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The SMART approach: Feasibility of Lentigo Maligna Superficial Margin Assessment with handheld Reflectance confocal microscopy Technology

Running Head: Lentigo maligna margin assessment with handheld-RCM

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ABSTRACT

Background: Lentigo maligna may be challenging to clear surgically.

Objective: To evaluate feasibility of using superficial skin cuts as RCM imaging anchors for attaining negative surgical margins in lentigo maligna.

Methods: Included patients presented with lentigo maligna near cosmetically-sensitive facial structures. We evaluated, with handheld-RCM, microscopic clearance of melanoma beyond its dermoscopically-detected edges. Evaluated margins were annotated using shallow skin-cuts. If a margin was positive at 'first-step' RCM evaluation, we sequentially advanced the margin radially

outward at that segment by 2mm intervals until an RCM-negative margin was identified. Prior to final surgical excision, we placed sutures at the outmost skin-cuts to allow comparison of RCM and histopathological margin assessments. Primary outcome measure was histopathological verification that RCM-negative margins were clear of melanoma.

Results: The study included 126 first-step margin evaluations in 23 patients, median age 70 years (range: 43-91). Seventeen patients (74%) had primary in situ melanoma and 6 (26%) invasive melanoma, mean thickness 0.3mm (range 0.2-0.4mm). Six cases (26%) showed complete negative RCM margins on 'first-step', 11 (48%) were negative at 'second-step', and 4 (17%) at 'third-step'. In two additional cases (9%), margins clearance could not be determined via RCM due to widespread dendritic cells proliferation. The RCM-negative margins in all 21 cases proved clear of melanoma on histopathology. Of the 15 cases that returned at one-year-follow-up, none showed any residual melanoma on dermoscopic and RCM examinations. Inter-observer reproducibility showed fair agreement between bedside RCM reader and blinded remote-site reader, with Spearman's rho of 0.48 and Cohen's kappa of 0.43; using bedside reader as reference, the remote reader's sensitivity was 92% and specificity 57% in positive margin detection.

Conclusions: Margin mapping of lentigo maligna with handheld-RCM, using superficial skin cuts, appears feasible. This approach needs validation by larger studies.

INTRODUCTION

Lentigo maligna, a subtype of melanoma arising on the sun-damaged facial skin may be challenging to clear surgically.[1, 2] This melanoma is notorious for presenting with ill-defined clinical borders and microscopic infiltration of neoplastic melanocytes along a flattened dermal-epidermal junction that frequently extends well into normal-appearing surrounding skin. The proximity of melanoma to functionally- and cosmetically-sensitive facial structures (e.g., eyes) makes radical excision prohibitive, while overly conservative surgery may lead to melanoma persistence or recurrence.[3] The recommended wide excision margins of 5mm are frequently insufficient for complete clearance

of lentigo maligna.[4] Thus, precise margin delineation is warranted to guarantee complete removal of the melanoma, while minimizing healthy-tissue removal.

Reflectance confocal microscopy (RCM) is an *in vivo* imaging technique that enables visualization of skin at cellular-level resolution**.**[11] RCM has shown high sensitivity for detecting proliferation of neoplastic cells in melanomas on sun-damaged skin.[12] For this reason, in difficult cases, researchers have proposed to integrate RCM-based margin mapping with excisional or Mohs surgery. Until recently, wide-probe-RCM (Vivascope 1500®, MAVIG GmbH, Munich, Germany) was used for margin-mapping of facial skin cancers. The wide-probe-RCM can generate large skin maps (up to 8X8mm^2), which correlate well with clinical and dermoscopic images, ensuring that areas identified by RCM as tumor-positive may be identified clinically or dermoscopically after the RCM probe is removed.[13-15] However, the wide-probe-RCM is bulky, relatively-cumbersome and technically difficult for contoured anatomic sites, so that margins delineation of large melanoma takes over an hour.

RCM skin imaging can also be obtained with Handheld-RCM (HH-RCM, Vivascope 3000®, MAVIG GmbH, Munich, Germany). While having a more limited field-of-view (0.85x0.85mm^2), [16, 17] HH-RCM allows for rapid assessment of multiple points at the margins of skin cancer. However, the lack of firm skin attachment hinders the identification of the precise area that is being imaged and limits clinical-RCM orientation. To that end, we devised a technique of using superficial skin cuts as clinical-RCM orientation anchors during HH-RCM pre-surgical margin mapping. [18,19] The cuts can be readily recognized with the naked eye, as well as during RCM imaging. In addition, the cuts can be readily seen and allow further orientation during histopathologic verification of margins.

In this study, we sought to evaluate and describe the feasibility of HH-RCM margin mapping using superficial skin cuts in a series of lentigo maligna.

MATERIALS AND METHODS

The study was conducted in two tertiary academic medical centers. Consenting patients, with primary or recurrent lentigo maligna, with difficult-to-assess clinical margins or located near cosmetically- or functionally-sensitive facial structures (**Fig. 1**), were consecutively enrolled. Prior to surgery, all patients underwent HH-RCM margin mapping using superficial skin cuts. The Institutional Review Board approved the study; written informed consent was attained from all participants.

Imaging procedure

Cases of biopsy-proven lentigo maligna or cases with clear-cut melanoma features on wide-probe-RCM were recruited. Dermatoscopic images were acquired with non-contact polarized light dermoscopy (Dermlite HR, 3Gen, San Juan Capistrano, CA, USA) and HH-RCM (Vivascope 3000®) images were attained using previously described methods.[16]

The margins were evaluated during live HH-RCM imaging and captured as a movie. The following were considered melanoma-specific RCM attributes: (1) proliferation of dendritic cells within disarrayed basal or spinous layers of epidermis, appearing as densely-crowded and tangled bright lines (dendritic processes), along with occasionally-visible nucleated cell bodies; (2) presence of multiple round or pleomorphic large nucleated cells at the basal layer or in Pagetoid spread; (3) dendritic or round nucleated cells infiltrating follicular infundibulum; and (4) aggregates of melanocytes (nests) at the dermal-epidermal junction.[12, 20] Margins were considered positive if there were ≥1 round cells >20μm in size, or ≥3 dendritic or round cells regardless of cellular size; and if the melanoma-specific attributes were identified within two fields-of-view (corresponding to 1.7mm) from the superficial skin-cut.

Our adapted technique consisted of the following steps (**Fig. 2**):

(i) Clinical and dermoscopic determination of visible margins. Along with clinical inspection, we used dermoscopy to delineate visible melanoma margins. We then marked with a dermographic pen the first-score ('Score I') perimeter, 2mm beyond the clinically- and dermoscopically-determined borders (**Fig. 2a**).

(ii) Margin scoring. We anesthetized the margins, by subcutaneous infiltration of 0.1% diluted carbocaine immediately before the procedure (in 14 patients) or by occlusive application of topical anaesthetic, EMLA Cream (Lignocaine 25mg/g, Prilocaine 25mg/g), one hour prior (in 9 patients). A superficial cut was made with either a scalpel (blade #11) or tip of a needle, overlying the dermographic pen line at four or more cardinal points (**Fig. 2b, eVideo1**). Bleeding was readily stopped with sterile gauze.

(iii) RCM imaging and margin exploration. The RCM probe was initially placed at the center of the lesion and maneuvered outwards in a linear direction until Score I was visualized.

(iv) ***Delineation of clear margins*****.** Once Score I was identified, the area beyond the cut was examined for features of melanoma. The margin was 'RCM-negative' when melanoma-specific findings were absent in at least two fields-of-view (>1.7mm) inward from the cut, and completely absent outward from the cut (**Fig. 2e, eVideo1**). The margin was 'positive' if melanoma features were seen within two fields-of-view (≤1.7mm) of the superficial cut at any direction, inward or outward (**Fig. 2f, eVideo1**). We ascertained that the two adjacent RCM fields did not overlap by using tissue structures (e.g. follicle, surface glyphics), located at the transition between consecutive RCM fields, as reference anchors. If the margin was positive, but did not extend beyond Score I, the RCM probe would be removed, and a further cut would be made 2mm away from Score I, and this became Score II. (**Fig. 2c**). However, if the margin was positive and extended outward beyond Score I, then the exact number of melanoma positive RCM fields-of-view beyond Score I were counted; Score II would be made at a radial distance of 2mm from the outermost positive RCM

field. For example, if the positive margin extended by two RCM fields-of-view beyond Score I, then Score II would be made at distance of 3.7mm (0.85mm [breadth of RCM field-of-view] X 2 + 2mm additional safety margin) from Score I. Subsequently, Score II would be examined with RCM in a similar manner. Again, if Score II was positive on RCM, Score III would be made, and so on, until negative RCM margin achieved. This process was repeated for every cardinal point of the tumor perimeter. A final RCM-based negative margin was drawn around the entire perimeter of the melanoma by connecting the outermost skin cuts (**Fig. 2d**).

Surgical procedure and patient follow-up

Patients proceeded to surgery with the final border marked. When the outlined border was close to important facial structures (e.g. eyes), then excision followed exactly the RCM-annotated borders. However, for lentigo malignas that did not encroach on facial structures, an additional safety margin of 3mm was attained beyond the RCM-defined borders. We added a safety margin since this was the first feasibility study of this mapping procedure in lentigo maligna. To allow comparison of RCM and histopathological margin status, numbered silk sutures marked the locations of the outermost superficial skin-cuts (**Figs 2g-h**).

All patients were scheduled for follow-up visit after 6 and 12 months. Follow-up visits included dermoscopic examination and RCM imaging of the area surrounding the scar to exclude recurrent or persistent melanoma.

Reproducibility study

To validate reproducibility of RCM reading of the procedure, we randomly-selected RCM imaging videos of 33 margin-segments from 10 patients that were retrospectively evaluated by an external reader (AS), blinded to any clinical information, and to clinical or dermoscopic images. The external reader was asked to 1) identify the superficial cut in RCM videos and annotate it (on RCM images obtained via "print-screen") and, 2) to evaluate RCM-margin clearance.

Statistical Section

Absolute and relative positive margins have been calculated for each stage.

For the reproducibility study, Spearman's rho correlation index and Cohen's kappa were calculated to evaluate inter-observer reproducibility, using the bedside reader as reference standard.

RESULTS

The study included 23 patients (10 females), median age 70 years (range: 43-91 years), enrolled between December 2014 and March 2016 at the Dermatology Department of University of Modena and Reggio Emilia (n=17) and at Melanoma Institute Australia (n=6). Sixteen patients (70%) had primary in situ melanoma (lentigo maligna); one (4%) - recurrent in situ melanoma; and 6 (26%) invasive melanoma (lentigo maligna melanoma), mean Breslow thickness 0.3 mm (range 0.2-0.4 mm). For each melanoma, precise anatomic location and clinical and dermoscopic attributes are summarized in **Table 1.** Notably, 18 melanomas (78%) displayed clinically-detected margins at a distance of ≤1cm from functionally- or aesthetically-sensitive facial structures.

For the first-step (Score I) RCM margin imaging, scoring of the entire peripheral perimeter, i.e. at 2mm margin beyond clinically- and dermoscopically-visible edges, required a median of 5 contiguous superficial linear cuts (mean=5.5, SD=1.3, range 4-8 cuts) (**Table 1**). In all, a median of 2 (mean=2.8, SD=1.5, range 0-6) RCM-positive margins were detected per lesion.

Of 126 Score I margin evaluations from 23 patients, 47 margins (37%) from 17 patients were RCM-positive; in 6 of the 23 patients (26%), the entire Score I perimeter, comprising of a total 25 margins showed RCM-clearance.

We proceeded to Score II evaluations in 15 of the 17 patients with positive Score I evaluation. However, in two of the 17 patients (**Table 1**, cases#10&22), we identified all 12 Score I margins as positive, and extending as far as 3cm beyond the Score I perimeter – hence, we did not proceed to Score II, since we were unable to identify RCM negative margins, because dendritic cells were visualized across all margins. In the remaining 35 Score II margin evaluations from 15 patients, 4

margins from 4 patients were still RCM-positive, while the remaining 31 margins from 11 patients showed clearance of Score II. The four remaining patients showed RCM-clearance of the four remaining Score III margins.

Overall, the time required to complete the SMART procedure ranged between 20 to 75 minutes and was proportional to the length of Score I perimeter and the number of positive margins imaged before identifying RCM-cleared margins.

Histopathological examination confirmed that margins were free of melanoma in all 21 cases in which negative RCM margins were attained. The minimal distance between the superficial linear incision and residual melanoma was 1.7mm, observed in one segment of a melanoma (patient#5) which extended to the lower eyelid (**Figs 2a&f**). A 6-month- and 1-year-follow-up was available for 15 of the 21 RCM-cleared melanomas – none showed residual or recurrent melanoma under clinical, dermoscopic and RCM examinations.

The two melanomas in which RCM failed to identify clear margins (**Table 1**, cases#10&22) were excised with 5mm margins around the first-step perimeter. Histopathological analysis, including Melan-A (MART-1) immunostaining, showed increased density of single melanocytes at the margins. The pathologist was unable to ascertain clearance of margins, in the absence of clear-cut distinction between melanocytic hyperplasia related to sun-damaged-skin and residual melanoma in situ. The area around the surgical scar was subsequently treated with adjuvant 5% imiquimod cream for 12 weeks. We continued to monitor the area around the scar, and at 12 months of follow-up, there was no evidence of local recurrence. Notably, following this experience, we now explore the surrounding skin beyond the dermoscopic border with RCM, before making the Score I cuts, to ensure the proliferation of melanocytes is confined and not widespread.

We tested inter-observer agreement on reading of RCM videos of the SMART procedure. The blinded evaluator correctly identified the superficial cut in 31 (94%) of the 33 RCM margin-videos. The blinded evaluator agreed with the bedside reader in RCM margin evaluation of 11 out of the 12 positive videos (sensitivity=92%) and in 12 of the 21 negative RCM margin-videos

(specificity=57%), obtaining Spearman's rho of 0.481 (p=0.005) and Cohen's kappa of 0.427 (p=0.006).

DISCUSSION

RCM offers new opportunities for precise margin mapping of melanoma on the face.[21] Because RCM images skin in the horizontal plane, the proliferation of atypical melanocytes can be readily detected *in vivo*, making RCM-based diagnosis of melanoma more accurate than dermoscopic diagnosis.[12, 22] Herein, we report on the applicability of using HH-RCM for detecting negative margins in melanomas of the face, beyond the clinically- and dermoscopically-determined margins. The current study focused on melanomas located adjacent to functionally- or cosmetically-sensitive facial structures. In these cases, attaining the recommended wide safety margins is difficult and hence, the practical aim is to achieve clear narrow margins via precisely-determined tumor borders, akin to the principle of Mohs surgery.

We found that dermoscopically-assisted clinical delineation was accurate in predicting precise tumor border in only 26% of cases. The paucity of dermoscopic clues at the melanoma margins is hardly surprising, given that the single cell proliferation of melanocytes along the flattened dermal-epidermal junction is insufficient to produce strong contrast under dermoscopy. However, with RCM, melanocytes as single cells and small aggregates appear sufficiently bright to be readily distinguished with good contrast from the background epidermis. In fact, RCM-based tumor border delineation could be determined in 91% of cases; the accuracy of RCM-determined margins was confirmed by histopathological examination, which showed clear margins in all RCM-negative margins. The superficial cut did not appear to produce false negative RCM findings (e.g., due to inflammation). Our findings are concordant with a recent HH-RCM-histopathology correlative study of the diagnosis of lentigo maligna-type melanoma on head and neck; the authors imaged with HH-RCM 63 sites from 17 melanomas and found a negative predictive value for RCM evaluation of 100% (i.e., an RCM negative site was negative, in all cases, for melanoma under

histopathology), with positive predictive value of 85%.[23] In a larger study of 33 consecutive lentigo maligna-type melanomas of the head, only 0.2% (6.7 mm of 3158 mm) of first-step lentigo maligna margins, whose surgical clearance was guided by HH-RCM imaging, were found to be positive under histopathology requiring wider surgical clearance.[14]

In two cases, it was not possible to determine the precise tumor borders with RCM because numerous dendritic cells were present far beyond the clinical borders. At present, we are unable to predict, in advance, which melanomas will fail RCM margin delineation because of dendritic cell proliferation. Sun-damaged skin[24] can display proliferation of dendritic melanocytes with increased density, simulating a malignant proliferation. In these cases, precise tumor margin delineation is difficult not only with RCM, but also in paraffin-embedded tissue sections.[25] Abundance of Langerhans cells, which have dendritic cellular outlines similar to melanocytes,[26] may also confound precise RCM margin mapping.

The SMART approach has advantages. First, margin mapping is non-invasive, and can be completed pre-operatively, so that surgery can be performed in one stage. Integrating RCM imaging with Mohs surgery may eventually save time by reducing the required number of Mohs stages. Second, RCM imaging allows not only preoperative assessment, but can potentially be extended to intraoperative search for residual cancer[27,28] and postoperative monitoring for local recurrence.[29] Third, our interobserver agreement study has shown that positive RCM findings for melanoma diagnosis, as well as the location of the superficial cut, can be readily identified.

The SMART approach also has potential constraints. First, pathology lab specimen cut-up protocols may need to be altered to accommodate the assessment of all RCM-determined margins and associated sequential cuts, thus communication with the pathology team is essential. Second, restraints applicable to RCM in general apply, such as high cost of equipment, the need for expertise with HH-RCM imaging and with assessment of melanoma margins. Additional cost and time restraints, suggest the SMART procedure is likely to be most beneficial for pre-operative or

intra-operative margin assessment of melanomas on the face, where it is anticipated wide margins will be difficult to attain. Finally, the HH-RCM probe cannot be sterilized and hence thorough cleaning after each procedure is required.

Our study has limitations. First, RCM examination is operator-dependent. The study was conducted at two centers with marked expertise in RCM imaging, and was done on a limited series of cases. Generalizability of the results need to be shown in more patients and across more centers. It is probable that SMART procedure may take longer, at least initially, in less experienced hands. Second, we need longer follow-up data on study participants concerning local recurrence of melanoma. Third, histopathological correlation of RCM margins was limited to the final superficial cuts at the outmost margins, to ensure negative margins were attained. However, a recent lentigo maligna margin mapping RCM-histopathological correlation study has shown that RCM is quite accurate in predicting histopathological clear margins; RCM underestimated the final histopathology-confirmed margin of staged surgery by a mean of 0.76 mm (95% CI: 0.67–0.84mm).[30]

In conclusion, while the present study should be considered preliminary, the SMART procedure appears to be feasible for margin mapping of melanomas on the face.

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TABLE 1: Clinical and dermoscopic characteristics and outcome of RCM margin evaluations

Case #	Sex / age (decades)	Histopathological characteristics	Location, Size, Color	Minimal distance from important facial anatomic structures	Dermoscopic findings	Score I: Total cuts / positive margins	Score II: Total cuts / positive margins
1	F/ 80s	Recurrent melanoma in situ	Right cheek, 0.4 x 0.3cm, Light brown	1.4cm from lower eyelid rim	Light brown pseudonetwork	8 / 2	2 / 0
2	M / 50s	Melanoma 0.21mm	Nose, 0.7 x 0.5cm, Dark and light brown	Located on nasal tip	Irregular pseudonetwork, annular granular pattern, irregular globules	6 /4	4 / 0
3	M / 70s	melanoma in situ	Scalp, 1.4 x 0.8cm, Dark brown	No important structures in proximity	Irregular pseudonetwork, asymmetric follicular pigmentation, rhomboidal structures	6 / 2	2 / 0
4	M / 70s	melanoma in situ	Right cheek, 2.8 x 1.8cm, Light brown	0.8cm from lower eyelid rim	Asymmetric follicular pigmentation, angulated	7 / 3	3 / 0

					pigmented lines		
5	F/ 50s	Melanoma 0.3mm	Left cheek, 1.4 x 0.8cm, Light brown	0.2cm from the lower eyelid rim	Irregular pseudonetwork, annular granular pattern	7 / 4	4 / 0
6	M / 60s	Melanoma 0.35mm	Nose, 1.3 x 1.2cm, Dark brown	Located on nasal tip	Rhomboidal structures, annular granular pattern	7 / 4	4 / 1

'TABLE 1 CONTINUED'

Case #	**Sex / age (decades)**	**Histopathological characteristics**	**Location, Size, Color**	**Minimal distance from important facial anatomic structures**	**Dermoscopic findings**	**Score I: Total cuts / positive margins**	**Score II: Total cuts / positive margins**
7	M / 90s	Melanoma 0.3mm	Left cheek, 1.1 x 1.0cm, Dark brown	1cm from lower eyelid rim	Chrysalis, peppering, streaks	6 / 2	2 / 0
8	M / 70s	melanoma in situ	Right retro-auricular region, 3.8x3.1cm, Dark and light	<0.1cm from retro-auricular fold	Rhomboidal structures, annular granular pattern, irregular pseudonetwork	6 / 2	2 / 0

			brown				
9	F/ 50s	melanoma in situ	Right cheek, 1.2 x 0.7cm, Light brown	0.4cm from lower eyelid rim	Annular granular pattern, irregular pseudonetwork	5 / 0	
10	F/ 40s	melanoma in situ	Left cheek, 1.2 x 1.0cm, Light brown	1cm from eyelid lateral canthus	Annular granular pattern, irregular pseudonetwork	6 / 6	Not applicable*
11	M / 70s	melanoma in situ	Pre-auricular, 1.1 x 1.0cm, Dark brown	0.5cm from tragus of ear	Rhomboidal structures, asymmetric follicular pigmentation	M / 70s	
12	M / 80s	melanoma in situ	Ear, 1.0x0.8cm, Pink / light brown	0.8 cm from antitragus	Not available**	4 / 2	2 / 0

'TABLE 1 CONTINUED'

Case #	Sex / age (decades)	Histopathological characteristics	Location, Size, Color	Minimal distance from important facial anatomic structures	Dermoscopic findings	Score I: Total cuts / positive margins	Score II: Total cuts / positive margins
13	M / 70s	melanoma in situ	Lip, 2.0 x 1.4 cm, Dark brown	On lip and oral mucosa	Not available**	4 / 0	
14	F / 60s	melanoma in situ	Nose, 3.0 x 1.2 cm, Light brown	0.7 cm from inner canthus	Brown globules, increased vascularity	4 / 0	
15	F / 50s	melanoma in situ	Nose, 1.0 x 0.8 cm, Pink	On nasal alar area, 0.1 cm from naris	Not available**	4 / 0	
16	M / 70s	Melanoma 0.4mm	Left cheek, 3.0 x 2.5 cm, Dark brown	*No relevant anatomical structures in proximity*	Dots within follicle, rhomboidal structures, asymmetric follicular pigmentation	4 / 0	
17	F / 80s	melanoma in situ	Nose, 2.5 x 1.5 cm, Dark brown	On nasal alar area, 0.1 cm from naris	Rhomboidal structures, asymmetric follicular pigmentation, annular granular pattern	8 / 2	2 / 1

'TABLE 1 CONTINUED'

Case #	Sex / age (decades)	Histopathological characteristics	Location, Size, Color	Minimal distance from important facial anatomic structures	Dermoscopic findings	Score I: Total cuts / positive margins	Score II: Total cuts / positive margins
18	F / 60s	melanoma in situ	Left cheek, 3.1 x 1.3 cm, Brown	0.3 cm from the lower eyelid rim	Irregular pseudonetwork, annular granular pattern	5 / 2	2 / 0
19	F / 40s	melanoma in situ	Left cheek, 0.8 x 1.1 cm, Pink	0.2 cm from the lower eyelid rim	Increased vascularity, peripheral homogeneous tan pigmentation	4 / 2	2 / 0
20	F / 80s	Melanoma 0.17mm	Forehead,2.1 x 1.7 cm, Pink/light brown	*No relevant anatomical structures in proximity*	Increased vascularity, areas of homogeneous tan pigmentation	5 / 2	2 / 1
21	M / 70s	melanoma in situ	Forehead, 0.4x0.3 cm, Pink	*No relevant anatomical structures in proximity*	Increased vascularity	5 / 1	1 / 1
22	M / 70s	melanoma in situ	Nose, 2.0 x 1.8 cm, Light brown	On nasal alar area, 0.3 cm from naris	Light brown irregular pseudonetwork	6 / 6	Not applicable
23	M / 60s	melanoma in situ	Scalp, 2.9 x 3.2 cm, Light Brown	*No relevant anatomical structures in proximity*	Light brown irregular pseudo-network, annular granular pattern	5 / 1	1 / 0

FIGURE LEGEND

Figure 1. Clinical, dermoscopic and RCM views of melanoma 0.31mm in thickness on the cheek (patient #5). (**a**) The lesion extends up to 0.2cm from the lower eyelid rim. (**b**) Dermoscopy displays ill-defined borders, pseudonetwork with irregularity of color and thickness of the lines, and sporadic dots. (**c**) Wide-probe RCM mosaic image (3X2mm^2) at the dermal-epidermal junction level showing irregular meshwork pattern with crowded junctional nests (asterisk) that show variability in brightness, thickness, shape and spacing, suggestive of melanoma (white bar = 500µm). (**d**) At higher magnification (1.25X1mm^2), the RCM image shows the proliferation of large roundish and dendritic atypical cells alongside the junctional aggregates (asterisk, white bar = 500µm).

Figure 2. Positive and negative RCM and histopathological margins of melanoma 0.31mm in thickness (patient #5). **(a)** Clinically and dermoscopically-determined margins were marked with red pen. **(b)** Seven short (3 to 5 mm) superficial linear skin cuts were made with a scalpel following the red pen mark. **(c)** The blue dotted lines correspond to second-step cuts performed in four segments that showed positive first-step RCM margins; all four second-step margins proved to be negative. **(d)** Black pen mark represents the new surgical RCM-confirmed margins, while the red line corresponds to initial dermoscopic margins. Of note, at the eyelid margins (#1-2), the final marking follows the RCM margins, while at the furthest margins (#5), 3mm safety margins were added beyond RCM-negative margin. **(e)** HH-RCM image at the basal to spinous epidermis showing the superficial cut and absence of melanoma features at first-step margin #2, immediately below the eyelid border (white bar = 100µm). **(f)** RCM image showing a melanoma-specific feature, namely proliferation of numerous dendritic cells, close to the first-step superficial cut at margin #3 (white bar = 100µm). **(g)** Histopatology section (Hematoxylin and eosin, 4x) corresponding to margin #2 showing the junctional proliferation of solitary melanocytes at a clearance distance of 1.7mm from the superficial skin cut. **(h)** Histopatology section (Hematoxylin

and eosin, 4x) corresponding to margin #3 showing the proliferation of atypical melanocytes abutting close to the superficial cut.

SUPPLEMENTARY MATERIAL

eVideo 1 (MP4): Margin scoring and RCM-based margin mapping. After local anesthesia, a series of contiguous superficial linear skin cuts, each 3-5mm in length, are made with a scalpel; the scores outline 'first-step' perimeter, follow the pen-marked contour that was based on clinical and dermoscopic inspection. Margin detection with the HH-RCM probe starts with visualization of the linear cut under RCM. The margin is 'RCM-negative' when melanoma-specific findings are absent in at least two fields-of-view (>1.7mm) inward from the cut, and completely absent outward from the cut. The margin is 'RCM-positive' when melanoma-specific features (e.g. proliferation of dendritic cells in the epidermis) are seen within two fields-of-view (≤1.7mm) of the superficial cut at any direction (inward or outward).