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SHORT COMMUNICATION

Stain-free Histopathology of Basal Cell Carcinoma by Dual Vibration Resonance Frequency CARS Microscopy

Norbert Kiss[1,2] · **Ádám Krolopp**[3] · **Kende Lőrincz**[2] · **András Bánvölgyi**[2] · **Róbert Szipőcs**[1,3] · **Norbert Wikonkál**[2]

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Abstract Basal cell carcinoma (BCC) is the most common malignancy in Caucasians. Nonlinear microscopy has been previously utilized for the imaging of BCC, but the captured images do not correlate with H&E staining. Recently, Freudiger et al. introduced a novel method to visualize tissue morphology analogous to H&E staining, using coherent anti-Stokes Raman scattering (CARS) technique. In our present work, we introduce a novel algorithm to post-process images obtained from dual vibration resonance frequency (DVRF) CARS measurements to acquire high-quality pseudo H&E images of BCC samples. We adapted our CARS setup to utilize the distinct vibrational properties of CH_3 (mainly in proteins) and CH_2 bonds (primarily in lipids). In a narrowband setup, the central wavelength of the pump laser is set to 791 nm and 796 nm to obtain optimal excitation. Due to the partial overlap of the excitation spectra and the 5–10 nm FWHM spectral bandwidth of our lasers, we set the wavelengths to 790 nm (proteins) and 800 nm (lipids). Nonresonant background from water molecules also reduces the chemical selectivity which can be significantly improved if we subtract the DVRF images from each other. As a result, we acquired two images: one for "lipids" and one for" proteins" when we properly set a multiplication factor to minimize the non-specific background. By merging these images, we obtained high contrast H&E "stained" images of BBC's. Nonlinear microscope systems upgraded for real time DVRF CARS measurements, providing pseudo H&E images can be suitable for in vivo assessment of BCC in the future.

Keywords Basal cell carcinoma · Nonlinear microscopy · Coherent anti-stokes Raman scattering · Pseudo HE images · Cancer detection

Introduction

Each year, more than 2 million non-melanoma skin cancers are diagnosed in the USA, of which approximately 4 in 5 cases are basal cell carcinomas (BCC). In fact, BCC is the most common type of cancer in Caucasian individuals, and is showing a worldwide increase in incidence. The most prevalent risk factor for BCC is exposure to UV light from the sun or indoor tanning beds. Other risk factors include immunosuppression and ionizing radiation. Although BCC metastasizes extremely rarely, it can be highly invasive locally, causing disfigurement as facial localization occurs frequently. Early diagnosis and careful management is essential to prevent tissue destruction and the progression of the disease [1].

An initial diagnosis of BCC can be established based on the clinical appearance of the skin lesion, but a skin biopsy is required for a definitive diagnosis. In the management of BCC, a wide range of treatment modalities are available. Nevertheless, the gold standard treatment is surgical excision, followed by a histopathological confirmation of clearance, largely based on haematoxylin and eosin (H&E) staining [2]. In order to achieve aesthetically acceptable post-surgical results, there is a high demand for novel, non-invasive imaging methods, which could be utilized by physicians to determine tumor-free margins.

✉ Róbert Szipőcs
r.szipocs@szipocs.com

[1] Wigner RCP, Institute for Solid State Physics and Optics, P.O. Box 49, Budapest H-1525, Hungary

[2] Department of Dermatology, Venereology and Dermatooncology, Semmelweis University, Budapest, Hungary

[3] R&D Ultrafast Lasers Ltd, P.O. Box 622, Budapest H-1539, Hungary

Nonlinear optical techniques have been formerly employed to investigate the morphological features of BCC [3]. However, the acquired images (second-harmonic generation (SHG) images of collagen and two-photon excitation autofluorescence (TFEF) images of NADH) do not correlate with the conventional H&E stained sections [4, 5]. Recently, Freudiger et al. developed a novel real-time method to visualize tissue morphology similar to H&E staining, based on CARS technique [6].

In our present work, we adapted our CARS microscope imaging system [7] for similar, stain-free histopathological investigations of human skin samples with BCC. To this end, we optimized our CARS imaging system for high contrast lipid and protein detection. The data obtained from the dual vibration resonance frequency (DVRF) CARS measurements was post-processed by a new algorithm to increase the chemical selectivity of our measurements and obtain pseudo H&E images of the BCC skin samples.

Methods and Techniques

In our experiments, we used a CARS imaging system comprising a femtosecond pulse, broadly tunable Ti-sapphire laser (FemtoRose 100 TUN NoTouch, product of R&D Ultrafast Lasers Ltd. (R&D), Hungary), an inherently synchronized, multi-stage Yb-fiber laser amplifier (CARS Stokes Unit, product of R&D), a FemtoCARS Laser Unit (product of R&D) and a commercial Axio Examiner LSM 7 MP laser scanning 2P microscope (product of Carl Zeiss, Germany), the detection optics of which had been upgraded for CARS measurements. Further technical details on the experimental setup can be found in Ref. 7.

For stain-free histopathology of BCC samples, we optimized our CARS imaging setup according to the CARS system presented by Freudiger et al.'s, who utilized the different vibrational properties of CH_2 (found mainly in lipids) and CH_3 bonds (most commonly found in proteins) in order to create H&E staining-like non-linear microscopic images. In their setting, merged images of CH_2 and CH_3-CH_2 were generated by a stimulated Raman scattering (SRS) microscopy. For SRS microscopy, Freudiger et al. used a picosecond pulse, dual wavelength laser system, the spectral bandwidth of which matches quite well that of the vibration resonance bandwidth of typical molecular bonds. In our CARS imaging system, however, typical pulse duration of the pump and Stokes pulses are in the 100–200 fs range, due to the fact that it had been upgraded from a commercial 2PEF imaging system [7]. The full width at half maximum (FWHM) spectral bandwidth of our lasers is in the 5–10 nm range, that is why the spectral resolution of our CARS imaging system is at least five times lower than in case of the cited work.

In an ideal (narrowband) case, when Stokes pulses are generated at 1030 nm (in an Yb-amplifier), central wavelength of the pump laser (in our case, the Ti-sapphire laser) has be set to 791 nm and 796 nm to obtain optimum excitation of the CH_3 and CH_2 bonds, respectively. Due to the partial overlap of the excitation spectra of proteins and lipids, and the 5–10 nm FWHM spectral bandwidth our lasers, we set these two wavelengths to 790 nm (for excitation of proteins) and 800 nm (for excitation of lipids). Besides the spectral overlap problem of proteins and lipids, there is another fact that reduces the chemical selectivity of CARS measurements, namely the nonresonant background signal originating from water molecules.

During the post-procession of our DVRF CARS images, we found that the chemical selectivity of the imaging can be considerably improved when we subtract the DVRF images from each other, since it eliminates the (wavelength independent) nonresonant background and increases the contrast for "protein" and "lipid" images as well. Subtraction of images was performed by ImageJ software (NIH, USA). As a result, we obtained two images: one for "lipids" and one for" proteins" when we properly set a multiplication factor (typically in the 0.9–1.1 range) for minimizing the non-specific CARS (and autofluorescence) background. This factor compensated for the changes in the laser pulse duration, the excitation power and the temporal overlap of the pump and the Stokes pulses between the two measurements. It is important to note that the time between taking the two images should be minimized in order to avoid any displacement of the sample either in the vertical or horizontal directions, otherwise subtraction can result in blurry images. To this end, our ex vivo human skin samples were water immersed to a microscope cover-glass during the imaging processes. For imaging planes parallel with the skin surface, it gave adequate results. However, for imaging planes vertical to the skin surface (in case of conventional pathological cuts, which is required for mosaic images to mimic conventional pathological microscopic slides), this method is not efficient enough since the interface between the sample and the cover-glass has a considerably smaller area. Novel spectral interferometric techniques [8] might help to minimize the time interval between the microscope exposures for the "protein" and "lipid" settings, which not only minimizes the problem of sample displacement but paves the way for video rate, pseudo H&E stained microscope imaging of pathological skin samples. For this ultimate goal, in our present work we investigated whether our DVRF imaging method is suitable for the histopathology of normal murine or human skin samples or human skin samples with basal cell carcinomas. We recorded images in both horizontal and vertical planes, the results of which are shown in the following section.

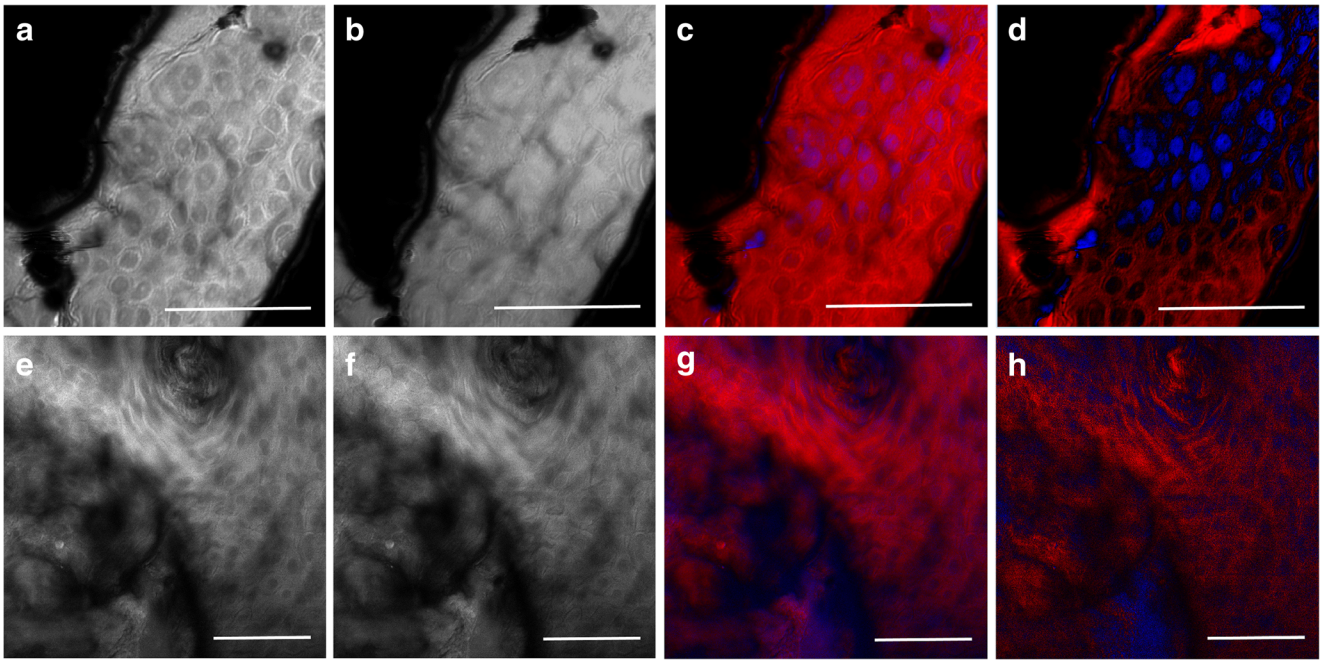

Fig. 1 CARS images of mouse (**a-d**) and human (**e-h**) epidermis, horizontal (**a-d**) and vertical (**e-h**) view. Scale bar represents 50 μm. a, e: CH_2 image; b, f: CH_3 image; c, g: merged color image (red: CH_2, blue: CH_3-CH_2); d, h: merged color image (red: CH_2-CH_3, blue: CH_3-CH_2)

Results

We recorded a large number of DVRF CARS images of different ex vivo murine and both normal and pathological human skin samples, the results of which are shown in Figs. 1 and 2. They also illustrate the distinctiveness of the images captured with "protein" (Figs. 1 and 2a, e) and "lipid" (Figs. 1 and 2b, f) wavelength settings. Fig. 1 displays images of mouse (a-d) and human epidermis (e-h). In Fig. 1d the nuclei of keratinocytes are clearly seen in blue while the cytoplasms appear in red, although in some cells, the nuclei are not visible. Fig. 1h shows images of human epidermis in a vertical view,

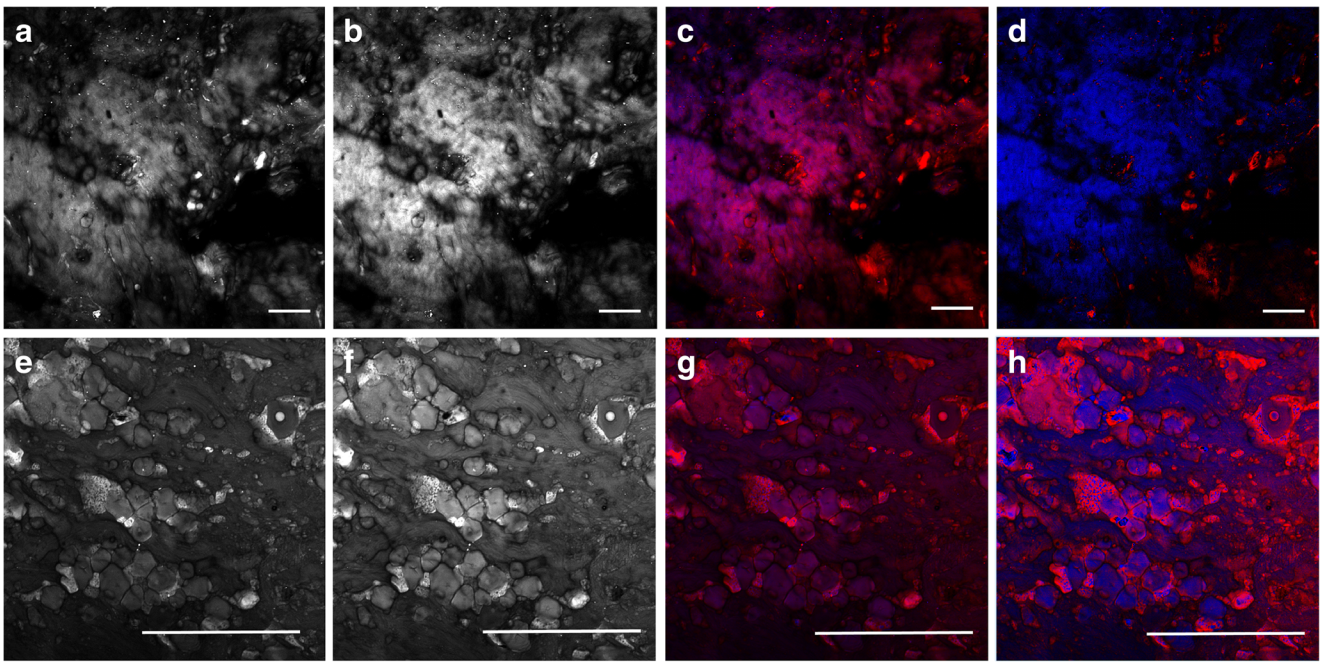

Fig. 2 CARS images of human normal dermis (a-d) and human basal cell carcinoma (e-h), horizontal view. Scale bar represents 50 μm. a, e: CH_2 image; b, f: CH_3 image; c, g: merged color image (red: CH_2, blue: CH_3-CH_2); d, h: merged color image (red: CH_2-CH_3, blue: CH_3-CH_2)

with the same characteristic colors. In contrast, in Fig. 1c, g the nuclei are only faintly visible and the cells are not clearly distinguishable. Fig. 1h has lower sharpness, which could be the result of a minuscule displacement of the sample, related to the vertical imaging. In Fig. 2, images of normal human epidermis (a-d) and basal cell carcinoma (e-h) are displayed. Fig. 2d illustrates that the dermis is mainly composed of a collagen-rich connective tissue, shown in blue color. In Fig. 2h, irregularly shaped and sized nests of tumor cells are seen, separated by collagen strands of the tumor stroma, displayed in blue. The displayed morphology is characteristic for infiltrative BCC, which was later verified by histopathology.

Discussion

Dual vibration resonance frequency (DVRF) CARS measurements were used to generate pseudo haematoxylin and eosin (H&E) "stained" ex vivo microscope images of basal cell carcinoma (BBC) human skin samples for their detection and identification. A novel image processing method was introduced, which provides higher contrast and sharper images than the method used previously [6], while does not involve a complicated algorithm.

A limitation of our current study is that we could not generate mosaics by merging individual images to display large areas of tissue. In order to capture high quality mosaic images, we need to upgrade our imaging setup to be able to change between the two different wavelength settings within a few seconds to avoid any displacement of the samples. Also, to create mosaics, automated sample positioning and an automatically delay controlled CARS signal (exhibiting a zero delay between the pump and Stokes pulses for hours) would be required, which we aim to employ in further experiments. Multimodal imaging with TPEF and SHG can also supplement our dual vibration resonance frequency CARS microscopy setup. Although, since clinicians are familiar with H&E stained histopathological images, pseudo H&E images alone may prove to be sufficient for diagnostic purposes, which may be confirmed in additional studies. Finally, we note that handheld, low repetition rate, Yb-fiber laser based nonlinear microscope systems [9] upgraded for real time DVRF CARS measurements (hence providing pseudo H&E images) can be suitable for in vivo, real time diagnostics of BCC in the future.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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