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► To cite this version:

Elisa Cinotti, Valerio Belgrano, Bruno Labeille, Damien Grivet, Catherine Douchet, et al.. In vivo and ex vivo confocal microscopy for the evaluation of surgical margins of melanoma. *Journal of Biophotonics*, 2020, 13, 10.1002/jbio.202000179 . hal-04822066

HAL Id: hal-04822066

<https://hal.science/hal-04822066v1>


Submitted on 6 Dec 2024

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FULL ARTICLE

In vivo and ex vivo confocal microscopy for the evaluation of surgical margins of melanoma

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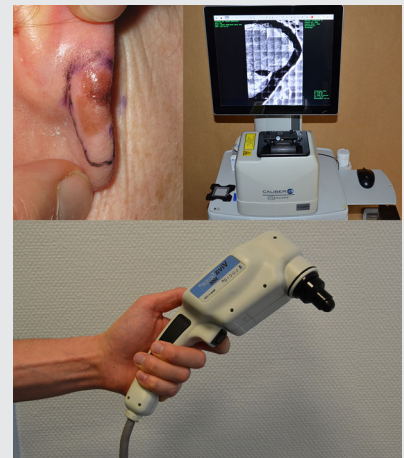
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Abstract

We report the first series of melanomas (MMs) where the surgical margins were evaluated both by ex vivo confocal microscopy (EVCN) and in vivo reflectance confocal microscopy (RCM). We evaluated the surgical margins of 42 cutaneous MMs of lentigo maligna/lentigo maligna melanoma type and 2 mucosal MMs with EVCN. Cutaneous MMs also underwent RCM mapping. Imaging results were compared with histopathology. The rate of correct identification of the tumor margins (invaded or not invaded) was 97.6% for RCM (evaluations of cutaneous MMs) and 95.5% for EVCN (evaluations of both cutaneous and mucosal MMs). Our study showed that the MM extension is visible under EVCN and that the combination of in vivo RCM and EVCN can be a new strategy for the evaluation of surgical margins of MMs.

KEYWORDS

ex vivo, histopathology, in vivo, lentigo maligna, melanoma, mucosa, reflectance confocal microscopy, surgery



1 | INTRODUCTION

In vivo reflectance confocal microscopy (RCM) and ex vivo confocal microscopy (EVCN) are two complementary imaging techniques that have been recently developed in the field of dermatology. In vivo RCM is

mainly used to help the clinical diagnosis of skin cancers noninvasively [1, 2] and it has also been applied to identify the surgical margins of clinically ill-defined melanomas (MMs) before surgery [3–7]. EVCN has been mainly developed to evaluate cutaneous tumor margins, in real-time, directly on freshly excised tissue in a perioperative

setting as a fast alternative to frozen histopathology during Mohs micrographic surgery (MMS) [8–10]. Although it has been demonstrated that the EVCM can image MM [11–15], its role in the definition of surgical margins has been investigated only in cutaneous carcinomas [8, 10, 16–19].

We report the first series of MMs where the surgical margins were evaluated with the association of *in vivo* RCM and EVCM for cutaneous MM and with EVCM for mucosal MMs, and imaging data were compared to the conventional histopathological examination.

2 | METHODS

2.1 | Patients and setting

We enrolled 43 patients with 42 lentigo maligna/lentigo maligna melanomas (LMs/LMMs) of the face at the Dermatology Department, 1 conjunctival MM at the Ophthalmology Department, and 1 vaginal MM at the Gynecology Department of the University Hospital of Saint-Etienne (France), between the 1 October 2013 and the 30 November 2016. One patient had two cutaneous MMs. All patients were scheduled for a follow-up visit every 6 months for at least 3 years. The study was realized in accordance with the Declaration of Helsinki.

2.2 | *In vivo* RCM

Cutaneous MMs underwent mapping of surgical margins by handheld *in vivo* RCM (Vivascope 3000, Caliber, NY, distributed in Europe by MAVIG GmbH, München, Germany) before surgery as previously detailed [4]. Marking began at the visual limits of LM, defined by the clinical examination and dermoscopy, and moved upward to obtain a first malignant cell-free examination. The following investigations were performed circumferentially, moving closer by 5 mm to the visual limits if the analysis was negative and moving away by 5 mm if malignant cells were found. This spacing of 5 mm corresponds to the size of the tip of the VivaScope 3000 camera of second-generation that was used in our study. When applied to the skin, this tip creates a temporary footprint that serves as a transitional benchmark for the next exploration, with the tip applied adjacent to the mark that the previous exploration left. A mark was made on the analyzed area using a demographic pen and then stained with a solution of fuchsin ink (1.25 g fuchsin, 25 g silver nitrate, 375 mL 90° alcohol, supplemented with water to obtain 480 mL of solution) to avoid any possible deletion during preoperative disinfection of the skin.

The mapping was performed together by two experts in RCM (J. L. P. and B. L.) in the Dermatology Department of the University Hospital of Saint-Etienne and lasted approximately 30 minutes per case. It was not performed for mucosal MMs due to the RCM probe size and the inability to mark the tumor margin on the conjunctiva. For mucosal MMs, the identification of margins was based on the clinical examination.

2.3 | *Ex vivo* confocal microscopy

The EVCM was performed in the Dermatology Department, where the cutaneous MMs were excised. All the mucosal MMs were transferred from the other Departments in a wet gauze. EVCM images were acquired from freshly excised samples using the EVCM dedicated to the skin (Vivascope 2500, Caliber, NY, distributed in Europe by MAVIG GmbH, München, Germany) with the reflectance mode [11, 20] (illumination with a diode laser, 830 nm wavelength). This device offers high-resolution mosaic images up to $20 \times 20 \text{ mm}^2$, consisting of individual images ($750 \times 750 \mu\text{m}^2$) stitched together.

Specimens with a diameter $< 20 \text{ mm}$ were directly observed under EVCM in the horizontal plane [20], whereas larger specimens were cut into four parts and the lateral margins of each quadrant were identified with an orientation ink.

The fresh surgical specimen was placed without prior preparation between two glass slides, allowing a horizontal analysis, parallel to the skin. A transparent ophthalmic gel (LaboratoiresThéa, Clermont-Ferrand, France) was placed under it. The two slides were attached by silicon (Turboflex, Light fast set, GACD, Paris, France). For each specimen, three horizontal mosaic images of the size of the sample, corresponding to the epidermal layer, the dermoepidermal junction (DEJ), and the upper dermis were acquired with the “*en face*” technique [20] and were evaluated in real-time by an expert (B. L.). About 10 minutes per case were required for EVCM acquisition and interpretation of the images.

2.4 | *In vivo* RCM and EVCM diagnostic criteria

Tumors were identified using the *in vivo* RCM diagnostic criteria for LM/LMs [4, 21] and for mucosal MMs [22, 23] and these criteria were also used for EVCM. Positive margins were considered in the presence of at least one bright, large ($> 20 \mu\text{m}$) round or dendritic cell either in the epidermis or the DEJ or the superficial dermis continuing from the tumor trailing edge.

2.5 | Histological examination

A conventional histopathological examination was later performed. After fixation, paraffin-embedded surgical specimens were sectioned perpendicularly to the major axis into 3 mm thick slices: 4 sections per slice (3 stained by hematoxylin–eosin and 1 stained by anti-MelanA antibodies), spaced 120 μm , were examined. Since there are few data about the use of *in vivo* RCM for the evaluation of surgical margins of MM and no studies about the evaluation of surgical margins by EVCM, the need for a re-excision in our series has been driven by the results of the conventional histological examination. The surgical reconstruction was performed after receiving the histopathological result for skin tumors and was immediate for mucosal tumors.

2.6 | Statistical analysis

Descriptive statistics, such as means, SD, medians, minimum and maximum values, and relative frequencies, were used to describe the patient, lesion, imaging and surgical characteristics. Moreover, the rate of correct classification for the imaging techniques compared to histopathology was evaluated and the agreement between EVCM and histopathology was calculated with Cohen's k .

3 | RESULTS

Details of the patients (sex/age), tumor localization, size and status (first excision or recurrent MM after previous surgical excision or second excision in case of positive histological margins) are listed in Table 1. Patients' mean age was 70 ± 9.6 years (range 49–87) and 65% were men. MM mean largest clinical diameter was 13 ± 7.5 mm (range 5–34). Concerning cutaneous MMs, 5 were LMMs and 37 were LMs (Figure 1) at the final histological examination. The cases that corresponded to secondary excision in case of positive histological margins, did not have their corresponding first excisions of MM included in this study.

EVCM was able to identify an atypical proliferation of melanocytes in all MMs. (Figures 2, 3 and 4). Most MMs (42 out of 44 MMs, 95.5%, and 41 out of 42 LM/LMMs, 97.6%) had tumor-free margins at histopathological examination. The rate of correct classification for *in vivo* RCM margin assessment of LM/LMM (Figure 1), when compared to histopathology, was 97.6%. It was not possible to calculate the agreement of *in vivo* RCM and histopathology with Cohen's k due to the presence of a class

with no frequencies (absence of positive margins under *in vivo* RCM). For EVCM margin assessment of MM when compared to histopathology, the rate of correct classification was 95.5% and the agreement with final histopathology evaluated with Cohen's was 0.47%. EVCM detected tumor invasion of the margins in the vaginal MM. Definitive traditional histopathological examination found tumor invasion of the margins in one additional LM (false-negative result). A second excision was performed in the latter case and the second surgical specimen was not included in this study. The rate of correct classification for EVCM margin assessment of LM/LMM was 95.2%. At a mean follow-up of 5 ± 0.74 years, there was only one local recurrence.

4 | DISCUSSION

Our study focused on LM/ LMM and mucosal MMs, types of MM that are in esthetic and functional areas and whose margins are often difficult to be identified with the consequent necessity of MMS or staged excisions. We found that EVCM associated with *in vivo* RCM can be a new strategy for the identification of the surgical margins of clinically ill-defined cutaneous MMs such as LM/LMMs, and that EVCM can also be used for the identification of surgical margins of mucosal MMs.

4.1 | *In vivo* RCM for the identification of the tumor margins of MM

We confirmed that preoperative margin mapping by handheld *in vivo* RCM is an effective procedure to reduce surgical stages because we found tumor-free margins in 97.6% of the LM/LMMs at histopathological examination after this procedure. Recent studies [3–5] showed that the conventional clinical and dermoscopy identification of surgical margins, even if it is performed by experts, it is not sufficient to identify the entire tumor extension in the majority of LM/LMM, and that *in vivo* RCM can reduce the surgical stages almost to one as found here.

4.2 | EVCM feasibility for MM imaging

We confirmed the feasibility of the use of EVCM to image LM/LMMs and mucosal MMs under EVCM. EVCM dedicated to the skin has been available on the market since 2013 [8]. It can display wide field-of-view mosaics of up to $20 \times 20 \text{ mm}^2$, making the examination of large lesions like LM/LMM possible, as shown by our study. Each EVCM mosaic image is created in a few minutes enabling

TABLE 1 Clinical, ex vivo confocal microscopy and histological features of the melanomas

Patient number	Sex	Age	MM status	MM location	MM size (mm)	Tumor free margins under EVCM	Tumor free margins under histological examination	Histological diagnosis
1	M	64	Second excision	Cheek	5 × 20	Yes	Yes	LM
2	F	81	First excision	Inferior eyelid	12 × 20	Yes	Yes	LM
3	M	85	Recurrent	Cheek	30 × 15	Yes	Yes	LMM
4	F	81	First excision	Cheek	15 × 32	Yes	Yes	LMM
5	M	74	First excision	Forehead	32 × 34	Yes	Yes	LM
6	M	69	First excision	Cheek	16 × 12	Yes	Yes	LM
7	F	68	First excision	Cheek	21 × 14	Yes	Yes	LM
8	M	83	Second excision	Nose	5 × 11	Yes	Yes	LM
9	M	68	First excision	Forehead	10 × 10	Yes	No	LM
10	M	80	First excision	Temple	16 × 15	Yes	Yes	LM
11	M	59	First excision	Cheek	15 × 15	Yes	Yes	LM
12	M	76	First excision	Forehead	20 × 20	Yes	Yes	LM
13	M	65	First excision	Conjunctiva	8 × 2	Yes	Yes	MM
14	F	62	First excision	Vagina	20 × 30	No	No	MM
15	F	62	First excision	Ear	12 × 8	No	Yes	LM
16	F	82	First excision	Forehead	5 × 6	Yes	Yes	LM
17	M	49	First excision	Cheek	7 × 9	Yes	Yes	LMM
18	F	67	Recurrent	Cheek	11 × 15	Yes	Yes	LM
19	F	72	First excision	Cheek	15 × 12	Yes	Yes	LMM
19	F	72	First excision	Cheek	12 × 10	Yes	Yes	LM
20	M	78	Recurrent	Nose	7 × 7	Yes	Yes	LM
21	F	64	First excision	Ear	8 × 15	Yes	Yes	LM
22	M	70	First excision	Ear	5 × 3	Yes	Yes	LM
23	M	55	First excision	Cheek	9 × 8	Yes	Yes	LM
24	M	65	First excision	Inferior eyelid	5 × 4	Yes	Yes	LM
25	M	66	First excision	Temple	11 × 11	Yes	Yes	LM
26	M	78	First excision	Cheek	6 × 8	Yes	Yes	LM
27	F	68	First excision	Cheek	6 × 6	Yes	Yes	LM
28	M	87	First excision	Temple	5 × 4	Yes	Yes	LMM
29	M	72	First excision	Cheek	8 × 6	Yes	Yes	LM
30	M	57	First excision	Cheek	16 × 8	Yes	Yes	LM
31	M	72	First excision	Cheek	10 × 6	Yes	Yes	LM
32	M	63	First excision	Nose	4 × 6	Yes	Yes	LM
33	F	83	First excision	Cheek	7 × 5	Yes	Yes	LM
34	F	72	First excision	Cheek	10 × 12	Yes	Yes	LM
35	M	69	First excision	Nose	3 × 7	Yes	Yes	LM
36	M	70	First excision	Cheek	7 × 9	Yes	Yes	LM
37	F	54	First excision	Ear	11 × 9	Yes	Yes	LM
38	M	74	First excision	Cheek	10 × 20	Yes	Yes	LM
39	M	50	First excision	Cheek	7 × 6	Yes	Yes	LM
40	F	61	First excision	Temple	9 × 5	Yes	Yes	LM

TABLE 1 (Continued)

Patient number	Sex	Age	MM status	MM location	MM size (mm)	Tumor free margins under EVCM	Tumor free margins under histological examination	Histological diagnosis
41	M	82	First excision	Cheek	7 × 7	Yes	Yes	LM
42	M	76	First excision	Cheek	9 × 6	Yes	Yes	LM
43	M	79	First excision	Cheek	12 × 12	Yes	Yes	LM

Abbreviations: EVCM, ex vivo confocal microscopy; LM, lentigo maligna; LMM, lentigo maligna melanoma; MM: malignant melanoma.

FIGURE 1 Clinical (A,B,D) aspect of lentigo maligna (LM) and correspondent in vivo reflectance confocal microscopy (RCM) examination (C,E,F). Figures B and D show the surgical margins of LM defined by RCM and marked by fuchsin ink. In vivo RCM images show large hyper-reflective dendritic (red arrows) and roundish (green arrow) cells in the epidermis (C,E) and confluent and elongated clusters of atypical cells budding from the hair follicles at the dermoepidermal junction called “medusa head like” structures (F). Hair follicles are indicated by stars. Scale bar: 200 μ m

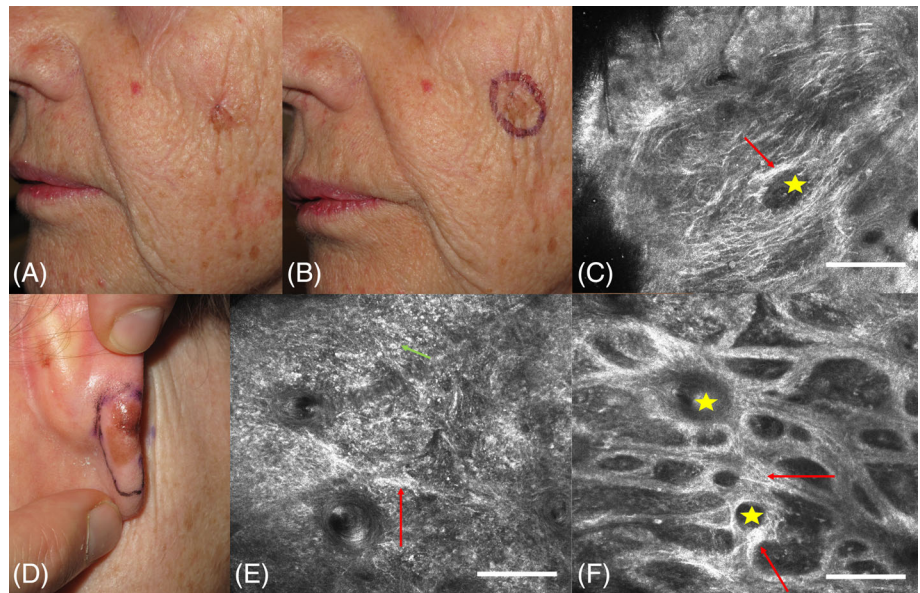
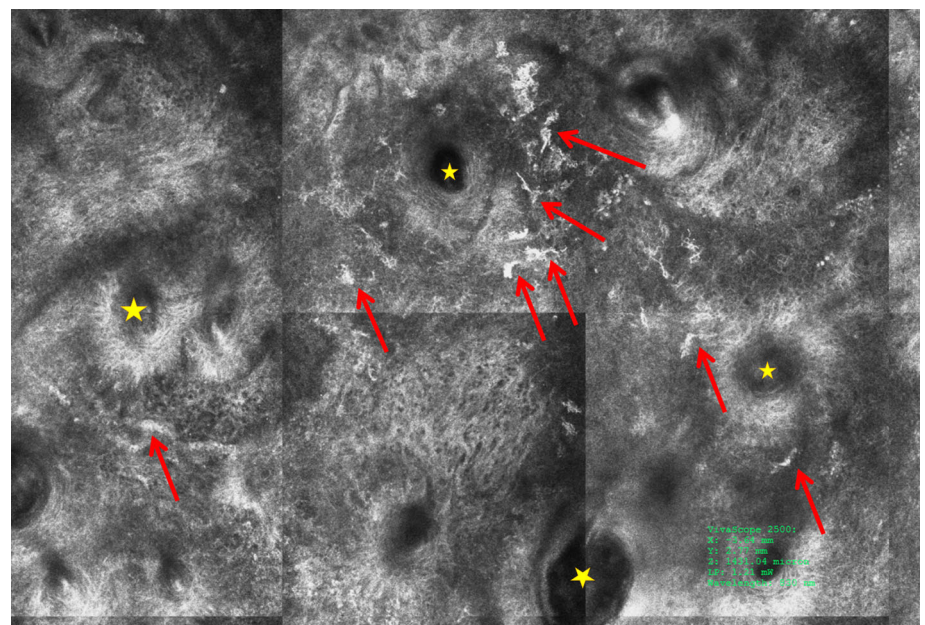


FIGURE 2 Ex vivo reflectance confocal microscopy examination of a lentigo maligna shows large hyper-reflective dendritic cells (red arrows) in the epidermis. Hair follicles are indicated by stars. Each square composing the mosaic has a diameter of 750 μ m



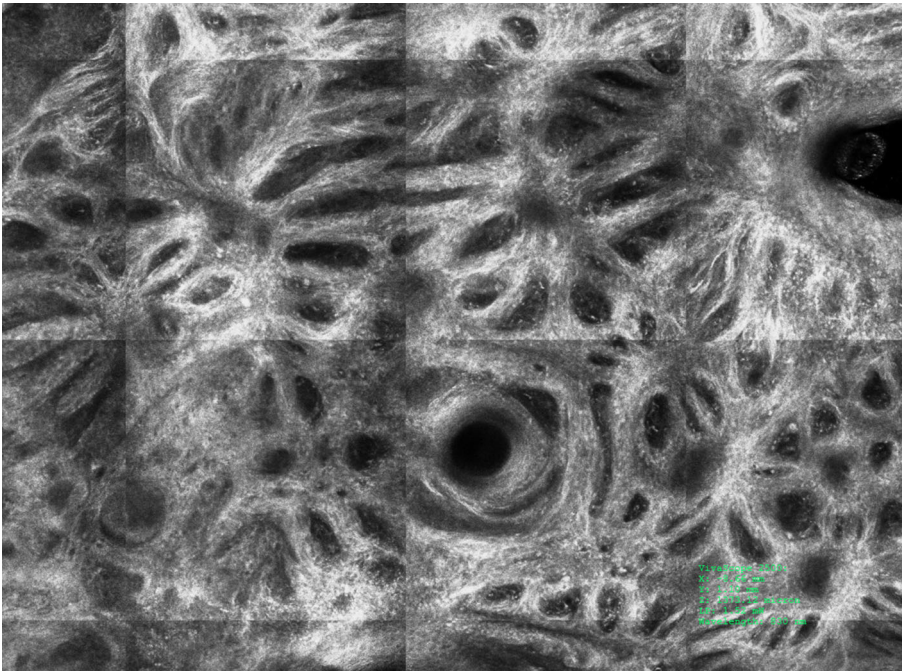


FIGURE 3 Ex vivo reflectance confocal microscopy of a lentigo maligna shows “medusa head like” structures. Each square composing the mosaic has a diameter of 750 μm

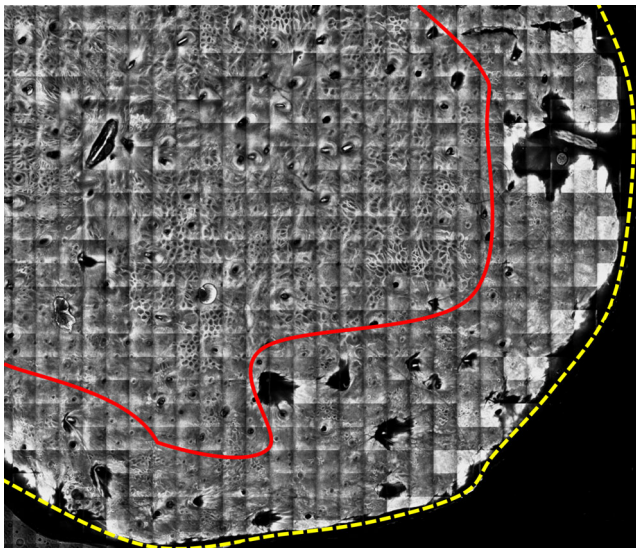


FIGURE 4 Ex vivo reflectance confocal microscopy of a lentigo maligna shows tumor free margins. The lesion is located on the left of the red line and the tumor margin is indicated by the yellow line. Each square composing the mosaic has a diameter of 750 μm

real-time bedside pathology. Up to date, only one LM [15] and two mucosal MMs [11, 24] were imaged under EVC. Differently from the previous cases, we demonstrated that is also possible to image these tumors in horizontal sections with the “en face” technique [20]. Horizontal EVC images were similar to those obtained under in vivo RCM and allow to better observe the horizontal spread of malignant melanocytes, that is,

particularly relevant in LM/LMMs and mucosal MMs, compared to the conventional EVC technique with vertical sections. [21]

MMs were observed directly under EVC with the reflectance mode, thanks to the spontaneous contrast provided by melanin, without needing any staining. However, fluorescence could improve MM identification using fusion imaging of the new EVC device that can merge reflectance and fluorescence signals or using fluorescent-labeled antibodies. [12, 14]

4.3 | EVC for the identification of the tumor margins of MM

Our study showed a good EVC rate of correct identification of the tumor margins with only one false-positive and one false-negative result. EVC allows an exhaustive examination of the margins of the tumor as MMS procedure. Differently from MMS, it can be performed extemporaneously on the fresh specimens, which do not need to be frozen, cut or stained. Notably, it is a non-destructive procedure, therefore enabling further histopathological examination. It has been demonstrated that EVC can be a fast alternative to MMS for skin carcinomas [8–10] and this could be even more relevant for MM because frozen-section processing often causes morphologic alterations of keratinocytes, which make them difficult to differentiate from melanocytes even with rapid immunostaining [25]. EVC could be particularly suitable when the clinical identification of MM margins is

difficult as in LM/LMM and mucosal MM. In our series, EVCM was concordant with the histopathological examination in all but two cases.

There was one EVCM false-negative case and the retrospective evaluation of the EVCM images showed some interruptions in the margins, which could explain the false absence of tumor cells due to a nonexhaustive examination. We, therefore, suggest paying attention to the positioning of the surgical specimen between the two glass slides, to ensure a homogeneous flattening of the specimens and complete visibility of the whole surgical margins. In our series, we put a transparent ophthalmic gel under the surgical specimen to facilitate the adaptation of the uneven surface of the tumor to the slides and new techniques are developing to achieve complete flattening of the specimens and to increase the quality of the EVCM images [8].

Our false-negative EVCM examination was our ninth case and it should be noted that for this technique a learning curve exists not only for the interpretation of the images as it is for in vivo RCM [4] but also for the mounting of the surgical specimen. We found one EVCM false-positive case and retrospective evaluation showed no MM signs on histopathology despite serial sectioning of the corresponding tissue. This false-positive result could be related to the presence of isolated atypical cells that can be observed in chronically photoexposed skin. It should also be noticed that EVCM could be also more accurate than conventional histological examination for the analysis of MM margins because pathologists have an overview of only one part of the tumor margins, unlike EVCM, which views the whole tumor margin in the horizontal plane and at different depth. Moreover, the false-positive case concerned the ear that is a topographically demanding location together with the nose and the eyelid due to the overall dimensions of the in vivo RCM camera and the difficulty of flattening cartilaginous areas for EVCM. Our study was performed with the penultimate generation of the in vivo RCM that has a camera tip of 0.5 cm in diameter and is not harmful for the eye [11], but the last version of the device has a larger camera tip and can damage the retina and therefore it cannot be used to explore areas that are difficult to be reached such as the ear concha or that are closed to the eye such as the eyelid margin [26].

4.4 | Limitations of the study and conclusions

The main limitation of our study in testing EVCM diagnostic accuracy is that the number of cases with positive histological margins was very limited because of the

addition of the in vivo RCM mapping as previously discussed. Another limitation is that EVCM is compared to conventional histological examination and not to longitudinal sections of the peripheral margins as it is done in MMS. However, follow-up was provided showing a recurrence in only one case over a long period.

In conclusion, our series suggests that MM extension is visible under EVCM using reflectance mode and virtual horizontal sections and that the combination of in vivo RCM and EVCM can be a new strategy for the evaluation of surgical margins of MMs. Large series are needed to validate our observations.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Cinotti E, Belgrano V, Labeille B, et al. In vivo and ex vivo confocal microscopy for the evaluation of surgical margins of melanoma. *J. Biophotonics*. 2020;e202000179. <https://doi.org/10.1002/jbio.202000179>