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Peri-operative delineation of non-melanoma skin cancer margins *in vivo* with handheld reflectance confocal microscopy and video-mosaicking

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Abstract

Background—The surgical removal of non-melanoma skin cancers (NMSCs) is guided by the pathologic examination of margins. However, the preparation of histopathology is time consuming, labor-intensive, and requires separate laboratory infrastructure. Furthermore, when histopathology indicates positive margins, patients must return for re-excisions. Reflectance confocal microscopy (RCM) with a new video-mosaicking approach can noninvasively delineate margins directly on patients and potentially guide surgery in real-time, augmenting the traditional approaches of histopathology.

Objective—To assess a new peri-operative RCM video-mosaicking approach for comprehensive delineation of NMSC margins on patients *in vivo*.

Methods—Thirty-five patients undergoing Mohs micrographic surgery (MMS) in the Mohs surgery unit at Memorial Sloan Kettering Cancer Center, New York, NY were included in the study. RCM imaging was performed before and after the first staged excision by acquiring videos along the surgical margins (epidermal, peripheral and deep dermal) of each wound, which were subsequently processed into video-mosaics. Two RCM evaluators read and assessed video-mosaics, and subsequently compared to the corresponding Mohs frozen histopathology.

Results—RCM videos and video-mosaics displayed acceptable imaging quality (resolution and contrast), pre-operatively in 32/35 (91%) NMSC lesions, and intra-operatively in 29/35 lesions (83%). Pre-operative delineation of margins correlated with the histopathology in 32/35 (91%) lesions. Intra-operative delineation correlated in 10/14 (71%) lesions for the presence of residual tumor and in 18/21 (86%) lesions for absence. Sensitivity/specificity were 71%/86% and

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86%/81% for two RCM video-mosaic evaluators, and overall agreement was 80% and 83% with histopathology, with moderate inter-evaluator agreement ($k=0.59$, $p=0.0002$).

Conclusions—Peri-operative RCM video-mosaicking of NMSC margins directly on patients may potentially guide surgery in real-time, serve as an adjunct to histopathology, reduce time spent in clinic and reduce the need for re-excisions. Further testing in larger studies is needed.

Introduction

The surgical removal of non-melanoma skin cancers (NMSCs) is guided by the pathologic examination of margins (1, 2). The clearance of margins is confirmed either with post-operative permanent pathology for standard surgical excision or with intra-operative frozen pathology of each staged excision during Mohs micrographic surgery (MMS). However, the preparation of histopathology is time consuming, labor-intensive, and requires separate laboratory infrastructure (3, 4). Furthermore, when histopathology indicates positive margins, the patient must return for re-excision. In the case of MMS, the patient must undergo additional staged excisions. A non-invasive real-time high-resolution optical imaging modality such as reflectance confocal microscopy (RCM) offers a complementary approach: peri-operative detection of margins directly on patients *in vivo*. RCM imaging diagnoses NMSCs *in vivo* with high sensitivity and specificity (5–7). Beyond diagnosis, we can detect margins, to potentially guide treatment and to augment the standard approaches of pathology.

Several pilot studies have reported the feasibility of RCM imaging for detecting basal cell carcinomas (BCCs) in wounds during and after surgeries (8–11), as well as for assessing risk of recurrence (12–14). In a recent study, RCM evaluation correctly delineated clinically and dermoscopically ill-defined margins of BCCs, and pre-operatively guided surgical re-excision, resulting in clear margins (14).

In all previous studies, however, the demonstrated feasibility and the imaging of margins were limited to a small number of discrete locations in the NMSC lesions. Furthermore, the sampling of the margins at each location was limited to either single images or small square-shaped areas, displayed in mosaics of images. (Mosaicking of a two-dimensional sequence of RCM images is currently a widely used approach to increase the sampling area.) (15). However, comprehensive delineation of margins requires imaging over larger freeform-shaped and contiguous areas. This is now possible with our newly developed RCM video-mosaicking algorithm (16). Instead of acquiring (and mosaicking) still images, we acquire videos with a handheld confocal microscope. The videos are subsequently processed into video-mosaics to display larger and contiguous freeform-shaped areas. Our approach for RCM video-mosaicking is similar, in concept, to capturing of a widefield panorama picture with a mobile phone camera.

In a clinical study, we tested a peri-operative RCM video-mosaicking approach for the delineation of NMSC margins directly on patients undergoing Mohs surgery. We report the results of this study, and discuss current limitations and future potential utility of RCM video-mosaicking for real-time delineation of NMSC margins *in vivo*.

Materials and methods

Study population

Patients undergoing Mohs surgery for NMSCs were recruited and consented under a research protocol approved by the Memorial Sloan Kettering Cancer Center (MSKCC) Institutional Review Board.

Instrumentation and imaging procedure

Imaging was performed with a handheld reflectance confocal microscope (Vivascope 3000; Caliber Imaging and Diagnostics, Rochester NY). The lens includes a window that provides a contact interface to skin. The window was gas sterilized between use on patients, with no loss of imaging quality. Imaging was performed on all patients before the start of Mohs surgery and immediately after Mohs stage I excision.

We utilized an imaging protocol that was previously developed for RCM imaging in Mohs surgical wounds (eFigure1 in the Supplement) (17). As per standard procedure, the Mohs surgeon ink-marked the entire surgical margin of each lesion, followed by infiltration of local anesthesia. A score was performed with a scalpel along the entire ink-marked margin, as well as at the 12, 3, 6 and 9 o'clock positions. A drop of Crodamol STS oil (Croda Inc., Edison, NJ, U.S.A.) was applied to the surface of the skin. We acquired videos along the scored surgical margin of the lesion in a clockwise fashion. Videos were acquired at a single depth (approximately 120–150 μm) at the dermal-epidermal junction. After pre-operative imaging, Mohs stage I excision was performed. After excision, we topically applied 35% aluminum chloride (AlCl_3) inside the wound, using sterile applicators, for 30 seconds. AlCl_3 has been shown to brighten nuclear morphology and enhance contrast of NMSC tumors in RCM images (8, 10, 17). Afterwards, the wound cavity was filled with sterile gel (Surgilube, Fougera, Melville, NY). Again, RCM imaging was performed, this time, inside the wound, along the surgical margin in a clockwise fashion.

Acquisition of Videos and Video-mosaics

Pre- and intra-operative imaging was performed in quadrants (17). Either one or two videos, depending on the lesion size, were acquired pre-operatively in each quadrant, starting at the 12 o'clock position and traversing in a 360° clockwise direction, along the scored surgical margin of each lesion. Similarly, videos were acquired intra-operatively in the surgical wound bed starting at the 12 o'clock position and traversing in a 360° clockwise direction, along the peripheral edge (epidermal and peripheral dermal margin) and including the base (deep dermal margins) of the wound.

In most cases, videos were taken from the 12 to 3, 3 to 6, 6 to 9 and 9 to 12 o'clock positions. For select small lesions of diameter less than 1 cm, only one video was acquired, starting and ending at the 12 o'clock position. Videos were acquired by two trained clinicians, a dermatologist (O.Y.) and a clinical imaging researcher (M.C.). Video acquisition was controlled for quality by gently pressing the lens and sterile window against the skin. Videos were acquired slowly and carefully to minimize blur and motion artifacts, by moving the microscope smoothly over the area of interest, while maintaining constant

depth throughout the imaging process. The total video acquisition time ranged from 1–2 minutes for small (diameter < 1 cm) wounds and 3–5 minutes for larger wounds. Each RCM video was processed into a video-mosaic, using our newly developed video-mosaicking algorithm (16). Additional details on the imaging procedure are available elsewhere (17).

Pre- and Intra-operative Assessment of RCM Videos and Video-Mosaics and histopathological correlation

RCM videos and video-mosaics were evaluated by our dermatologist and our imaging researcher (O.Y. and M.C., respectively). The overall quality of each video and video-mosaic was assessed as clinically acceptable or not. If the quality was acceptable, the video and video-mosaic were further evaluated for resolution, contrast and visualization of nuclear and morphologic detail. The nuclear and morphologic features that have been defined in our previous studies (10, 17) were compared to those seen in histopathology.

Histopathology was available for comparison to the RCM videos and video-mosaics. Mohs stage I excisions were processed into frozen sections with hematoxylin & eosin staining, as per standard protocol. These sections were in the *en face* orientation, similar to that of the RCM images. Diagnoses for initial diagnostic biopsies and all Mohs frozen sections were obtained from pathology reports.

Statistical analysis

All descriptive and comparative data analyses were performed using STATA software (version 12.0; StataCorp LP). Parameters for imaging quality and presence or absence of residual tumor were stratified by NMSC subtype (either BCC or SCC). Sensitivity and specificity and the overall agreement between RCM videos and video-mosaics and histopathology for presence or absence of residual tumor were determined for our two evaluators (M.C. and O.Y.). Inter-evaluator agreement between the two of them was calculated using the Cohen's kappa statistic. A two-sided p-value of <0.05 was considered significant.

Results

We imaged a total of 45 patients (41 with biopsy-proven BCC, 4 with biopsy-proven SCC). The lesion diameter ranged from 0.2cm to 2cm (mean=0.7cm). The lesions were located on various body sites including the arm (n=3), hand (n=1), face (n=17), neck (n=4), scalp (n=2), chest (n=4), shoulder/back (n=7) and leg (n=7).

Training set (n=10) for acquisition of videos and video-mosaicking

Imaging on the first 10 patients served as a training set for our dermatologist and imaging researcher (O.Y. and M.C.) to test the use of AlCl₃ (swabbing times up to 1 minute) and test the acquisition of videos and processing into video-mosaics. The objective was to test a previously developed RCM imaging and video-acquisition protocol (details available elsewhere) (17), and to establish a standardized approach for this study.

We acquired RCM videos, both pre- and intra-operatively, on 10 patients (9 with biopsy-proven BCC and 1 with biopsy-proven SCC). We determined that in 4 patients with nodular and infiltrative subtypes of BCC lesions, imaging at the dermo-epidermal junction was too superficial and had to be performed at a deeper level for similar lesions in the test set. For the remaining 6 superficial subtypes of lesions (superficial BCCs, SCCs), pre-operative imaging at the dermo-epidermal junction was determined to be adequate. We determined that the use of AlCl₃ (concentration 35% for topical application of 30 seconds) produced consistent and uniform contrast with minimal saturation, and allowed consistent visualization of nuclear morphology in the videos and video-mosaics.

Test set (n=35) for delineation of margins

In the remaining 35 patients (32 with biopsy-proven BCC and 3 with biopsy-proven SCC), the standardized imaging protocol and topical use of AlCl₃ was implemented. Pre- and intra-operative imaging was performed on all 35 patients. For the 16 superficial lesion subtypes, pre-operative imaging was performed at the dermo-epidermal junction (approximately 100–125 µm). For the remaining 19 nodular and infiltrative subtypes of lesions, pre-operative imaging was performed at a relatively deeper depth (approximately 125–150µm).

Pre-operative evaluation of lateral margins with RCM (n=35)

Thirty-two (91%) of 35 biopsy-proven NMSCs (29 BCCs, 3 SCCs) were observed with acceptable imaging quality, resolution and contrast (Table 1). All 32 videos allowed for visualization of nuclear and morphologic features. The remaining 3 videos were not acceptable, mainly due to motion blur artifacts. However, visualization of normal nuclear detail such as sebaceous glands, inflammatory cells and eccrine ducts was still possible. All acceptable 32 lesions exhibited the following normal features: honeycomb pattern (dark nuclei within bright cytoplasm), dermal rings, eccrine ducts, sebaceous glands and hair follicles. For BCC lesions, the following features were observed: erosion, streaming, atypical honeycomb pattern, dark silhouettes, thickened and bundled collagen, tumor nests containing palisading cells, clefting, cord-like structures with palisading, melanophages and small inflammatory cells in dermis (eFigure2 in the Supplement). For SCC lesions, the following features were observed: erosion, parakeratosis, atypical honeycomb pattern, epidermal disarray, keratinocyte pleomorphism and inflammatory cells (Fig. 1, example).

Intra-operative evaluation of surgical wounds with RCM (n=35)

Twenty-nine (83%) of 35 surgical wounds were observed with acceptable imaging quality, resolution and contrast, with respect to the videos and video-mosaics (Table 1). All 29 videos could be converted into video-mosaics. The remaining six videos were considered unacceptable due to the presence of motion blur artifacts and thus could not be converted to video-mosaics.

In the videos and video-mosaics (Fig. 2, example) that exhibited acceptable quality, nuclear and cellular structures at the epidermal, peripheral and deep dermal margins were identified:

- 1. Epidermal Margin –at the peripheral edge of the surgical wound—**At the periphery of the wound, bright nuclei within small polygonal cells (corresponding to

epidermal keratinocytes) were seen in a cobblestone pattern. Each cell was separated by a light reflective border. The direct exposure to aluminium chloride, and thus nuclei brightening, was due to the lack of a stratum corneum in the surgical wound. In contrast, the epidermal keratinocytes in the surrounding intact skin were seen as polygonal cells with dark nuclei and bright thin cytoplasm displayed in a honeycomb pattern. The difference between the cobblestone and honeycomb patterns was easily distinguished and defined the peripheral edges of each surgical wound.

2. Peripheral Dermal Margin –at the peripheral (papillary) dermis of the surgical wound—Deeper level images showed the dermo-epidermal junction (DEJ) and dermal papillae surrounded by a rim of bright cells, contrasting with the dark background. Hyper-reflective structures forming web-like structures corresponding to papillary collagen bundles within dermal papillae were seen at deeper levels of the wound cavity.

3. Deep Dermal Margin - at the base of the surgical wound—The base of the shaved wound cavity appeared dark, containing hyper-reflective fibrillar structures, which corresponded to reticular collagen bundles.

RCM videos and video-mosaics and histopathological correlation

Pre-operative imaging—Image quality (resolution, contrast) and visualization of nuclear detail was acceptable in 32 of 35 NMSC lesions (Table 1). In these lesions, we also observed correlation between the RCM videos and video-mosaics and pre-operative diagnoses for presence of tumor. BCC and SCC features were visualized in the RCM videos and video-mosaics of 29 biopsy-proven BCCs and 3 biopsy-proven SCCs. In 24 of the 32 acceptable videos and video-mosaics, visualization of tumor-specific features was observed to be unequivocally BCC or SCC. For the remaining 8 lesions, the videos and video-mosaics needed to be reviewed twice to confirm the features.

Intra-operative imaging—Among the 14 NMSCs with histopathologically-confirmed residual tumor, we observed correlation between the RCM videos/video-mosaics and the corresponding Stage I histopathology for presence of tumor in 10 lesions (9 residual BCC, 1 residual SCC) (Table 2) (Fig. 3, example). The observed features in the RCM videos/video-mosaics were consistent with those seen on histopathology. In the 21 lesions with absence of residual tumor, we observed correlation for absence of tumor in 18 lesions (Table 1). Overall image quality (resolution, contrast and visualization of nuclear detail) was acceptable in 29 of 35 NMSC lesions (Table 1).

RCM evaluator and histopathology correlation and inter-evaluator agreement (n=35)

Sensitivity/specificity for our two RCM evaluators (O.Y. and M.C.) was 71%/86% and 81%/86%, respectively (Table 2). Overall agreement between each evaluator and histopathology was 80% and 83%, respectively. Inter-evaluator agreement was found to have moderate correlation ($k=0.59$, $p=0.0002$).

Discussion

Our paper reports a novel advancement in RCM imaging for potentially guiding surgery of NMSCs, with a new video-mosaicking approach that can peri-operatively delineate margins on patients *in vivo*. Video-mosaics can display NMSC margins in freeform- shaped and contiguous areas that can be determined by the user in real-time “on the fly” during imaging (Figs 1–3; eFigures 2–4).

We found that examination of the surgical margin utilizing both videos and video-mosaics in a synergistic manner was critical for an accurate and consistent assessment for the presence of residual tumor. This was our experience in the 29 cases with acceptable videos and video-mosaics (Table 1). Among the intra-operative videos and video-mosaics, features that appeared bright round, cord-like, palisading, elongated and nodular were often marked as suspicious for residual BCC. In 3 such videos, BCC structures were later determined to be normal structures (hair follicles, sebaceous glands, and eccrine ducts) in histopathology, thus leading to false positives. Similarly, in one video, features that included an area with clefting with palisading cells were read as positive for residual tumor, but later determined to be negative. Video-mosaics were useful for orientation along the surgical margin and initial assessment for the presence or absence of residual tumor, while in ambiguous cases, videos were useful for additional confirmation. Only a small percentage (9% pre-operative; 17% intra-operative) of lesions presented unacceptable video quality and could not be processed into video-mosaics (Table 1). In these videos, there was too much motion blur and/or tissue distortion and warping, and/or incomplete coverage with “jumps” (due to sudden changes in depth or topography) and/or significant variations in image brightness (saturation due to too much AlCl₃ and/or too much illumination) (eFigures 3 and 4 in the Supplement). Further development of the video-mosaicking approach may minimize such errors.

Prior knowledge of the diagnosis allowed the user to adjust the imaging depth during pre-operative imaging. Typically, our imaging researchers (M.C., O. Y.) set the level of imaging at the dermo-epidermal junction, which does not always capture deeper infiltrative features that are seen in some NMSCs. In our study, RCM identified tumor-specific structures in 11 of 14 lesions with residual NMSC tumor (13 BCC, 1 SCC). However, for the 3 lesions that exhibited both superficial and infiltrative features, several videos at multiple depths would have been necessary, to capture both superficial and infiltrative BCC. Although the user can change depth while imaging with the handheld microscope, too much change in depth does not allow for creating seamless and contiguous video-mosaics. For superficial BCCs and SCCs, the depth was more superficial as opposed to micronodular or infiltrative BCCs, which required deeper imaging. In the future, the combination of RCM with optical coherence tomography (OCT), which images deeper (18) and can detect deeper margins (19), may be useful for assessing depth of infiltrative features.

At present, the main limitation of the RCM video-mosaicking approach is inherent in the algorithm itself. Depending on the size of the lesion, video acquisition can be quick (1–2 minutes per quadrant, up to 8 minutes per lesion), but the subsequent processing is relatively slow (25–45 minutes per lesion, depending on number of videos). We are developing a faster approach, based on machine learning with deep neural networks, to process and produce

video-mosaics in real-time. Other approaches toward quicker detection of margins may be to synergistically combine RCM video-mosaicking with either a faster but lower-resolution approach such as OCT (19, 20, 21), or faster but widefield approaches such as multimodal polarization-enhanced reflectance and fluorescence imaging (PERFI) and optical polarization imaging (OPI) (22, 23). The ability of OCT imaging to quickly delineate normal skin, and then guide RCM imaging toward detection of BCCs in the remaining suspicious areas was recently reported (18, 19). Similarly, PERFI or OPI may delineate unequivocally normal or positive areas, and then guide RCM imaging toward detection in the remaining suspicious areas.

Interpretation of RCM videos and video-mosaics, itself, appears to be relatively straightforward. When assessing the videos and video-mosaics, we found that our evaluators' sensitivity and specificity were both reasonably high (Table 2), when visualizing nuclear detail for the presence or absence of residual tumor, despite artifacts due to scoring and surgery and distortions of the anatomy due to the procedure. Overall agreement between the evaluator's and histopathology was good, and agreement between them was moderate (Table 2). These levels of sensitivity, specificity and agreement are reasonably promising, and suggests that improvements in the video-mosaicking algorithm, imaging procedure and reading of video-mosaics may provide a clinically useful tool at the bedside.

In conclusion, delineation of NMSC margins directly on patients with RCM video-mosaicking may potentially serve as an adjunct to traditional histopathology, by providing increased and comprehensive sampling (overcoming the limitations in previous approaches), and helping improve accuracy for clearing margins at the bedside. With further development and prospective validation in larger studies, handheld RCM video-mosaicking may become useful to guide surgery in real-time. In the Mohs surgery setting, this may allow for better patient counseling, fewer staged excisions and reduction of time spent in clinic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Pre-operative RCM video-mosaic of biopsy-proven SCC. Features observed along the scored surgical lateral margins of this lesion: (a) epidermal disarray and atypical honeycomb pattern from 12 to 2 o'clock position in the first quadrant (inset area in blue rectangle), and (b) shown in a magnified view of the inset (area in red circles). Seen, at approximately the 9 o'clock position is a break or discontinuity (labeled "JUMP") in the video-mosaic.

Figure 2.

Intra-operative RCM video mosaic showing absence (clearance) of NMSC tumor. The entire video-mosaic in the third quadrant, from 9 to 12 o'clock, is seen (a), along with a magnified view of the inset area in the yellow rectangle (b). The video-mosaic shows three depths of wound margins: 1. Deep dermal margin - showing near the base of a dark shaved wound cavity containing hyper-reflective fibrillar structures corresponding to reticular collagen bundles (yellow arrows). 2. Peripheral dermal margin - showing polygonal-shaped cells (keratinocytes) with bright nuclei arranged in a cobblestone pattern surrounding the shaved wound cavity (green arrows). Deeper level images showed the dermo-epidermal junction (DEJ) and dermal papillae surrounded by a rim of bright cells, contrasting with the dark background (orange arrow). Hyper-reflective structures forming web-like structures, corresponding to papillary collagen bundles within dermal papillae, were seen at deeper

levels of the wound cavity (red arrow). 3. Epidermal margin and the wound edge (yellow dotted line) - At the periphery of the wound, bright nuclei within small polygonal cells, corresponding to epidermal keratinocytes, were seen in a cobblestone pattern (blue arrow). Each cell was separated by a light reflective border. The direct exposure to AlCl_3 , and thus nuclei brightening, was due to the lack of a stratum corneum in the surgical wound. In contrast, the epidermal keratinocytes in the surrounding intact skin appeared as polygonal cells with dark nuclei and bright thin cytoplasm displayed in a honeycomb pattern. The difference between the cobblestone and honeycomb patterns was easily distinguished and defined the peripheral edges of each surgical wound.

Figure 3.

Intra-operative RCM video mosaic showing presence of residual BCC tumor along the surgical wound, along with histopathology correlation. Observed features are (a) a tumor island (inset area in yellow rectangle), (b) showing presence of a nodular BCC in a magnified view (red arrows), and (c) confirmed by the corresponding histopathology.

Table 1.

Evaluation of pre- and intra-operative RCM imaging

	Pre-operative			Post-operative			
	BCC (n=32)	SCC (n=3)	Total (n=35)	Residual BCC (n=13)	Residual SCC (n=1)	Absence (n=21)	Total (n=35)
Acceptable quality (video & video-mosaic)	29	3	32 (91%)	9	1	19	29 (83%)
Unacceptable quality (video & video-mosaic)	3	0	3 (9%)	4	0	2	6 (17%)
Total	32	3	35 (100%)	12	1	22	35 (100%)

Table 2.

Summary of intraoperative RCM and histopathological correlation

Histopathology	Reader 1		Reader 2	
	Presence	Absence	Presence	Absence
Presence of BCC residual tumor ($n=13$)	9	4	11	2
Presence of SCC residual tumor ($n=1$)	1	0	1	0
Absence of residual tumor ($n=21$)	3	18	4	17
Sensitivity	71%		86%	
Specificity	86%		81%	
Overall agreement	80%		83%	