SHORT REPORT

Evaluation of digital staining for ex vivo confocal laser scanning microscopy

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Abstract

Background Ex vivo confocal laser scanning microscopy (CLSM) is a novel diagnostic tool for the fast examination of native tissue. However, CLSM produces black/white/green images, depending on the refraction indices of the tissue structures, complemented by nuclear fluorescence staining, which the vast majority of Mohs surgeons and dermatopathologists are not trained to interpret. Digital staining is applicable to ex vivo CLSM investigations to simulate the images of conventional slides stained with haematoxylin and eosin (H&E).

Objectives The aim of our study was to evaluate in detail the appearance of human skin structures using digitally stained ex vivo CLSM images and compare the results to that of conventional H&E slides of the same specimen.

Methods After providing informed consent, 26 patients donated their Burow’s triangles (healthy skin) that resulted from plastic reconstruction after the RO excision of skin tumours. After being investigated by ex vivo CLSM, including automated digital staining (VivaScope 2500M-4G, MAVIG GmbH), the specimens were fixed in formalin, embedded in paraffin and stained with H&E.

Results Almost all skin structures in the digitally stained ex vivo CLSM images morphologically resembled the structures in the histopathological images acquired from H&E slides. Due to the high refraction index of melanin, the hair shafts appeared bright pink, and the melanocytes and melanophages were poorly imaged, resulting in a strong pink appearance that vastly differed from the appearance of conventional H&E-stained histopathology.

Conclusions Digital staining of ex vivo CLSM images is an easy and highly useful tool to facilitate the interpretation of black-field images generated by confocal laser scanning microscopy for dermatopathologists and Mohs surgeons who are familiar with H&E staining. Unlike the pigmented structures, the cutaneous and subcutaneous structures had excellent visualization with only minimal differences from their appearance on H&E slides.

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Conflicts of interest

The VivaScope 2500M-G4 microscope was provided free of charge for the duration of the study from MAVIG GmbH.

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Introduction

Confocal laser scanning microscopy (CLSM) is a novel technique that allows for high-resolution imaging of the skin. CLSM has shown promising results in the fast examination of native tissue during Mohs surgery for basal cell carcinoma (BCC) and, recently, squamous cell carcinoma (SCC). In vivo CLSM allows for a non-invasive optical biopsy up to the upper dermis and may be used to set macroscopic margins for BCC, lentigo maligna and, most recently, SCC prior to surgery. Ex vivo CLSM generates a horizontal scan of the whole sample, which allows for fast margin control during Mohs surgery in a traditional vertical view of the tissue sample.

CLSM produces black/white/green images, depending on the refraction indices of the tissue structures, complemented by nuclear fluorescence staining, which the vast majority of Mohs surgeons and dermatopathologists are not trained to interpret. In confocal laser scanning with the VivaScope 2500M-G4 microscope, two lasers with different wavelengths [488 nm (blue) and 785 nm (infrared)] are simultaneously used. Both reflectance and fluorescence signals are acquired and correlate in real time. A fluorescent dye is first applied to the tissue to highlight the cell

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structures. The resulting images can be digitally stained to mimic the appearance of conventional histopathology with haematoxylin and eosin. This may facilitate the introduction of confocal microscopy into the clinical realm.7

Furthermore, the VivaScope 2500M-G4 is equipped with a digital camera that provides a bright-field image of the specimen and allows for the easy navigation and selection of regions of interest within the tissue.

Similar to the images acquired with microscopy, these images can be examined at any magnification, ranging from displaying the whole sample up to a 550-fold magnification.

The aim of this study was to evaluate the appearance of normal skin and its appendages using digitally stained ex vivo CLSM images acquired with the VivaScope 2500M-G4 and compare the appearance to that of the corresponding conventional H&E slides of the same specimen.

**Materials and methods**

The study was approved by the ethics committee of the medical faculty of University of Leipzig (No. O93/18-ek).

After providing informed consent, 26 patients donated their Burow’s triangles from various donor sites that resulted from plastic reconstruction after R0 excisions of skin tumours. Immediately after resection, the skin was placed into saline (0.9% NaCl) until further workup to avoid desiccation.

The skin was then incubated for 20 s in 0.04 mg/mL resp. 0.12 mmol/L acridine orange solution followed by a 10 s rinse in phosphate buffered saline (PBS, pH 7.4). All samples were scanned in vertical mode during ex vivo CLSM, including automated digital staining (VivaScope 2500M-4G, Mavig GmbH, Munich, Germany, Fig. 1), to best correlate with traditional histology images. After CLSM imaging, the skin was fixed in formalin, embedded in paraffin and stained with H&E. Comparisons of the digitally stained confocal laser scan microscopy and histological examination images were performed by two independently trained dermatopathologists.

**Results**

Almost all skin structures in the digitally stained ex vivo CLSM images morphologically resembled those in the histopathological images acquired from the H&E slides (Fig. 2a–j). The epidermal layers were well defined, and keratinocytes presented as cuboidal and polygonal cells with a smooth transition into the stratum corneum. Granularity of the keratinocytes of stratum granulosum was less prominent in the ex vivo CLSM image than in the H&E image, and the basal membrane was not marked as distinct structure as in the H&E specimen (Fig. 2a). The dermis showed a network of reticulated fibres with sharp imaging of collagen in pink, which was similar to the H&E images, and the elastic fibres appeared in bright purple (Fig. 2c). Due to acridine orange staining, the nuclei of adipocytes of the subcutaneous fat tissue were precisely discriminable, while in the H&E image, only the isolated cell nuclei appeared visible in lipocytes. In the digitally stained confocal image, almost every lipid cell clearly presented a flat marginal nucleus with dark violet coloration (Fig 2g). The skin appendages including hair follicles and sebaceous and eccrine glands showed fine definition, with colorations almost perfectly mimicking those in H&E staining (Fig. 2b,e,f).

The muscles, nerves, vessels and cartilage were also visible (Fig. 2h,i). Even the extracellular matrix and perichondral tissue were perfectly differentiated (Fig 2d,h).

However, due to the high refraction index of melanin, the hair shafts appeared in bright pink, and the melanocytes and melanophages were poorly imaged, thus resulting in a strong pink and blurred appearance that differed highly from the appearance of conventional H&E-stained histopathology (Fig 2j). The acridine orange stain used for fluorescence signals did not interfere with the subsequent histopathological workup and H&E staining process.
Discussion
Processing frozen histology is labour and time intensive. Confocal microscopy is a promising diagnostic tool that casts new light on confocal-guided bedside surgery. Confocal microscopy can minimize the patients’ interoperative waiting time and length of hospitalization by reducing the time needed for pathological imaging down to a few minutes while avoiding long and cost-intensive tissue sampling processes. To date, CLMS has been used either in vivo in reflectance mode to achieve imaging by different refractive indices of ‘endogenous chromophores’, such as keratin and melanin, or ex vivo in fluorescent mode by prior nuclear staining with a fluorophore. In both techniques, black and white CLMS images are produced. Understanding how the device functions and interpreting the resulting images requires an intense and dedicated learning process. To simplify the evaluation process for dermatopathologists and Mohs surgeons who are familiar with

Figure 2  *Ex vivo* CLSM image of healthy skin (left column) and their histological features that correlate with haematoxylin and eosin (H&E) staining (right column). (a) Epidermis with a smooth transition of keratinocytes into stratum corneum but missing granularity of keratinocytes and visibility of the basal membrane. (b) Hair follicle with a bright pink appearance in CLSM image due to high reflection of melanin. (c) Dermis with distinctive solar elastosis of the upper dermis visible with both techniques and images of the lymphatic and capillary vessels as they appear with H&E staining. (d) Connective tissue with a pink appearance of collagen similar to that seen with H&E staining, and the elastic fibres are presented in bright purple. (e) Sebocytes and the structure of the sebaceous glands are well defined with coloration similar to that from H&E slides. (f) Eccrine glands with a purple appearance and a light purple reflection from the gland lumina. (g) Subcutaneous fat with precisely discriminable nuclei due to acridine orange staining. (h) Elastic cartilage with chondrocytes, extracellular matrix and perichondral tissue. (i) Muscle fibres with precisely discriminable nuclei due to acridine orange staining. (j) Melanocytes/naevi with strong reflection from melanin, which covers the majority of other signals and results in a ‘blurred’ image of cells.

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H&E staining, digital staining of confocal mosaics was developed to simulate conventional histopathology.7

In this feasibility study, we evaluated the consistency between conventional H&E slides of normal skin and its appendages and digitally stained ex vivo CLSM images produced by VivaScope 2500M-G4. This laser scanning microscope combines confocal fluorescence and reflectance to create a multimodal pseudo-colour image that mimics the appearance of histopathology with haematoxylin and eosin.7

Impressively, our study showed a very high correlation between the digitally stained images of confocal mosaics and the corresponding images of H&E-stained specimens. All skin structures, including the appendages, were easily recognizable and morphologically resembled those seen on the H&E slides. Due to high nuclear contrast, the cells were well defined. Some tissue structures (e.g. adipocytes, hair follicles) were even more visible in the digitally stained CLSM images than in H&E-stained images. There was perfect labelling of the cytoplasm and dermal collagen; the elastic fibres were prominently marked in purple, which may be due to a non-specific agglomeration of acridine orange at the fibres. In the past, reflectance mode alone was shown to be inferior to H&E images in contrast and sensitivity since CLSM could not detect small BCC tumour strands due to the modest contrast of the nuclei caused by the background reflectance of keratin and collagen.8

The VivaScope 2500M-G4 improves the sensitivity and specificity of CLSM by combining fluorescence imaging, which shows nuclear detail (comparable to haematoxylin in histopathology), and reflectance signals, which are sensitive to collagen and the cytoplasm without the need for staining (similar to eosin in histopathology).

For nuclear staining, a fluorophore must be applied to the specimen. In the VivaScope 2500M-G4 standard, filter sets are integrated for acridine orange and fluorescein (blue laser) as well as for indocyanine green (ICG – infrared laser). We used acridine orange, which is the most commonly used fluorophore in the clinical setting because acridine orange provides excellent contrast for the cells while even providing additional information regarding fat tissue.9

By using specific fluorescent dyes with selective binding to the structures of interest, they might be used in ex vivo CLSM, similar to the way immunochemistry is used, in the future.10 Previous studies have shown that there is good contrast between melanin and melanocytes with bright and large nuclei in reflectance confocal microscopy (RCM) due to the high reflectance of melanin in contrast to that of keratinocytes.10 In vivo CLSM was successfully used to set macroscopic margins for lentigo maligna prior to surgery.2 Other studies that consecutively used the reflectance and fluorescence modes have not only shown good discrimination between the main subtypes of naevi (junctional, compound, dermal and dysplastic) but have also been able to differentiate naevi from melanoma with excellent correlation to histopathology.11

Conclusion
Digital staining with ex vivo CLSM images is an easy and highly useful tool to facilitate the interpretation of black-field images generated by confocal laser scanning microscopy for dermatopathologists and Mohs surgeons who are familiar with H&E staining. The following cutaneous and subcutaneous structures are excellently displayed in the CLSM images, with only minimal differences compared to the H&E slides: Epidermis with all layers, collagen and elastic tissue, lymphatic and capillary vessels, appendages including hair follicles, sebaceous and eccrine glands, subcutaneous fat, elastic cartilage and muscle fibres.

In contrast, due to their intensive reflecting pigmented structures, such as hair shafts, melanocytes and melanophages appeared pink and blurred in digital stained ex vivo CLSM images, which is different from conventional H&E slides and until now, a major limitation of the technique.

In addition to gaining knowledge of the regular skin features, further studies with structured comparisons of ex vivo CLSM images with H&E slides for each tumour entity are needed before applying CLSM to daily clinical routine to avoid misinterpretations and ensure the best results.

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