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Co-localized line-field confocal optical coherence tomography (LC-OCT) and confocal Raman microspectroscopy for *ex vivo* analysis of skin tissues

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ABSTRACT

Line-field confocal optical coherence tomography (LC-OCT) is an optical technology developed for *in vivo* skin imaging. Combining the principles of OCT and reflectance confocal microscopy, LC-OCT generates high-resolution three-dimensional (3D) images with an isotropic spatial resolution of 1.3 micron and up to 500 microns in depth. Confocal Raman microspectroscopy is a label-free optical technique which allows for point measurement of the molecular content of a sample with micrometer resolution. While LC-OCT provides morphological information, Raman spectroscopy brings chemical information but lacks image guidance for targeting specific points of interest in the sample. Combining the two modalities would therefore provide complementary information and guidance for Raman measurements. We present a method to co-localize LC-OCT and Raman acquisitions for *ex vivo* applications. This co-localization approach allows acquisition of Raman spectra at specific locations targeted in a 3D LC-OCT image, with an accuracy of $\pm 20 \mu\text{m}$. The co-localization method was developed using a LC-OCT device designed for *ex vivo* imaging and a custom Raman system. The principle of co-localization relies on both the use of a specific sample holder that can be positioned under each device with high repeatability and of a coordinate-based calibration between the two devices. Co-localization was validated using pig skin samples containing tattoo ink of known composition. LC-OCT images allowed to target specific regions in the samples where the presence of tattoo ink was revealed by detection of a specific Raman signature.

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

1. INTRODUCTION

LC-OCT is an optical technique applied to *in vivo* skin imaging, which combines the principles of two optical modalities, optical coherence tomography (OCT) and reflectance confocal microscopy (RCM) using illumination of the skin with a line of light and detection using a line camera. LC-OCT is capable of generating both two-dimensional (2D) horizontal images ($x \times y$) and 2D vertical images ($x \times z$), as well as three-dimensional (3D) images with an isotropic resolution of $\sim 1 \mu\text{m}$.¹⁻⁴ LC-OCT images provide morphological information arising from variations of the refractive index within the skin. LC-OCT has been used for examination and diagnosis of various skin pathologies, from tattoo adverse reaction⁵ to skin cancers.⁶⁻⁸

Confocal Raman microspectroscopy (CRM) is a label-free technique which provides information on the molecular content of the analyzed sample, often referred to as the sample molecular fingerprint.⁹ By rejecting light scattered from out-of-focus regions, CRM provides point-wise biochemical information. CRM is used in dermatology for skin cancer screening,¹⁰ surgery¹¹ and research.¹² It is also a tool of great interest in dermocosmetics, for studying effects of cosmetic formulations on the skin¹³ and penetration of cosmetic actives into the skin.¹⁴ CRM was also applied to tattoo characterization¹⁵ where it is capable of providing tattoo ink composition.

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However, CRM lacks image guidance for targeting specific points of interest (POIs) in the sample, which can make CRM acquisitions time-consuming and fastidious.

The combination of LC-OCT and CRM would therefore provide molecular information complementary to the morphological information contained in the LC-OCT images, and guide CRM acquisitions. Since both modalities have a high spatial resolution, the coupling of LC-OCT and CRM would allow correlation of morphological and molecular information at the cellular level. In this paper, we introduce a method to co-localize LC-OCT and CRM acquisitions performed on *ex vivo* samples, using two separate devices. LC-OCT provides 3D morphological overview images in which POIs can be located for molecular analysis using CRM. The principle of co-localization is based on the use of a specific sample holder that is successively positioned under each device with high repeatability and a coordinate-based calibration between the two devices. To demonstrate the value of the developed approach for *ex vivo* skin tissues, we present results of co-localization experiments carried out on a pig skin sample containing tattoo ink of known composition.

2. EXPERIMENTAL SET-UP

The LC-OCT device is a custom system developed for *ex vivo* applications, which is close to the device designed for *in vivo* skin imaging.²⁻⁴ The LC-OCT device is based on a Linnik-type interferometer with a high numerical aperture objective (20x 0.5-NA) in each arm for imaging with a high lateral resolution. It uses a supercontinuum laser as a broadband source, providing a high axial resolution, and a cylindrical lens for focusing light along a line in the sample. 2D vertical imaging is achieved using a piezoelectric stage for depth scan of the line of light. A mirror galvanometer is employed for lateral scan of the line, allowing 2D horizontal imaging.

In the *ex vivo* device, the sample is placed in a sample holder under the microscope objective of the LC-OCT sample arm. The sample holder is shown in Fig 1(a). 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 the background Raman signal superimposed 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. Silicone oil is added on top of the sample holder as an immersion medium. Silicone oil matches the mean optical refractive index of skin (~ 1.4), which ensures that the coherence plane and the focus of the microscope objective of the LC-OCT sample arm remain superimposed during depth scan.¹⁶

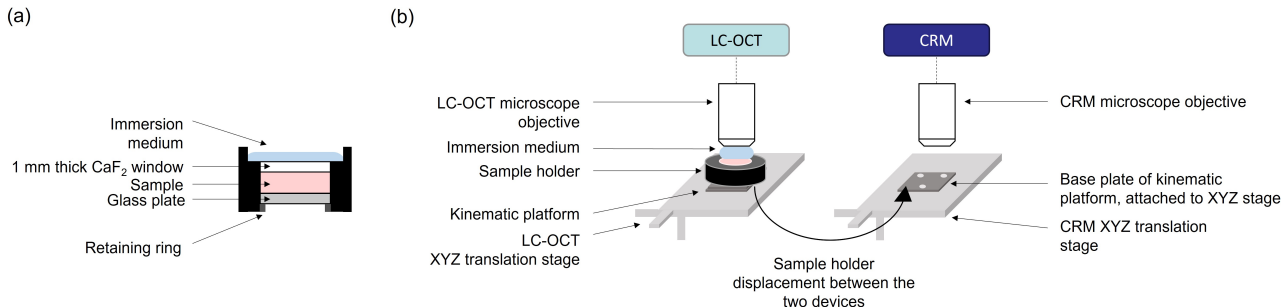


Figure 1. (a) Schematic view of the sample holder dedicated to co-localization experiments. (b) Schematic of the co-localization set-up.

The CRM system is a custom-built set-up using a 785 nm continuous-wave laser as Raman excitation source and a 40x 0.8-NA water-immersion microscope objective to focus the beam on the sample and collect the Raman signal. The Raman signal is isolated from the excitation source using a set of dichroic filters and injected into a 105 μm -core diameter, 0.22-NA, multimode optical fiber connected to a spectrometer equipped with gold-coated mirrors and a back-thinned FFT-CCD detector with spectral resolution of 11 cm⁻¹. Water is used as immersion medium in the CRM device in order to minimize optical aberrations that would distort the volume probed. The

CRM set-up allows to probe volumes of $\sim 1 \times 1 \times 30 \mu\text{m}$ ($x \times y \times z$). Dimensions of the probed volume, determined by the microscope objective and the collection fiber, were chosen as a compromise to collect a sufficient amount of Raman signal while allowing accurate location of the depth probed with the CRM system within an LC-OCT image. The acquisition time for the Raman measurements was set to 10s with a 6-fold accumulation and the laser power at the sample level was 50 mW. Raman spectra were subtracted from background autofluorescence by fitting a fifth order polynomial function on the acquired spectra.

The set-up used for the co-localization experiments is illustrated in Fig 1(b). Identical XYZ translation stages are placed under the LC-OCT microscope objective of the sample arm and under the CRM objective. A calibration is performed prior to co-localization experiments, using a specific calibration sample. During the co-localization experiments, the sample is first imaged with LC-OCT; XYZ translations used to navigate within the sample and locate POIs. Once POIs are identified in the sample, a 3D LC-OCT image is acquired, associated with given XYZ coordinates on the stage. The sample holder is then manually moved on the XYZ stage under the CRM objective. Using the prior calibration of both XYZ stages, the user is able to compute the XYZ coordinates to be reached on the CRM stage to target POIs previously identified in the LC-OCT image, based on the XYZ coordinates associated with the image and the position of the POIs in the 3D LC-OCT image. For each POI, the sample is moved to the computed XYZ coordinates where a Raman spectrum is acquired. This method provides local biochemical information in a 3D LC-OCT image with an accuracy of $\pm 20 \mu\text{m}$. A kinematic platform is used to interface the sample holder with the XYZ translation stages. The kinematic platform ensures that the sample holder is placed on each XYZ stage with high repeatability ($\leq 2 \mu\text{m}$), thus preserving the calibration throughout the co-localization experiments.

3. CHARACTERIZATION OF TATTOOED PIG SKIN SAMPLE

We applied the co-localization method to the characterization of pig skin samples containing tattoo ink. Porcine skin is often used as a human skin model for Raman spectroscopy since its structure and chemical composition are close to the ones of human skin.¹⁷ Fresh pig skin was obtained from a local butcher and cut into $\sim 0.5 \text{ cm}^2$ samples. Yellow tattoo ink with known composition (Sunflower ink, Eternal Ink EUROPE UG, Germany) was inserted in a pig skin sample using a standard needle.

Fig 2 reports on results of co-localization experiments performed on the pig skin sample tattooed with the yellow ink. A 3D LC-OCT image of this sample was acquired, in which POIs were targeted. For better visualization, these POIs are displayed on 2D horizontal and vertical images extracted from the 3D image and are materialized by yellow ellipsoids. The ellipsoids have dimensions of $40 \mu\text{m} \times 40 \mu\text{m} \times 30 \mu\text{m}$ and represent the volume in which the Raman acquisition is included, due to uncertainty of the colocalization procedure and spatial resolution of the CRM system.

As seen in Fig 2, the pig skin sample exhibits a thin epidermis and a superficial dermis composed of numerous and well-defined fibers. In the 3D LC-OCT image of the sample, bright regions can be observed, likely corresponding to tattoo ink. These areas start from the epidermis where the ink was injected with the needle, with hyper-reflective sharp structures. They penetrate into the dermis where they form diffuse bright areas, with intensities close to dermis fibers. Thus, identification of ink-containing regions in depth based on the LC-OCT image alone can be ambiguous; CRM is able to provide biochemical information necessary to differentiate ink from dermis fibers. Three POIs were chosen at different locations within the pig skin sample: POIs 1 and 2 were chosen in bright regions located at different depths ($120 \mu\text{m}$ for POI 1 and $360 \mu\text{m}$ for POI 2). POI 3 was chosen in a region adjacent to POI 1, at $120 \mu\text{m}$ depth, composed of well-defined dermal fibers.

Using the co-localization procedure, Raman spectra were acquired at each POI. In agreement with the LC-OCT image, the spectrum of POI 3 shows Raman bands typical of pig skin at $815, 853, 933, 1001, 1252, 1450$ and 1662 cm^{-1} (highlighted in Fig 2 by blue arrows overlaid on the spectrum).^{15,18} The spectra of POIs 1 and 2 show sharp and intense Raman peaks at $1252, 1287, 1331, 1397$ and 1594 cm^{-1} . These peaks correspond to the molecular signature of the yellow tattoo ink inserted in the sample, and are more precisely assigned to the yellow pigment C.I.21095 of the ink (depicted by yellow arrows on the spectra).^{15,19} Despite the strong Raman signal of the pigment, a few bands corresponding to the dermis around $1001, 1450$ and 1662 cm^{-1} can still be observed in the spectra of POIs 1 and 2 (blue arrows) since POIs 1 and 2 indeed also include dermal tissue. One

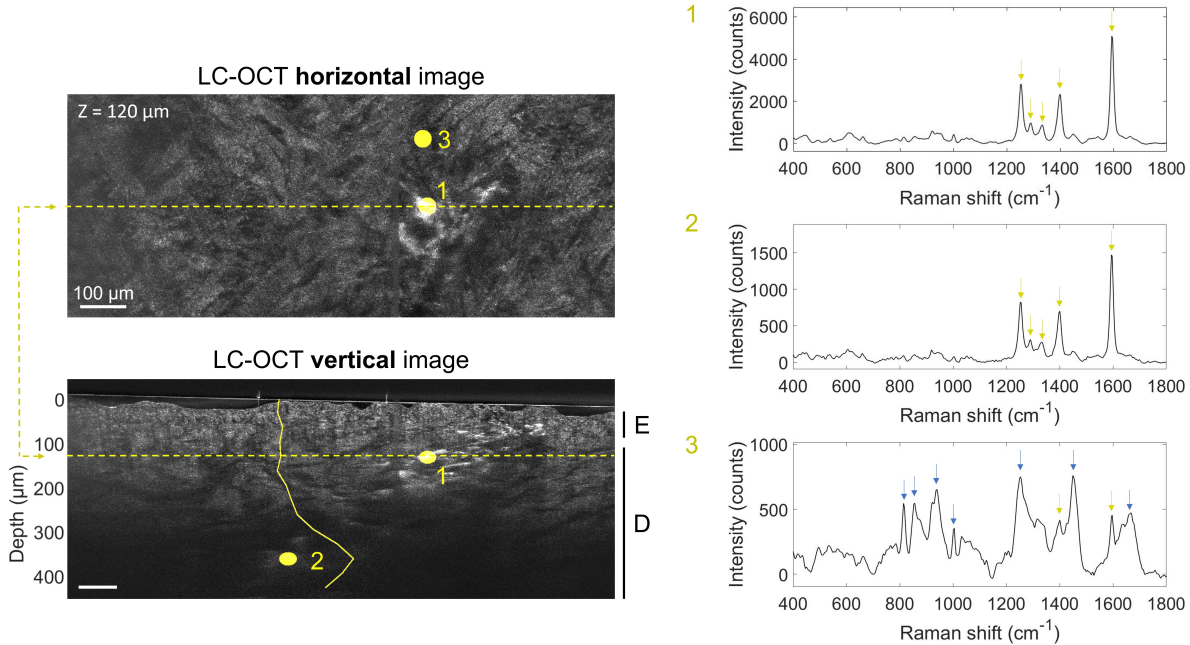


Figure 2. LC-OCT images (orthogonal views) of a pig skin sample tattooed with yellow ink, with associated Raman spectra obtained from CRM for three POIs identified in the 3D LC-OCT image and materialized by ellipsoids. LC-OCT provides images of the two main layers of the pig skin, the epidermis (E) and the dermis (D). 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. The solid curve on the vertical image represents the 1D distribution of yellow ink with depth at the level of POI 2. Blue arrows on Raman spectra depict Raman bands corresponding to pig skin dermis and yellow arrows show Raman peaks assigned to yellow ink.

can also notice that the strongest peaks of the yellow pigment (at 1397 and 1594 cm^{-1}) are slightly visible in the spectrum of POI 3. However their intensity is negligible in comparison with their intensity in POIs 1 and 2, suggesting that a very small amount of ink, which does not appear in the LC-OCT image, might be present within POI 3. This finding could be expected since POIs 1 and 3 are only ~ 150 μm apart. A curve representing the Raman intensity of the yellow pigment along the Z axis through POI 2 is overlaid on the 2D vertical image. This curve is obtained by acquiring Raman spectra from the skin surface down to 420 μm depth with a 30 μm step through POI 2 and computing the intensity of the 1594 cm^{-1} Raman peak associated to yellow ink with depth. The curve shows a maximum at 360 μm depth, in agreement with the depth of the bright zone spotted in the LC-OCT image, which highlights the complementarity of information provided by LC-OCT and CRM.

4. DISCUSSION AND CONCLUSION

In this paper, we introduced a method to perform co-localized acquisitions using separate LC-OCT and CRM devices. The co-localization method allows to acquire the Raman spectra of POIs precisely located in a 3D LC-OCT image, with an accuracy of ± 20 μm . The LC-OCT/CRM co-localization approach provides complementary information at the cellular scale, without requiring any hardware modification of each modality. Co-localization relies on a coordinate-based calibration performed prior to experiments using a dedicated calibration sample, and a specific sample holder that is manually switched from the LC-OCT device to the CRM device.

The potential of co-localization was demonstrated on a sample of tattooed pig skin. Co-localization allowed to locate tattoo ink in bright regions of the dermis, based on the Raman signature of the yellow pigment contained in the ink. Precise identification of the ink pigment used was provided by CRM, while LC-OCT allowed to locate the origin of the Raman signal within the sample. 1D spatial distribution of the tattoo ink down to 400 μm can be obtained using CRM, supported by a morphological image with cellular resolution. In addition, CRM

removed potential ambiguities between regions containing ink and regions comprising only dermal fibers observed in depth with LC-OCT, since these regions have similar appearance and intensities in the 3D LC-OCT image.

Application to sample of tattooed pig skin illustrates the complementarity of the two modalities, each bringing benefit to the other, and shows the value of our co-localization method for morpho-molecular characterization of *ex vivo* skin samples. The co-localization method could thus be applied to the characterization of tattoo biopsies collected for example in the case of adverse tattoo reactions. The combination of morphological and biochemical information would allow a more complete characterization of the reaction and potentially provide a better understanding of the underlying reaction mechanisms. Multiple other applications could be investigated in dermatology for the characterization of pathological lesions, including suspected skin cancers.

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