear as very bright particles in the upper dermis, with irregular or spindle shapes,^{26,27} findings which are consistent with the morphological features of these particles in LC-OCT images. Moreover, the Raman spectrum obtained at POI 3 matches well the Raman spectrum of macrophages reported by Tetard *et al.*²⁸ which shows a broad band centered at 1400 cm^{-1} , and a peak centered at 567 cm^{-1} . These results are in agreement with findings obtained from immunohistochemistry, reporting the presence of lymphocytes, which are also inflammatory cells.

4. CONCLUSION

A biopsy of a red-colored tattoo with adverse tattoo reaction was characterized using co-localized LC-OCT and CRM acquisitions. The combination of morphological and molecular information revealed the presence of red tattoo ink in diffuse hyperreflective areas of the dermis, with higher abundance in brighter regions of the dermis. The LC-OCT-CRM co-localization provided new information on the type of ink pigment used, complementing previously reported examinations of the biopsy.¹² LC-OCT identified highly reflective structures in the biopsy, which CRM identified as potential inflammatory cells, in a manner similar to immunohistochemistry but without requiring specific preparation of the biopsy. The morpho-molecular characterization provided by the combination of LC-OCT and CRM could allow better management of tattoo reactions, and contribute to warn against the use of certain pigments in tattoos. LC-OCT/CRM co-localization could also be of great interest in the field of onco-dermatology for the characterization of skin cancers, both for diagnostic purposes and clinical research.

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