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Optical imaging of collagen fiber damage to assess thermally injured human skin

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Abstract

Surgery is the definitive treatment for burn patients who sustain full-thickness burn injuries. Visual assessment of burn depth is made by the clinician early after injury but is accurate only up to 70% of the time among experienced surgeons. Collagen undergoes denaturation as a result of thermal injury; however, the association of collagen denaturation and cellular death in response to thermal injury is unknown. While gene expression assays and histologic staining allow for ex vivo identification of collagen changes, these methods do not provide spatial or integrity information in vivo. Thermal effects on collagen and the role of collagen in wound repair have been understudied in human burn models due to a lack of methods to visualize both intact and denatured collagen. Hence, there is a critical need for a clinically applicable method to discriminate between damaged and intact collagen fibers in tissues. We present two complementary candidate methods for visualization of collagen structure in three dimensions. Second harmonic generation imaging offers a label-free, high-resolution method to identify intact collagen. Simultaneously, a fluorophore-tagged collagen-mimetic peptide can detect damaged collagen. Together, these methods enable the characterization of collagen damage in human skin biopsies from burn patients, as well as ex vivo thermally injured human skin samples. These combined methods could enhance the understanding of the role of collagen in human wound healing after thermal injury and potentially assist in clinical decision-making.

1 | INTRODUCTION

Non-fatal thermal injuries are a leading cause of morbidity worldwide, with roughly 40 000 hospitalizations a year required in the United States.[1] While considered largely preventable, burns can leave patients with debilitating injuries and scarring.[2] A major challenge lies in the variable healing time for burns that are less than full-thickness, along with the disparity between clinical identification of burn depth, histopathologic determination of cellular and extracellular matrix (ECM) damage, and the regenerative potential of the tissue.[3] More

severe, full-thickness burns that damage the subcutaneous layer require excision and autologous skin grafts while dermal burns may heal spontaneously if thermal damage is confined to the more superficial dermis.[2,4] Surgical removals of necrotic tissue is necessary to remove the inflammatory burden, allowing viable cells to regenerate. Conversely, over-excision of burns can result in scarring and morbidity in the form of donor site wounds, as well as increase the cost of care incurred by the patient.[2] In an attempt to understand the potential for wound healing, tissue excised from the initial surgical intervention may be analyzed through gene expression assays and histological staining. Gene expression can identify the types of collagen expressed but this method does not provide spatial information regarding collagen structure. Histologic stains like Picrosirius red[5] and Masson's trichrome[6] can identify collagen structures and organization but only on a thin, single slice of tissue ex vivo. These methods are not applicable for identifying damaged collagen on a patient in a clinical setting. In addition, the exact depth of burn injury that will heal without surgery and the association between cellular and collagen damage is unknown. There is a critical need for developing robust minimally-invasive techniques that are clinically translatable to quantify the extent of thermal damage to the collagen in the ECM in three dimensions, and specifically visualizing denatured collagen is a significant first step.

Collagen is the most abundant protein in the body, comprising 75% of the dry weight of the skin.[7] Collagen provides the structural scaffold of the ECM upon which cellular epithelialization takes place. It also serves a significant role within the dermal layer of the skin for cell adhesion and migration.[8,9] Our group has shown that this migration potential can be hijacked during metastasis in breast and ovarian cancer, such that collagen structure and alignment can be a prognostic indicator of cancer progression,[10,11] implicating collagen as a significant cell behavior mediator. During wound repair, deposition of collagen occurs alongside regeneration of the ECM thereby playing an important role in determining morphological skin properties.[9] Thermal injuries result in collagen denaturation and structural changes.[12] Despite efforts to visualize architectural changes of collagen in the ECM in wound models, the clinical impact of collagen assessment for determining burn severity or depth after burn injury is still relatively unexplored.[13] Characterization of the wound healing response from thermal injury in a zebrafish model indicates a dramatic loss of collagen fibers associated with delayed healing when compared to a transection wound.[14] With better visualization of collagen damage in vivo and an understanding of the relationship between this damage and cellular viability, therapeutic development could be directed toward collagen repair. The current inability to assess the role of collagen in thermal injury is due largely to the lack of available methods to visualize damaged collagen in vivo and a lack of human models of thermal injury.

Clinically, understanding the changes to collagen architecture due to thermal injury is important, as collagen plays a major role in the mechanical integrity of the skin and the resultant functional outcomes and scarring. Tanaka et al performed second harmonic generation (SHG) imaging on in vivo samples to observe changes in dermal collagen fibers in living rat burn models.[12] SHG occurs when two incident photons interact with the non-centrosymmetric triple-helical structure of collagen and combine to form a single emitted photon of exactly half the wavelength or twice the energy, offering a label-free, quantitative measure of intact collagen with high spatial resolution.[10-12] However, SHG has several issues that limit its clinical application, including being expensive, bulky, utilizes non-eye-safe class IV lasers and does not currently have US Food and Drug Administration (FDA) approval.[11] Therefore, exploring other imaging techniques to specifically identify denatured collagen may prove useful during burn excision.

Currently, approximately 80% of burn surgeons in the United States determine burn depth by visualization alone, an assessment method that is accurate in only 64% to 76% of cases.[2,15] Furthermore, indeterminate burn depths—the burn depth at which decisions about surgical intervention necessity is least clear—are even less accurately identified.[16,17] The next most common method to determine the healing potential of a thermal injury is Laser Doppler imaging (LDI),[18,19] which has a 95% to 100% positive predictive value for prognosing healing within 3 weeks, increasing over time as does the visual interpretation.[20] The use of LDI is limited by day-to-day and intra-operator differences resulting from training variability.[20] Furthermore, LDI is not useful intraoperatively during excision when the wound is excised under tourniquet as the blood flow is artificially decreased. Optical coherence tomography (OCT) offers high-resolution, multi-sectional imaging but the long acquisition times limit the applicability.[21] Fluorescence-guidance-based techniques can be used to asses wounds, but the availability of specifically targeted probes and instrumentation is limited. The clinically approved fluorescence agent Indocyanine green (ICG), is used for determining vascular flow during reconstructive surgery. However, it can be challenging to interpret when applied for burn cases.[22] The use of perfusion as a readout for viability is particularly challenging where tourniquets or vasoconstriction injections are used, as is common intraoperatively. Furthermore, ICG does not provide any information regarding collagen structural damage to the ECM.[3,23] Visualization of ICG requires injection and is more invasive than potentially non-intravenous alternatives that may identify thermal collagen damage more explicitly, such as a fluorescently tagged collagen-mimetic peptide.

Collagen-mimetic peptides (CMPs) are synthetic chains of amino acid residues that replicate the strands in natural collagen fibers.[7] CMPs can bind to damaged collagen and have been used for several purposes, including identifying atherosclerotic plaques.[24] In previous work, we synthesized a CMP that contains three types of residues: (2*S*,4*S*)-4-fluoroproline (flp), (2*S*,4*R*)-4-hydroxyproline (Hyp), and glycine. This CMP, $(flpHypGly)_7$,[25] anneals to damaged collagen but not to itself.[26] By appending a cyanine 5 (Cy5) dye, we imaged damaged collagen in a burned rat tendon by excitation (λ_{ex} = 652 nm) using either multiphoton or single-photon fluorescence microscopy, and capturing emission (λ_{em} = 672 nm).[26] We validated the binding of the Cy5-CMP conjugate specifically to damaged collagen through SHG imaging, which generates a signal only for intact collagen, and observed that Cy5-CMP does not bind to adjacent intact collagen.[26]

Here, we probe collagen damage following thermal injury in human skin by leveraging signals obtained from the fluorescence of Cy5-CMP adhered to denatured collagen, combined with SHG imaging of intact collagen. We show that Cy5-CMP binds specifically to regions of denatured collagen in human in vivo clinical burn biopsies and provides contrast from normal tissue. The images add insight alongside histologic staining with lactate dehydrogenase (LDH) for cellular viability in an ex vivo thermally injured skin model. Finally, we demonstrate the potential for Cy5-CMP to be visualized in the clinic through the use of single-photon fluorescence imaging.

2 | METHODS

2.1 | Cy5-collagen mimetic peptide synthesis, preparation, and validation

A Cy5-CMP conjugate, Cy5-Gly-(Ser-Gly)$_2$-(flpHypGly)$_7$, was prepared as described in Dones et al.[26] Briefly, the peptide was synthesized by microwave-assisted solid-phase peptide synthesis, purified by reversed-phase high-performance liquid chromatography, and conjugated at its N terminus to Cy5. The specific binding of Cy5-CMP to damaged collagen was validated by using a compositional isomer (CI) conjugate, Cy5-Gly-(Ser-Gly)$_2$-(HypflpGly)$_7$, or Cy5-CI, as a negative control. Cy5-CI contains the same three amino acid residues as does Cy5-CMP but arranged in an order that does not allow for specific annealing to damaged natural collagen.[26] Washing of residual unbound Cy5-CMP or Cy5-CI from human skin samples was performed with 1× PBS (phosphate buffered saline solution) (Sigma, St. Louis, Missouri) (see Supplemental Data, Figure S1).

2.2 | Clinical in vivo patient selection and tissue preparation

Clinical tissue samples were obtained through the University of Wisconsin Hospital as described previously.[3] This study was approved by the University of Wisconsin Human Subjects Committee Institutional Review Board in compliance with the 1975 Declaration of Helsinki. Briefly, samples were obtained from patients with indeterminate depth burn injury at the time of surgery, 6 to 13 days after burn injury following a period of observation to assess healing trajectory. Intraoperatively, burn wound depth was determined by the burn surgeon using visual assessment prior to and during excision.[3] Normal skin tissue used as a control was obtained from non-burn related elective reconstructive surgery. Control non-burned and burned tissue samples were stored for up to 1 hour in normal saline (Baxter) prior to further processing. A 6-mm biopsy punch was taken from the skin samples and 100 μL of 100 μM Cy5-CMP or 100 μL of 100 μM Cy5-CI was applied directly onto the superficial side of the biopsy, fully submerged in dye solution, and incubated in a petri dish covered with aluminum foil at room temperature for 1 hour. The samples (n = 6 burn pairs) were washed three times in rapid succession with 1× PBS (Sigma) prior to imaging and kept covered in 1× PBS. Images were collected from both superficial and deep surfaces of the biopsies immediately following rinsing.

2.3 | Tissue sample preparation

Normal skin tissue for use in the ex vivo model was obtained from patients receiving elective surgery at the University of Wisconsin Hospital (Madison, Wisconsin). Discarded de-identified tissue was transported in normal saline after surgical excision.

2.4 | Thermal injury, tissue culture, and Cy5-CMP labeling

A novel burn device was created in collaboration with the Morgridge Institute for Research FabLab group (Madison, Wisconsin) (unpublished results). Briefly, the device generated a burn on the ex vivo skin with an 8 mm circular face applied with uniform pressure of 0.12 kg/cm^2 at 150°C for 3 seconds to simulate a partial-thickness burn. After a thermal injury, the tissues (including non-burn control skin) were biopsied using a 6 mm punch and cultured in Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% bovine calf serum (Hyclone), 0.625 μg/mL amphotericin B (Gemini Bio Products), and 100 μg/mL of Pen/Strep (Gibco by Life Technologies) at 37°C for 48 hours prior to Cy5-CMP application and imaging. After 48 hours, the necrotic epidermis on the 3 seconds samples was mechanically debrided with a forceps to expose the dermis. All biopsies received 100 μL of 100 μM Cy5-CMP, were fully submerged and incubated for 1 hour, and washed three times with 1× PBS, as described above. After Cy5-CMP application, incubation, and washing, the 6 mm ex vivo thermally injured and debrided biopsies were bisected. One-half of the biopsy was used for multiphoton imaging of Cy5 and SHG, the other half was cryopreserved and sectioned for LDH staining.

2.5 | Lactate dehydrogenase staining on histological sections

LDH staining was performed on cryosections of ex vivo tissue sections to assess cell viability. Staining was performed as described in Gibson et al.[27] Briefly, sections were air-dried for a minimum of 3 hours (range 3-18 hours) then washed twice with PBS for 5 minutes each time. Sections were incubated with freshly prepared LDH solution containing 5% Polypep (Sigma); 2 mM Gly-Gly (Sigma); 0.75% NaCl (Fisher, Hampton, New Hampshire); 60 mM lactic acid (Dot Scientific, Burton, Michigan); 1.75 mg/mL β-nicotinamide adenine (Sigma, St. Louis, Missouri); and 3 mg/mL Nitroblue Tetrazolium (Sigma) pH 8.0, for 3.5 hours at 37°C. Slides were washed twice for 2 minutes each with 50°C tap water, followed by two washes with PBS for 2 minutes each. Tissues were counterstained with aqueous eosin (Newcomer Supply, Middleton, Wisconsin) for 4 minutes. Slides

were washed with PBS for 1 second, dehydrated with acetone for 30 seconds followed by acetone: xylene (1:1) for 1 minute, and finally with xylene alone for 1 minute. Slides were then cover slipped with Cytoseal 60 (Thermo Fisher Scientific).

2.6 | Multiphoton excitation of Cy5 and second harmonic generation

All imaging was performed on the Bruker Ultima IV multiphoton microscope system described in Dones et al, 2019.[26] A Ti:Sapphire pulsed laser (Spectra-Physics Insight DeepSee) was tuned to 820 nm to excite the Cy5 fluorophore of Cy5-CMP, using a 690 (50) nm emission bandpass filter (Chroma Technology Corporation, VT) to isolate the fluorescence emission. SHG of intact collagen was performed on all tissues on the same microscope by tuning the laser wavelength to 890 nm and using a 445 (50) nm bandpass filter (Semrock Inc., Rochester, New York). Samples were imaged using a 10× CFI Super Fluor objective lens (Nikon, NA = 0.50). Images generated were acquired as *z*-stacks from superficial to deep at 40 μm intervals spanning 800 to 1200 μm in depth. Slices were summed and stitched to capture the full field of view using FIJI.[28] A high pass Gaussian filter was used to correct for gradients in the images in Figures 3 and 4, which were taken with a 4× Super Fluor objective (Nikon, NA = 0.20).

2.7 | Widefield fluorescence with CMOS camera

Widefield fluorescence imaging was performed on Cy5-CMP applied to a 6-mm ex vivo human skin biopsy that was burned as described above. A PhotoFluor II mercury halogen lamp (Chroma) excited the Cy5-CMP fluorescence through a Cy5.5 dichroic cube (Chroma) consisting of a 685 nm long pass beam splitter, a 650 (50) nm bandpass excitation filter, and a 720 nm (50) nm bandpass emission filter. The resulting signal was captured with a complementary metal-oxide-semiconductor (CMOS) camera (Digital Sight DS-U3, Nikon) using a 4× Super Fluor objective (Nikon, NA = 0.20) on the same Bruker Ultima microscope as the multiphoton imaging was performed. A non-burned biopsy labeled with Cy5-CMP was used as a control. Images generated were stitched to capture the full field of view using FIJI.[28]

3 | RESULTS

3.1 | Collagen mimetic peptides are specific to sites of collagen denaturation in excised human burn tissue

Tissues obtained from patients who sustained thermal injuries, diagnosed at the time of excision (6-13 days after burn injury) as deep partial-thickness,[3] were imaged after treatment with Cy5-CMP (n = 6). Cy5-CI washes from the denatured collagen and the fluorescence intensity of Cy5-CMP was 25 times greater at the burn site compared to neighboring intact collagen as identified by SHG imaging. Multiphoton images taken of the deep dermal surfaces of human burn tissue reveal that Cy5-CMP binds specifically to regions of denatured collagen as validated by the inverse relationship of these images to those obtained using SHG imaging, which detects only intact collagen (Figure 1A-C). Burn depth is heterogeneous within a given wound; therefore, during surgical excision of burn wounds, it is expected that the deep surface of excision will have a mixture of intact and denatured collagen present. Figure 1B illustrates this phenomenon where the SHG imaging identifies the intact collagen, while the Cy5-CMP image correlates with the low-signal regions in the SHG image (Figure 1A,B, yellow circles), representing regions of denatured collagen that remained after surgical excision. In this case, Cy5-CMP was able to clearly identify and confirm that regions of collagen damage were residually present. Figure 1D-F show that the control Cy5-CI does not exhibit specific binding to the region identified in Figure 1E with low SHG (yellow region), as the Cy5 fluorescence signal is also low in this region in Figure 1D (yellow region). These data show the potential for Cy5-CMP to be used to identify regions of damaged collagen resulting from thermal injury in human patients.

3.2 | Cy5-CMP and SHG on ex vivo thermally injured human tissue

Using an ex vivo human burn injury model, we qualitatively evaluated Cy5-CMP penetration on thicker sections in experimentally generated burns by generating a histogram of pixel intensities from Cy5 fluorescence throughout the sample as measured in FIJI,[28] as well as determined the relationship between collagen denaturation and cell viability with LDH-staining (Figure 2). Deep partial-thickness thermal injuries were generated in ex vivo human skin. Burned tissue was cultured for 48 hours prior to Cy5-CMP application and imaging to simulate the clinically relevant period of time in which initial depth determination for operative planning occurs. The non-burned control (Figure 2A) shows very little Cy5 signal, with no specific binding to the intact dermal collagen, as determined by the correlation with the SHG signal (Figure 2B). LDH-staining shows cell viability that is blue color, throughout the depth of the tissue (Figure 2D). The 3 seconds burns show higher Cy5-CMP uptake where SHG signal is low and, conversely, low Cy5-CMP binding where SHG is brighter (Figure 2E-G). This region of intact collagen appears to localize near the region with viable cells identified with LDH-staining (Figure 2H). This suggests that following thermal injury, the location of viable cells correlates with the areas of intact collagen.

3.3 | Widefield fluorescence excitation of Cy5-CMP can distinguish between normal skin and thermally injured skin

To demonstrate that visualization of Cy5-CMP would not be limited to multiphoton excitation, which would greatly curtail any clinical

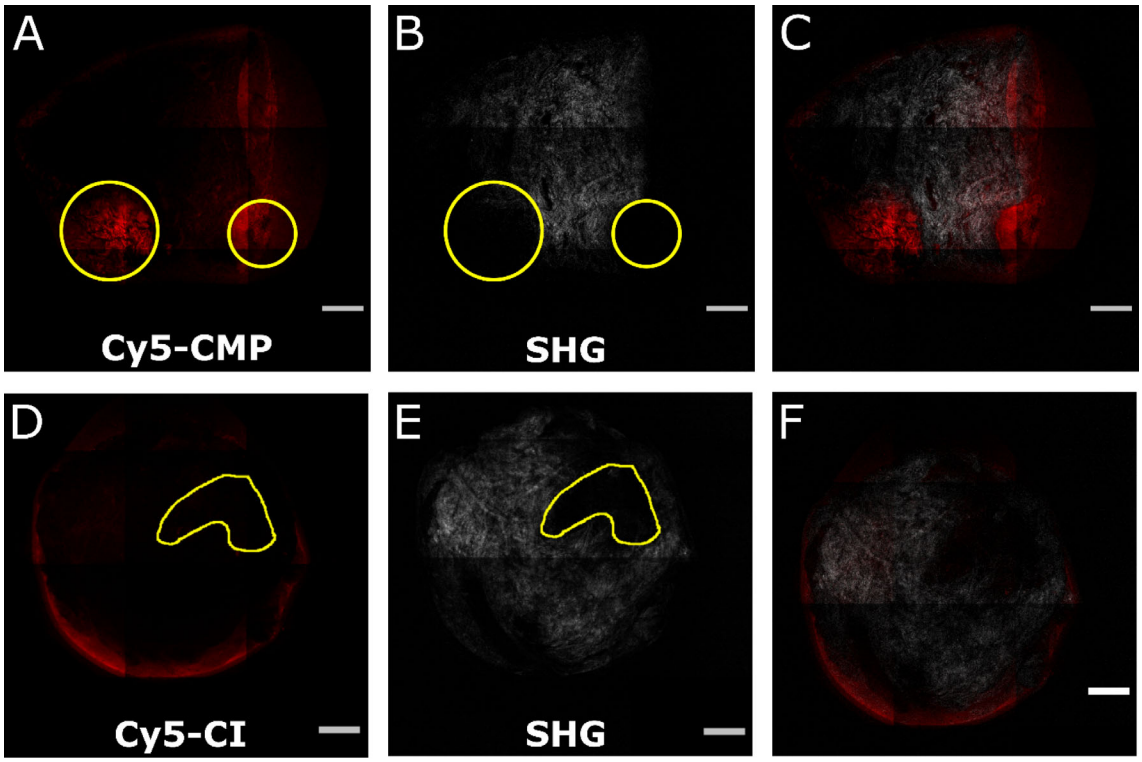

FIGURE 1 The specificity of Cy5-CMP to denatured collagen in patient burn tissue. Stitched *z*-projections of multiphoton micrographs of (A) Cy5 fluorescence image with regions of interest (ROIs) (yellow circles) showing Cy5-CMP labeling on the deep surface (en face view) of 6 mm biopsies of tangentially excised deep partial-thickness burn tissue, and (B) absence of SHG signal in the same ROIs (yellow circles). (C) Composite image of Cy5 fluorescence and SHG. The region of brighter Cy5 fluorescence near the right side of the image corresponds to a portion of the cut edge of the biopsy where we see some fluorescence signal from the superficial side of the biopsy penetrating through the *z* stacked images. (D) Burn tissue sample labeled with Cy5-CI (negative control) imaged with Cy5 parameters and (E) SHG parameters. Yellow ROIs and (F) the composite image of tissue labeled with Cy5-CI indicating that the dark regions in panel E are not fluorescent in panel D. The Cy5 fluorescence on the circumference of the biopsy corresponds to non-specific binding around the superficial cut edge of the biopsy. Scale bars = 1 mm. The color version is available in the online copy [Color figure can be viewed at wileyonlinelibrary.com]

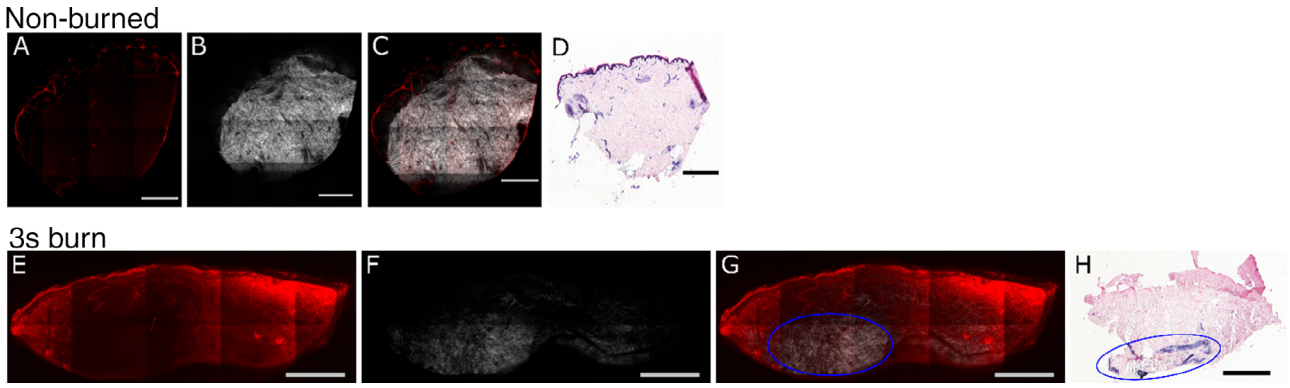

FIGURE 2 Cy5 excitation of Cy5-CMP reveals collagen damage with depth on cross-sectional views. Shown are the side profiles of a 6 mm biopsy of (A-D) normal non-burned skin and (E-H) skin burned for 3 seconds at 150°C with a custom burn device. (A) Multiphoton excitation of Cy5-CMP shows little fluorescence, while (B) SHG image reveals intact collagen throughout the normal biopsy. In contrast, (E) Cy5-CMP extensively labels the burned sample while the (F) SHG signal is minimal. SHG signal exhibits an inversion of signal intensity to the Cy5 channel, highlighted by the composite images of (C) Cy5-CMP + SHG for normal skin and (G) 3 seconds burned skin. LDH images show that viable cells (blue) are present throughout the (D) non-burned skin and in the (H) deep portion in the 3 seconds burned skin, correlating with intact collagen. Scale bar = 1 mm. The color version is available in the online copy [Color figure can be viewed at wileyonlinelibrary.com]

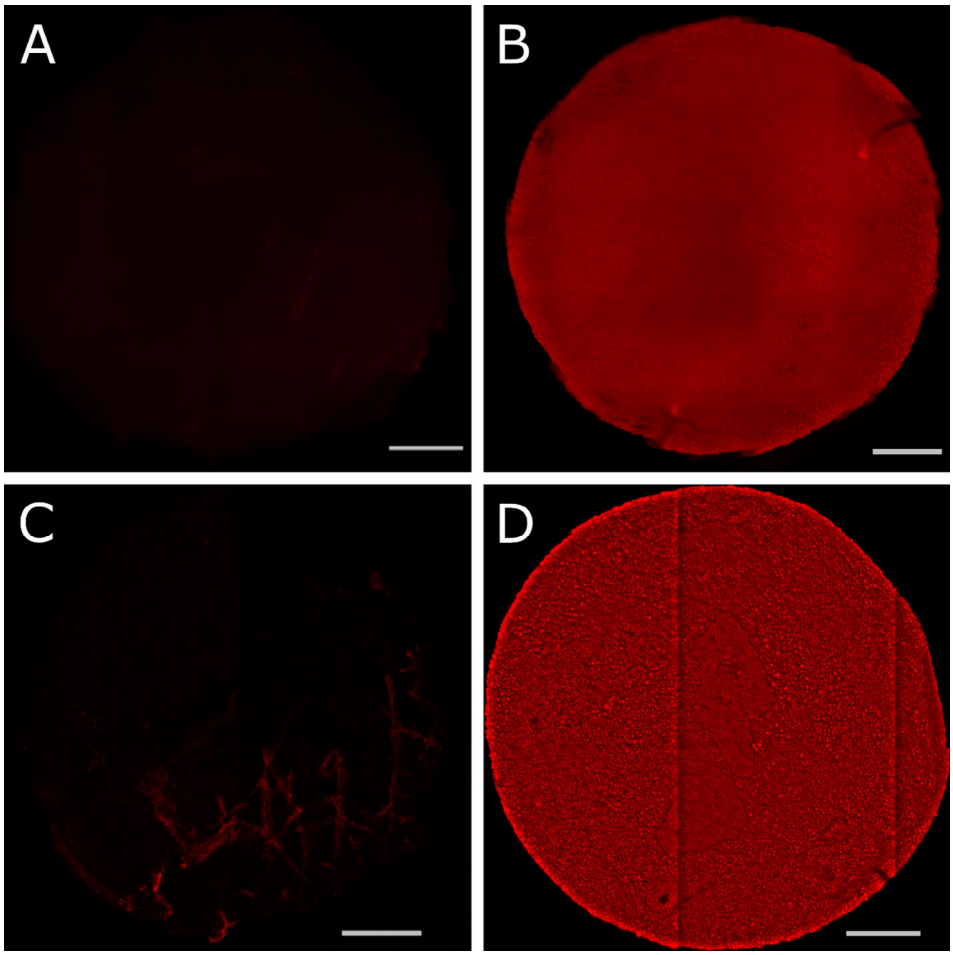

FIGURE 3 Assessment of Cy5-CMP labeling using widefield fluorescence microscopy. Fluorescence of an ex vivo 6 mm biopsy from (A) normal non-burned skin and (B) burned skin relative to the same samples imaged with multiphoton microscopy in (C) non-burned and (D) burned skin. Scale bar = 1 mm. The color version is available in the online copy [Color figure can be viewed at wileyonlinelibrary.com]

application given the cumbersome methods needed for image acquisition, we imaged the superficial surface of a control non-burned and ex vivo thermally injured human skin sample with Cy5-CMP using widefield fluorescence excitation (Figure 3). Using a mercury-arc light source, we found intensely bright Cy5 fluorescence on the superficial face of an ex vivo thermal injury post-Cy5-CMP application when compared to the non-burned control (Figure 3A,B). Alongside the wide field images, we captured images on the same microscope platform with multiphoton microscopy for the same sample conditions of non-burned control and thermally injured skin (Figure 3C,D). Widefield fluorescence excitation provides sufficient contrast to substantially excite Cy5-CMP and discriminate burned from non-burned human skin.

4 | DISCUSSION

Collagen plays a significant role in maintaining the structural integrity of human skin as it provides a scaffold for cells to migrate and regenerate in the wound healing process.[7-9] Currently, there are few clinically viable imaging techniques to probe collagen, and fewer still that permits real-time visualization of regions of denatured collagen. SHG offers the ability to image intact collagen without exogenous contrast at a high spatial resolution. Tanaka et al assessed whether a change in SHG signal could be associated with a burn degree, where the vanishing of the signal corresponds to denatured collagen and proposed a quantitative parameter for burn-assessment based on the depth profile of the mean SHG intensity across the entire image.[12] Pierce et al used polarization-sensitive OCT to show that partial-thickness burns had a loss of collagen birefringence due to denaturation.[29] These previous studies suggest that collagen denaturation can be quantified with SHG and OCT and correlated to burn severity. Still, both techniques can only image intact collagen rather than identifying regions of damage. Our results show that in clinical burn tissue, Cy5-CMP specifically labels damaged collagen. SHG coupled with multiphoton fluorescence excitation provides an opportunity to validate the Cy5 signal from Cy5-CMP on the same multiphoton microscope platform.

We aimed to test whether a thermal injury can be assessed using Cy5-CMP and whether the binding of Cy5-CMP correlated with injury using an ex vivo skin model of burn injury. The use of a dye to localize denatured and potentially non-viable tissue will allow more precision with the removal of necrotic tissue in real-time during surgical excision or bedside wound debridement compared to the current standard of care, which is a visual evaluation of tissue characteristics, thought further testing would be necessary to facilitate clinical

implementation. Future studies would be directed to understand the mechanism of how the temperature of the wound affects collagen damage and how this damage correlates further with cellular viability in physiological models. It is likely that, at certain temperatures, cells are more sensitive to damage than collagen is to denaturation, but the basis for this observation requires future investigation. Ex vivo skin also lacks the vascular flow of blood and lymphatics present in the clinical in vivo patient samples. This perfusion undoubtedly plays a crucial role in wound repair and may affect collagen response to injury. In addition, burns are highly heterogeneous even within the adjacent regions on the same patient. Because of this, we reported on relative contrast in fluorescence signal between non-burned regions and burned regions, as a numerical rubric for image intensity may not directly correlate to clinical relevance. Further, it would be beneficial to explore other dye delivery solvents, such as dimethyl sulfoxide, that might improve the ability for the superficial application of Cy5-CMP to penetrate the skin surface.

Widefieldfluorescence imaging offers the capability to visualize subcellular structures and dynamic biological processes with high spatial resolution in the clinic.[30] While multiphoton excitation successfully allowed us to visualize Cy5-CMP-labeled damaged collagen, in conjunction with the intact collagen structures provided by SHG, neither modality is currently clinically available. Therefore, we showed that the far-red fluorescence of Cy5-CMP can be captured using the more readily available widefield fluorescence with a mercury arc lamp and appropriate filters with a CMOS camera system. Such a widefield imaging instrumentation setup is potentially suitable for fluorescence-guided surgery, whereas, as mentioned previously, SHG is not a clinically available modality. Cy5-CMP may provide a clinically viable labeling methodology for assessing the extent of collagen damage following thermal injury, and commercial fluorescence-guided imaging systems can potentially capture a Cy5-CMP signal.[30]

5 | CONCLUSION

Cy5-CMP anneals to the damaged collagen of thermally injured skin while washing away from the skin with intact collagen. This peptide could be beneficial in conjunction with current histologic methods to assist in visualizing locations of collagen denaturation as a result of thermal injury. This integrative approach would allow the correlation of collagen and cellular damage, which could help to illuminate the molecular mechanism of injury progression and wound repair following thermal injury. Moreover, Cy5-CMP could serve as the basis for the intraoperative labeling of thermally damaged tissue to be removed during burn excision. The utility of seeing a signal corresponding directly to denatured collagen is that the directed excision of necrotic tissue would be an improvement to the visual characteristics that currently lead to over-excision of burn wounds.

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CONFLICT OF INTEREST

Kevin W. Eliceiri is a co-founder of OnLume, Inc. a commercial company manufacturing imaging instrumentation for fluorescence-guided surgery. Ronald T. Raines is a Scientific Advisory Board member and shareholder of Stuart Therapeutics, Inc., which is developing collagen mimetic peptide technology for ophthalmology.

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SUPPLEMENTAL DATA DISCUSSION: COLLAGEN MIMETIC PEPTIDES BIND WITH GREATER FLUORESCENCE INTENSITY THAN CI ON BURN PATIENT TISSUE

To better simulate a clinical application we also tried Cy5-CMP binding with human burn patient tissue to visualize between bright Cy5-CMP fluorescence on a burn site vs non-burned normal skin on the same patient. We tested Cy5-CMP vs Cy5-CI, which is a compositional isomer,[26] to validate the specificity of Cy5-CMP for binding to damaged collagen. Briefly, multiple 6 mm biopsies were taken from the excised burn tissue of a patient (n = 2) 11 days post-burn and from normal skin (n = 2) to be used for grafting. Cy5-CMP was applied onto the superficial side of one of each of the burned and non-burned biopsies, while Cy5-CI was applied to the other set of burned and non-burned biopsies. Biopsies were incubated 1 hour before washing 3× with 1× PBS to remove unbound dye. Multiphoton imaging was first performed on the two burned samples to excite Cy5, followed by the two normal samples. White light photographs with a digital camera were then taken of the biopsies showing the retention on burned skin and washing from normal uninjured skin.

We found that when applied onto biopsied burned human skin, Cy5-CMP is retained and shows higher fluorescence intensity than does Cy5-CI (n = 6). Figure S1 includes biopsies from normal uninjured skin and burned skin from the same patient. These data demonstrate that the washing of both Cy5-CMP and Cy5-CI from non-burned skin is nearly complete (Figure S1A,B), while the Cy5-CMP binds to the clinical thermal wound (Figure S1C), but the Cy5-CI control does not (Figure S1D). The gross visual comparison of the blue dye associated with Cy5-CMP and Cy5-CI in ambient room lighting confirms that all tissues took up the dye; however, the Cy5-CMP in the burned tissue has the highest signal intensity (Figure S1G).

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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