Optical spectroscopy as an effective tool for skin cancer features analysis: applicability investigation

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ABSTRACT

Skin carcinoma is one of the most frequent and spreaded type of skin cancers. Its diagnosis and the resulting surgical treatment are complicated due to the lack of precise surgical margin delineation approaches. Optical methods are very promising tools for effective solution of this problem because of their non-invasiveness and high informativity. Spatially resolved multi-modal spectroscopy (SRMMS) provides *in vivo* information with depth resolution about cancer specific features with high precision by analyzing skin diffuse reflectance (DR) and autofluorescence (AF) spectra at specific locations on tissue. Due to multiple light scattering and absorption of the photons in the biological tissue it results in very poor photons penetration into the deeper areas and, consecutively, low depth sensitivity. Potential solution of this problem is optical clearing technology. In this work, we have analyzed the time kinetics of DR and multiply excited AF spectra collected from top of *ex vivo* skin/gel hybrid model following topical application of OCA at 4 different acquisition distances and 5 different excitation wavelengths. We have also investigated the possible impact of probe pressure as well as drying of the skin sample on the spectroscopic signals, besides the optical clearing effect. The results obtained have showed that the studied OCA solutions have reduced autofluorescence intensity of the skin and improved the depth sensitivity of the spectroscopy applied to the skin. Another notable effect is the strong increasing in collected exogenous fluorescence of a bottom layer in a "dry" condition (absence of immersion).

Keywords: reflectance, multi-modality, skin carcinoma, fluorescence, optical clearing, optical clearing agent, spatial resolution

1. INTRODUCTION

From more than two decades now, the number of applications of optical methods in medicine for *in vivo* diagnosis, therapy and surgery-guidance has constantly increased. They are represented by numerous approaches such as optical coherence tomography, diffuse optical imaging, multispectral imaging, confocal microscopy, diffuse reflectance, autofluorescence or Raman spectroscopy, imaging techniques, etc...[1,2] All these methods are based on a non-invasive approach. They exploit the light propagation through the biological tissue, to extract morphological and geometrical parameters of the tissue under investigation, from the detected light. This contribution can be focused on skin tissue in the frame of non-invasive in vivo optical characterization for improvement of the skin carcinoma peroperative surgical guidance[3,4].

Most carcinomas arise from skin sites chronically exposed to sun actinic (i.e. ultraviolet) radiation during the patient's lifetime. Ninety percent (90 %) of patients are diagnosed with skin carcinomas when they are 40 years of age or older. During carcinogenesis metabolic and morphological changes in skin tissue take place. These changes also affect the skin intracellular and extracellular components which are the main fluorophores that contribute to the skin autofluorescence signal. For instance, collagen fluorescence is decreased and NAD(P)H and porphyrin fluorescence are increased. The resulting autofluorescence of skin affected by carcinogenesis is different from that of healthy skin[5]. Thus, in order to improve the accuracy of skin cancer diagnosis multiple excitation light induced AF spectroscopy with several

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wavelengths can be applied to separately obtain different AF spectra corresponding to the spectral regions of the various main skin fluorophores.

Spatially resolved multi-modal spectroscopy (SRMMS) is an optical biopsy technique combining spatially resolved DR and AF intensity spectra measurements. Spatial resolution is achieved by using multi-fiber optical probe with several source-to-detector separations (SDS) – several measurement distances from excitation fiber to the detection fibers, in order to acquire data from the different depths[6]. Combination of a multiple excitation wavelengths with the depth resolution could provide comprehensive information about carcinogenesis process.

However, because the transport of photons into biological tissues of interest is weakened due to losses caused by multiple light scattering and absorption, the depth resolution and photon penetration depth of optical methods still need to be improved. It has been shown by a lot of studies[7-15], that the optical properties of biological tissues can be controlled using special biocompatible hyperosmotic agents or optical clearing agents (OCAs). The latter might penetrate inside a tissue, interact with and modify its natural high-scattering formation through different ways[16-19]. Three main mechanisms of the optical clearing (OC) technique are suggested: tissue dehydration, refractive index matching, and reversible dissociation of collagen fibers. But for non-invasive topical application of such agents their penetration through the skin is weakened due to the epidermal layer features that prevent penetration in and out of liquids, thus acting like a natural barrier. Consequently, special biocompatible chemicals that may overcome such barrier have been proposed as enhancers in combination with OCAs to improve its efficiency[11-15,20,21].

The main features of carcinogenesis are the thickening of epidermal layer and keratinocytes misorientation. These features can be assessed without OC (no scattering reduction). But other features, such as the dermal modifications (fibrosis, elastosis) are more difficult to assess because of the relative in-depth location of these areas of interest. Moreover, one of the main limitations of optical spectroscopy in general, and SRMMS in particular, is the photons absorption by melanin[22] i.e. patients' phototype. Aforementioned OCA may improve photon penetration depth and therefore may help spectroscopy to probe deeper skin layers to obtain a complete diagnosis tool.

The aim of this work was to study the modifications in depth resolution of the proposed SRMMS method by combining it with an optical clearing approach and to estimate the applicability of this method for future clinical use.

2. MATERIALS AND METHODS

2.1 Spatially Resolved Multi-Modal Spectroscopic acquisition system

The SRMMS device ("SpectroLive"), used to acquire spatially resolved DR and multiply excited AF spectra on skin, includes a multiple optical fiber probe made of one central 600 µm diameter excitation fiber (numerical aperture NA = 0.22), surrounded by the 4 rings of 6 collecting fibers (200 µm core diameter, NA = 0.22) each (located at SDS D1=400 µm, D2=600 µm, D3=800 µm, and D4=1000 µm). The latter probe, in contact with the skin epidermis surface (Figure 1) allows for collecting spectroscopic data with the separation by depth[6].

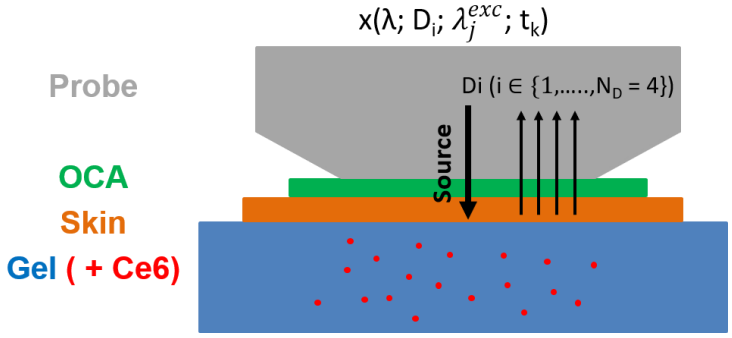


Figure 1. Schematic figure of the experimental model. "SpectroLive" probe head placed in a contact with the OCA applied on the skin site of skin/gel hybrid model. Fluorescence spectra are collected at 4 SDS distances and 5 excitation wavelengths every 4 minutes within 36 minutes starting at T0 = 0 min.

In the AF measurements the central fiber was used for AF excitation. Five bandpass filtered LED light sources (Full Width at Half-Maximum ~ 10 nm) were used to emit 5 excitation wavelengths (λ_{exc}) centered at 365, 385, 395, 405 and 415 nm, consecutively. Different skin fluorophores are therefore targeted, and different AF signals obtained, depending on their locations and absorption wavelength bands. For DR measurements, the central fiber was used as a white light source and corresponding DR spectra are registered by the 4 rings of collecting fibers.

2.2 *Ex vivo* skin-based hybrid experimental model and OC protocol

For experimental study, a hybrid *ex vivo* model was used. It was made of 2 layers, where the top layer was a human skin strip sample (with an average thickness of 901±282 µm) and the bottom layer was a fluorescing and scattering gel-based layer made of agarose (1% weight/vol), intralipids-20% (5% vol/vol), Indian Ink (0.02% vol/vol) and Chlorin e6 (Ce6, 500 µM) as exogenous fluorophore. The skin strips were obtained from abdominal skin postoperative wastes of fair-skinned (phototype 2) female aged 37 years old and dark-skinned (phototype 3-4) female aged 37 years old. Skin thicknesses of each skin sample were determined as average values over 10 evenly distributed points of measurement on a Hematoxylin and Eosin stained histological slide. The purpose of the bottom layer was to have a substrate, containing fluorophore, with well-known optical properties that might get used in further analysis. The thickness of the gel layer was ~15 mm.

Two combinations of OCA and enhancer solutions were implemented: the first one (S1), was a mixture of sucrose (Sigma-Aldrich), Polyethylene Glycol 400 (PEG 400, Sigma-Aldrich) and Polypropylene Glycol (PG, Sigma-Aldrich) with ratios of 50%/45%/5% (vol/vol) and the second one (S2), was a mixture of PEG-400 and Dimethyl Sulfoxide (DMSO, Sigma-Aldrich) in ratios of 80%/20% (vol/vol). Saline was used as a substitution of the OCAs for control experiment. The selection of the chosen OCAs was based on a literature data and preliminary experiments. All selected agents and enhancers are FDA-approved[23]. The OC protocol was as follows: 3 ml of a chosen OCA was applied at initial time point T0 on the epidermis surface of the skin sample lying on the gel substrate of the hybrid model. The SRMMS's probe was placed in gentle contact with the skin epidermis in the OCA-topical application area. Then, the DR and AF spectroscopic data were acquired at four SDSs and at each of five λ_{exc}. To get the kinetics information, the spectroscopic data were collected every 4 minutes within 36 minutes (T0+4i with i = {1,2,3,4,5,6,7}). The same sequence of action was applied in the control protocol (using saline instead of OCA) and in the "dry" condition experimental protocol in which neither saline nor OCA solutions were applied. The objective of the latter "dry" condition was to estimate the potential mechanical impact on a spectroscopic data. At every time point, the spectroscopic data were acquired 3 times to make an averaging and improve the signal-to-noise ratio.

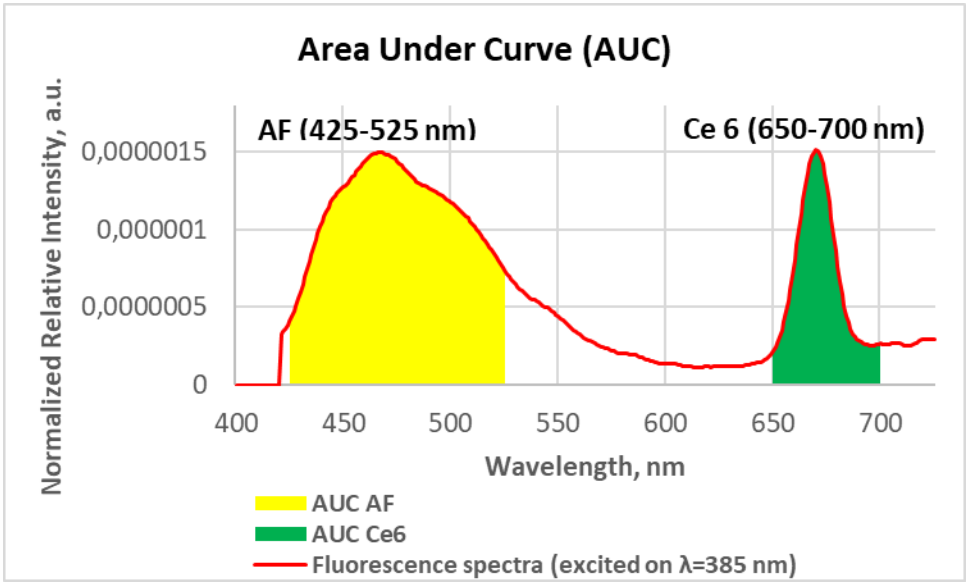
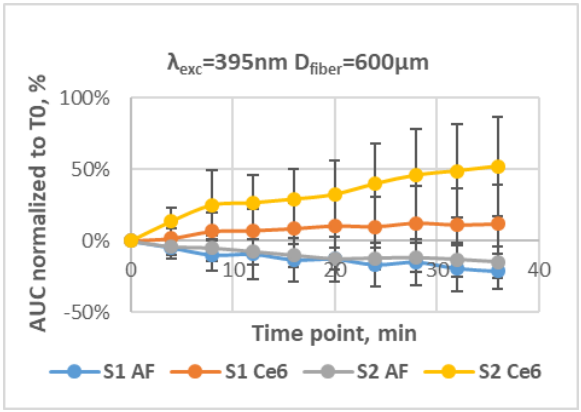


Figure 2. Fluorescence emission spectra (excited on λ=385 nm and collected by D2 ring of collecting fibers) and corresponding integrals of Areas Under the Curve (AUC) for autofluorescence (425-525 nm) and Chlorin e6 fluorescence (650-700 nm).

Then, the collected data set was preprocessed (smoothing and noise filtering, background subtraction, spectra averaging) and the Area Under the Curve (AUC) values were calculated for the specific wavelength bandwidths of interest for the skin-AF and Ce6-fluorescence spectra obtained at every of the 5 excitation peaks and 4 SDS (Figure 2). For AF-AUC, the 425-525 nm interval of integration was chosen, that corresponds to the main skin fluorophores emission spectra. For Ce6-AUC, the 650-700 nm interval was chosen as the well-known emission bandwidth of Ce6. Finally, AUC values were all normalized to their initial (at T0) values to get the kinetics curves of the spectroscopic signals.

3. RESULTS AND DISCUSSION



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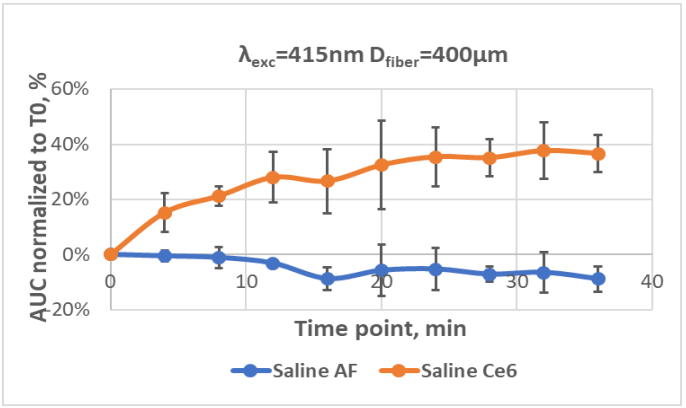

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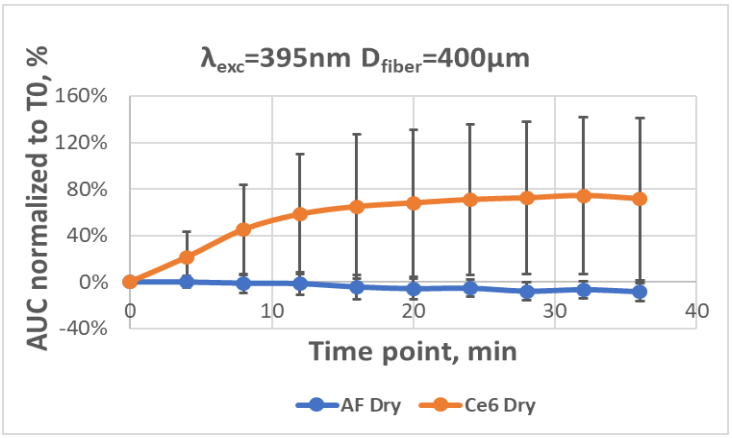

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Figure 3. (a) Normalized AUC kinetics of AF and Ce6 spectra, excited on λ=395 nm and collected by D_{fiber}=600 µm, for two OCA (S1 and S2); (b) Normalized AUC kinetics of AF and Ce6 spectra, excited on λ=415 nm and collected by D_{fiber}=400 µm, for saline application; (c) Normalized AUC kinetics of AF and Ce6 spectra, excited on λ=395 nm and collected by D_{fiber}=400 µm, for "dry" condition.

Time kinetics of skin-AF and Ce6-fluorescence spectra AUC showed that under 36 minutes of OCA application it is possible to observe certain enhanced light penetration effect through an increasing of normalized Ce6-fluorescence intensity (arising from bottom gel layer) and a slight decreasing of normalized skin-AF intensity (from upper layer of the hybrid model) (Figure 3(a)). Each SDS distance allowed to obtain an information from deeper tissue layers, depending

on the distance between the collecting fiber and the excitation fiber. Average maximum decreasing of AF signal for S1 solution after 36 minutes of application for all λ_{exc} at D1 was ~21%, and for S2 solution it was ~15%. The use of ANOVA statistical analysis has shown an insignificance of differences between these values ($P<0.2$). But the average maximum increasing of Ce6 fluorescence for S1 solution after 36 minutes of application for all λ_{exc} at D1 was ~36%, and for S2 solution it was ~59%, which varies by more than 1.5 times. Thus, it was possible to observe that optical clearing led to increased depth resolution with time. Moreover, the effect of clearing itself was observed to be dependent on the chosen OCA. Despite the relatively high values of standard deviation in our data set, an obvious difference between the two OCA's effect was clearly assessed. ANOVA test has shown that the differences between the Ce6 fluorescence maximum under the two OCA solutions effect are statistically significant ($P<0.2$). Application of the saline solution didn't cause significant changes in normalized autofluorescence intensities (average maximum decreasing after 36 minutes of application for all λexc at D1 was ~5%). But the effect of increasing in normalized acquired Ce6 fluorescence with time (Figure 3(b)) has been observed (average maximum increasing after 36 minutes of application for all λ_{exc} at D1 was ~42%). This increasing is probably due to immersion and hydration of *ex vivo* skin sample resulting into the better photon penetration.

The result of "dry" condition experiment highlighted that while there were slight changes in AF spectrum amplitudes (average maximum decreasing of AF signal after 36 minutes for all λexc at D1 was ~12%), a strong increase of Ce6 fluorescence emission intensity (average maximum increasing of Ce6 fluorescence after 36 minutes for all λ_{exc} at D1 was ~86%) was acquired from the bottom gel layer (Figure 3(c)). This phenomenon, probably due to clearing effect, might be caused by drying, compaction and shrinking of the *ex vivo* skin sample, which cannot maintain its properties like in *in vivo* conditions, or due changes caused by pressure that is applied by the acquisition device's probe[24,25].But the most probably, there is contribution of both effects.

4. CONCLUSION

Spatially resolved multiply excited autofluorescence and Chlorin e6 spectra were acquired on an *ex vivo* hybrid skin/gel model. Kinetics of these spectra during optical clearing by a 2 different OCAs, during saline action and dry condition were investigated. The analysis of obtained results allowed concluding that both of investigated OCAs improved the depth resolution of a spectroscopic approach by reducing skin scattering and absorption and, thus, increasing excited and collected Ce6 fluorescence of a gel layer. Besides, the results of dry condition kinetic measurements showed that absence of any applied liquid on a skin surface caused significant increasing of collected Ce6 fluorescence with time. This effect was probably occurred as the result of drying of an *ex vivo* skin sample combined with pressure applied by the spectroscopic probe, that was resulted into more tight and compact skin compounds configuration and, as the result, reduced scattering and increased photon penetration. Future experiments should be aimed, among other things, at explaining and understanding of this effect.

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