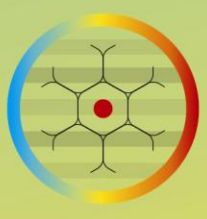


diagnoptics

Project Acronym: DIAGNOPTICS
Grant Agreement number: 621066
Project Title: Diagnosis of skin cancer using optics

D.5.5 Clinical protocol for the use of the ex vivo confocal device

Revision: 2

Authors: Dott. Sara Bassoli, Dott. Alice Casari, Dott. Chiara FIorentini, Dott. Barbara Ferrari, Prof. Giovanni Pellacani (Unimore), Ms. Raphaela Kaestle (Mavig)

Project co-funded by the European Commission within the ICT Policy Support Programme		
Dissemination Level		
P	Public	X
C	Confidential, only for members of the consortium and the Commission Services	

Revision History

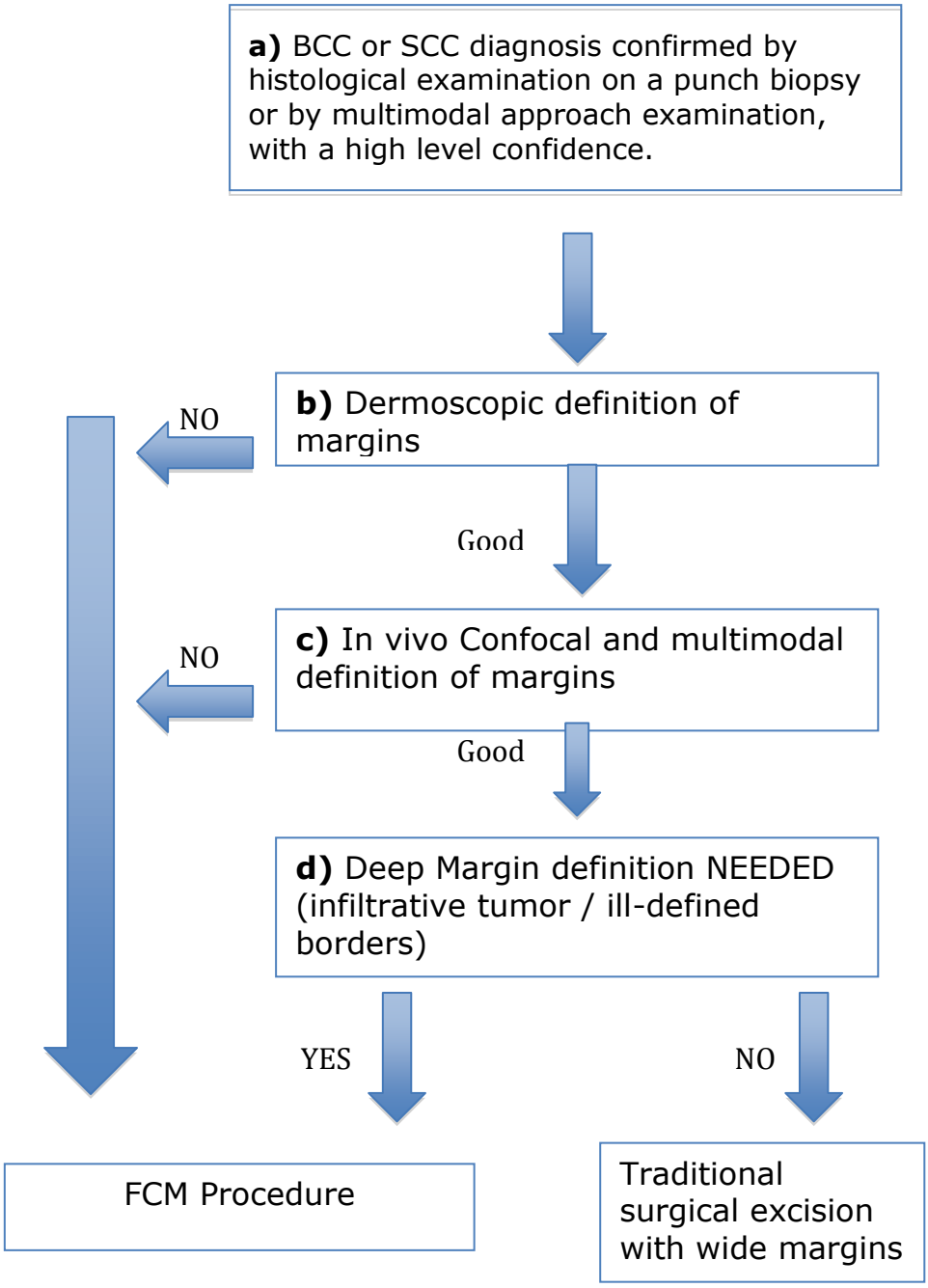
Revision	Date	Author	Organisation	Description
1	30/06/2015	Ms. Sara Bassoli et al.	UNIMORE	Original version
2	02/07/2015	Ms. Raphaela Kaestle	MAVIG	Revision of minor issues. Final version

Statement of originality:

This deliverable contains original unpublished work except where clearly indicated otherwise. Acknowledgement of previously published material and of the work of others has been made through appropriate citation, quotation or both.

INDEX

1.1 Flow Chart of the Clinical Protocol



1.2 Involved personnel and procedures

a) Clinicians of the Skin Cancer Unit (Dermatoscopy Unit) for the choice of the lesions to image / Technician for the image acquisition

Collection of all lesions referred with the suspicion of malignancy at the Skin Cancer Unit at Unimore. Handheld and digital dermoscopes (Visiomed MD4®) are currently used in the clinical practice. Dermoscopy is performed by applying water or gel between the lesion to examine and the glass of the dermoscope, allowing the vision of the skin under the surface. With digital dermoscopy a magnified image is projected on the computer screen and can be saved in a digital file.

The presence of arborizing vessels, blue ovoid nests, areas of erosion / ulceration, and/or pink-white shiny areas with small linear vessels are suggestive of BCC, whereas dotted vessels / hairpin vessels / glomerular vessels, associated to a variable grade of keratinization (whitish areas and keratin plugs) are suggestive of a SCC.

For all lesions suspected to be a BCC or SCC, dermoscopy and RCM / multimodal examination is performed in order to confirm the diagnosis.
Upon confocal microscopy, tumor islands / cords or dark silhouettes in the upper dermis, palisading of cells and dilated vessels are suggestive for the diagnosis of a BCC. SCC is suspected when altered keratinocyte morphology, mainly in presence of targetoid cells and atypical keratinocytes, tongues of irregular epithelial cells protruding into the dermis, and/or atypical vascularization are seen.

For those lesions with not confirmed diagnosis by the in vivo examination, an incisional biopsy is performed to have the histological report.

b/c) Technician for image acquisition / Clinicians skilled in confocal microscopy for image reading

If dermoscopic / confocal features are clearly confined to a portion of skin, then the tumor margins can be clearly defined, and no further examination is required before therapeutic procedure.
In case of not clearly defined borders, a careful examination of the peripheral portions of the lesion, through the exploration of dermoscopic and confocal features, is specifically performed along the clinically detectable periphery of the lesion, for a more accurate pre-surgical margin detection.

d) Surgical Team

In case of ill-defined borders and incapability to determine lateral margin after focused dermoscopy and confocal exploration, and / or invasive / ulcerated tumours, ex vivo confocal examination is performed.

Ex vivo confocal microscopy is an emerging nondestructive procedure that allows, in a few minutes, complete imaging of tumour margins directly in fresh tissue excisions from Mohs surgery with nuclear-level resolution that is comparable to that of histopathology. The microscope was modified for use in fluorescent mode (fluorescence confocal microscopy, FCM) with the use of several possible contrast agents. Among them, acridine orange is a well-known contrast agent that specifically stains nuclei and increases the contrast between the nuclear morphology and the surrounding background dermis. (Longo, Brit J Dermatol 2014)
Currently, FCM can image freshly excised Mohs tissue measuring up to 20 mm X 20 mm for the newest instruments.
Reported imaging time is now less than 3 min for a 10 mm x 10 mm mosaic, based on technological advances in optics, electronics and mechanics.

On the occasion of the surgical excision, FCM will be used in order to achieve a better estimation of the margins involvement.

1.3 The ex vivo confocal examination

1.3.1 Technical aspects

Confocal mosaics are acquired using a newer version ((VivaScope® 2500, MAVIG GmbH, Munich, Germany)(CDRH Class II, Europe Class 2, EC.)
Currently, there are three laser in the ex vivo microscope: 830 nm for reflectance; 488 nm and 658 nm for fluorescence. Imaging is with a 30X, 0.9 numerical aperture water immersion lens which provides optical sectioning of ~1.5 microns and resolution of ~0.4 microns at the 488 nm wavelength.

1.3.2 Staining procedures and image acquisition

Specimens are freshly excised lesions of Mohs surgery.

A drawing with the schematic representation of the anatomic area (i.e. face) is provided along with the orientation of the specimen that is usually done by using silk suture at one or more poles of the tissue. Briefly, all margins are numbered using o'clock procedure and labeled as A, B, C, D and E (the centre of the tumor and the deep margin) at the I° staging of Mohs surgery.

1^ Step: The tissue of 2-3 mm-thickness is immersed in a 0.6 mM solution of acridine orange dye for 10 to 20 seconds, and then the excess dye removed by wiping with paper. Acridine orange provides a strong contrast between nucleus and dermis because it specifically stains the DNA and RNA of nucleated cells. With FCM, only weak fluorescence is collected from the dermis and subcutaneous fat, therefore increasing the contrast of epithelial cells 1000-fold, including epidermis, adnexal structures, and BCC cells. Equally important is the fact that acridine orange

immersion affects neither subsequent frozen sections nor the quality of formalin-fixed histo-pathologic sections.

2^ Step: The tissue is sandwiched between two glass slides that are fixed with silicon glue (Unimore), or Blu Tack ® Bostik (Barcelona Unit), and then positioned onto the platform of the inverted microscope. The button "Scan" is set up. The imaging acquisition can be done as "A/C" (automatic contrast) or set up manually by using the best fluorescent contrast of the tissue.

3^ Step: Each case is numbered consecutively by using a code (i.e. EX001) and a separate excel spreadsheet is filled in with all patient information (last and first name, age, sex, tumour location). Within the patient's folder, all margins are numbered. For each skin specimen (one layer/margin), a two-dimensional sequence of images were acquired and stitched together into a mosaic that displayed the entire area of the tissue. The size of the mosaic ranges from 7 mm x 7 mm to 20 x 20 mm. According to the size of a given specimen, different size can be chosen either for X and Y axis (i.e. 20 mm x 7 mm). The time needed for acquiring images and creating a single mosaic is about 7 minutes for the largest size. Each image consists of 1000x1000 pixels and the complete mosaic is stored in standard bitmap format.

4^ Step: After Fluorescence Confocal Microscopy (FCM) imaging, the "sandwich" can be easily broken and the specimen processed for frozen-sections or conventional histopathology.

5^ Step: Each case can be exported by right clicking on the number of the case and choosing "export all images" in "Vivascope E". This step will allow the Reader to obtain all images as bitmap format that can be read on PC or MAC by using any type of imaging software.
The tissue is examined at low magnification by using the "pan zoom" modality (a 12 mm x 12 mm mosaic on FCM roughly correspond to 2x magnification on a

conventional light microscope) followed by zooming-in at higher magnification to analyze cytologic details and microstructures.

1.3.3 Images evaluation

The following features for the distinction between BCC and healthy skin, and for the definition of the tumour subtype, will be evaluated. (Longo et al. Brit J Dermatol 2014).

Table 1 Fluorescence confocal microscopy descriptors of normal skin and basal cell carcinoma

	Fluorescence confocal microscopy
Normal skin	
Epidermis	Bright polygonal cells with nucleus/cytoplasm ratio in favour of nucleus; the rete ridge is clearly visible
Fat tissue	Hypofluorescent large lobules with eccentric and peripheral nuclei compressed against the plasma membrane
Eccrine glands	Snake-like bright tubular structures with two rows of cells, located in the dermis
Sebaceous glands	Round to oval aggregates of large cells with weak fluorescent cytoplasm corresponding to lipid-containing lobules and large fluorescent nucleus centrally located within the cell; they can be observed within the pilosebaceous unit or individual glands in the dermis
Muscle	Weakly fluorescent elongated fibres with brighter peripheral nuclei
Basal cell carcinoma	
Fluorescence	Area of higher fluorescence compared with a darker background; fluorescence is typically seen as forming structures/aggregates
Clefting	Hypofluorescent space surrounding basaloid islands; it partially outline the islands in micronodular and infiltrative basal cell carcinoma subtype whereas it is more evident in superficial and nodular tumours
Peripheral palisading	Highly fluorescent basaloid tumour cells with the peripheral palisading of nuclei
Basaloid islands	Highly fluorescent well-defined mass of basaloid cells with variable shape, showing peripheral palisading
Stroma reaction	Multiple bright spots basically corresponding to the nuclei of the inflammatory infiltrate (lymphocytes)
Basal cell carcinoma subtype	
Superficial	Proliferation of atypical basaloid cells that form an axis parallel to the epidermal surface and demonstrate slit-like retraction of the palisaded basal cells from the subjacent stroma (cleft-like spaces)
Nodular	Small to large nodules with peripheral palisading and clefting
Micronodular	Monotonous small round and well-defined islands with roughly the same shape and contour
Infiltrative	Columns and cords of basaloid cells one to two cells thick with sharp angulation, enmeshed in a densely collagenized stroma; palisading and clefting are not always present

As standard procedure, we will first analyze the centre of the tumour (specimen E) to define the histologic BCC subtype (superficial, nodular, micronodular, infiltrative, morphea-like). The definition of the BCC subtype is mandatory before checking the clearance of the margins to avoid under-diagnosis in case of tiny strands of basaloid islands.

In case of positive margin (i.e. presence of tumour islands), a II° staging is performed and labeled accordingly (i.e. margin "A" II° staging). The staging is performed until all margins are free of any tumoural proliferations.

1.4 *Future perspectives*

Although lack of literature, the same protocol will be also tested on squamous cell carcinoma and different epithelial tumours, in order to test its generalizability.

Melanoma margins are not currently evaluable since a stain specific for melanocytic cells is still not available.
A future development of FCM could include an applicative for an immediate melanoma thickness evaluation and histological subtype definition.

Any changes in FCM application in the field of the current Diagnoptics European Project will be promptly communicated in next study reports.