Quantifying Age-Related Changes in Skin Wound Metabolism Using *In Vivo* Multiphoton Microscopy

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Objective: The elderly are at high risk for developing chronic skin wounds, but the effects of intrinsic aging on skin healing are difficult to isolate due to common comorbidities like diabetes. Our objective is to use multiphoton microscopy (MPM) to find endogenous, noninvasive biomarkers to differentiate changes in skin wound healing metabolism between young and aged mice *in vivo*.

Approach: We utilized MPM to monitor skin metabolism at the edge of full-thickness, excisional wounds in 24- and 4-month-old mice of both sexes for 10 days. MPM can assess quantitative biomarkers of cellular metabolism *in vivo* by utilizing autofluorescence from the cofactors nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD).

Results: An optical redox ratio of FAD/(NADH+FAD) autofluorescence and NADH fluorescence lifetime imaging revealed dynamic changes in keratinocyte function during healing. Aged female mice demonstrated an attenuation of keratinocyte proliferation during wound healing detectable optically through a higher redox ratio and longer NADH fluorescence lifetime. By measuring the correlation between NADH lifetime and the optical redox ratio at each day, we also demonstrate sensitivity to the proliferative phase of wound healing.

Innovation: Label-free MPM was used to longitudinally monitor individual wounds *in vivo*, which revealed age-dependent differences in wound metabolism.

Conclusion: These results indicate *in vivo* MPM can provide quantitative biomarkers of age-related delays in healing, which can be used in the future to provide patient-specific wound care.

Keywords: multiphoton, microscopy, aging, wounds, redox, proliferation

INTRODUCTION

MULTIPHOTON MICROSCOPY (MPM) is a versatile optical imaging modality ideal for 3D *in vivo* skin imaging applications. MPM has an inherent depth-sectioning ability, more efficient light collection, and better depth penetration compared to confocal microscopy.¹ Through the use of infrared light, MPM is also capable of efficiently exciting naturally

present fluorophores in skin, such as nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD), melanin, and keratin.^{2–5} In addition to these fluorophores, fibrillar collagen can be visualized through a frequency-doubling phenomenon called second harmonic generation (SHG).^{6,7} The fluorescence of NADH and FAD is of particular interest, because these cofactors are integral

to cell metabolism. They cycle between oxidized (NAD^+ and FAD) and reduced states (NADH and FADH_2) as they carry electrons between protein complexes during various metabolic processes. However, only the reduced form of NADH and the oxidized form of FAD produce any measurable fluorescence. Through an optical redox ratio of $\text{FAD}/(\text{NADH}+\text{FAD})$ autofluorescence intensity, the relative balance between glucose catabolism and ATP production has been assessed for a variety of cell types.^{8–11}

In addition to the optical redox ratio, fluorescence lifetime imaging microscopy (FLIM) of NADH can be used to provide additional optical biomarkers of skin metabolism. By measuring the time a fluorophore spends in an excited state before emission, the fraction of free to protein-bound NADH^{12–14} can be quantified from its fluorescence lifetime decay. Various studies have demonstrated that an optical redox ratio and NADH fluorescence lifetime measurements are able to detect metabolic changes during cellular events such as hypoxia, proliferation, and biosynthesis.^{15–19} Recently, it has been demonstrated that a combination of NADH and FAD autofluorescence measurements can be used to discriminate a variety of cell types and disease states such as proliferative and metastatic cancers, diabetes mellitus, and cardiovascular disorders, which cause shifts or perturbations in normal cell metabolism.²⁰ However, the combined use of an optical redox ratio and NADH FLIM to evaluate skin wound healing *in vivo* remains unexplored.

CLINICAL PROBLEM ADDRESSED

Up to 6 million patients in the United States alone suffer from chronic, ulcerative wounds arising from conditions such as ischemia, diabetes mellitus, venous stasis, and prolonged pressure.^{21,22} However, chronic wounds disproportionately afflict elderly patients with ~85% of nonhealing ulcers occurring in individuals 65 years of age or older.^{21,23} As aging progresses, intrinsic changes in cell function and metabolism result in dysfunction during the different stages of the healing process.²⁴ During the inflammatory stage of wound healing, aged macrophages have a decreased phagocytic ability and lower growth factor production compared to young macrophages.²⁵ Reepithelialization is also delayed as the capacity of keratinocytes and dermal fibroblasts to proliferate and migrate is significantly attenuated with advanced age.^{26–28} These changes cumulatively predispose the elderly to injury, infection, and the formation of chronic ulcers.^{26,29}

However, it is difficult to discriminate the effects of advanced age on skin wound healing from other comorbidities that impair wound closure. Recently, a workshop hosting the association of Specialty Professors, the National Institute on Aging, and the Wound Healing Society stressed that “substantial research is needed to identify, evaluate, and validate biomarkers related to wound healing both in general and specifically in older adults.”²⁹ While established techniques such as histology and immunohistochemistry have elucidated many mechanisms of impaired healing, they require invasive sample collection as well as substantial processing times and reagents. Therefore, there is a critical need to develop noninvasive, quantitative biomarkers capable of detecting and monitoring age-related delays in healing to supplement current standards of care and assist in the clinical management of wounds in vulnerable elderly populations.

Recently, we have demonstrated that *in vivo* MPM can be used to discriminate changes in keratinocyte function during diabetic wound healing.³⁰ The goal of this study is to evaluate if the endogenous fluorescence from NADH and FAD is capable of noninvasively monitoring age-related differences in wound healing. To accomplish this, we monitored skin metabolism at the edge of full-thickness, excisional wounds in 24- and 4-month-old mice of both sexes for 10 days. Through the combined use of an optical redox ratio and the NADH fluorescence lifetime, we characterized the effects of age, sex, and postwound time on keratinocyte function. Our findings provide evidence that autofluorescence imaging is capable of detecting metabolic dysfunction and wound healing delays associated with intrinsic aging in mice.

MATERIALS AND METHODS

Aged animal models of wound healing

All experiments were approved and performed according to the University of Arkansas IACUC (Protocol No. 17063). C57BL/6J mice at 4 months ($n=8$; four male and four female) and 23–24 months ($n=15$; eight male and seven female) of age were anesthetized with 2–5% isoflurane for induction and maintained at 1–3% for the remainder of wound application or imaging. Each mouse received a subcutaneous injection of an analgesic (Carprofen, 5 mg/kg) before the creation of a 6mm full-thickness, excisional wound on the dorsum using a sterile biopsy punch. The excised tissue was embedded in Tissue-Tek[®] optimal cutting temperature compound (Sakura Finetek; Tokyo,

Japan) and flash frozen at -80°C . Wound size was traced over acetate paper immediately after the creation of the wound on day 0, and at all following imaging time points. Tracings were digitized and wound size was computed in MATLAB. All wound size data following the day 0 measurement were normalized relative to the initial wound size. All wounds were bandaged with a primary covering of Tegaderm™ (3M; Maplewood, ME) and secondary layer of surgical tape.

***In vivo* multiphoton fluorescence intensity imaging**

Mice were anesthetized and imaged on days 1, 3, 5, 7, and 10 postwounding for no longer than 90 min. Image stacks were acquired with a $20\times$, 1.0 numerical aperture water-immersion objective (Olympus, Tokyo, Japan) at the wound edge using a Bruker Ultima Investigator laser scanning microscope (Middleton, WI) and Ti:sapphire laser (Spectra-Physics, Santa Clara, CA). Fluorescence emission was collected using two separate GaAsP PMTs (Hamamatsu; H10770PB-40) with emission filters at $460\pm 20\text{ nm}$ (Chroma, ET460/40m-2p) and $525\pm 25\text{ nm}$ (Chroma, ET525/50m-2p), respectively. At 755 nm excitation, NADH autofluorescence was isolated in the 460 nm channel; at 900 nm excitation, FAD autofluorescence was isolated in the 525 nm channel and SHG from dermal collagen was collected in the 460 nm channel.³¹ Intensity image z-stacks were acquired en face at three locations along the wound edge (superior, inferior, and right lateral edge) per animal at each time point (Fig. 2B). The stacks comprised 100 individual images (512×512 pixels; $584\times 584\text{ }\mu\text{m}$; and 13-bit depth) acquired at z-steps of $2.5\text{ }\mu\text{m}$ spanning from the top of the epidermis down $250\text{ }\mu\text{m}$ into the tissue. Rapid image acquisition ($\sim 70\text{ ms}$ per image slice; 7 s per stack) was employed by a piezo motor and resonant galvanometric scanning system enabling 50 sequential image stacks to be acquired in $\sim 6\text{--}7\text{ min}$ per site. By acquiring large sets of image data by rapid acquisition, breathing/motion artifacts were limited to a small subset of the total acquired images, and could be removed later during image processing as described below. Pixel dwell times during resonant scanning were limited to $0.4\text{ }\mu\text{s}$, while the cumulative dwell time after all 50 z-stack acquisitions was $20\text{ }\mu\text{s}$.

Image processing of *in vivo* intensity image stacks

Fluorescence intensity images from all 50 image stacks were processed to register images and remove any that contained a significant motion artifact based on previously published methods.³⁰

Briefly, images out of alignment due to animal motion were either registered or removed by examining the cross correlation of all 50 images that corresponded to the same depth. Only registered images were averaged together to create high-contrast image stacks. On average, $8\%\pm 0.5\%$ of the images were discarded per stack due to excessive motion artifacts. This processing enabled stacks of NADH two-photon excited fluorescence (TPEF), FAD/keratin TPEF, and collagen SHG to be created without mechanical restraint of tissue, while still providing resolution of individual keratinocytes. The final averaged image stacks of the 755 nm ex./460 nm em. channel (NADH TPEF) and 900 nm ex./525 nm em. channel (FAD/keratin TPEF) were registered together using a 3D cross correlation algorithm and combined to create the final high-contrast multicolor wound edge stacks. Fluorescence intensities from the averaged stacks were normalized by laser power and PMT gain calibrated to fluorescein concentrations as described in previous studies.^{11,32} Pixel-wise calculations of an optical redox ratio of $[\text{FAD}/(\text{NADH}+\text{FAD})]$ were computed using the normalized fluorescence intensities. Resulting redox ratio values were assigned to a jet color map in MATLAB and upper and lower color limits were set for ease of visualization. The keratinocyte region within the stack was digitized using a manual tracing function in MATLAB to produce a mask of the epithelium based on the intensity image stack. The epithelial region was defined as the area that contained keratinocytes between the dermal collagen SHG signal and the highly fluorescent stratum corneum. When tracing the keratinocyte region of interest, hair and hair follicle fluorescence were avoided with a buffer of $\sim 5\text{--}10$ pixels. The average epithelial redox ratio for each z-stack at the wound edge was calculated from the redox ratio values within the traced regions of interest in the images at 15/100, 35/100, 50/100, 65/100, and 85/100 of the total stack depth.

***In vivo* fluorescence lifetime imaging**

NADH fluorescence lifetime image data were acquired immediately following acquisition of each fluorescence image intensity stack on days 1, 3, 5, 7, and 10 postwounding. NADH lifetime images were acquired $125\text{ }\mu\text{m}$ deep from the surface of the epidermis corresponding to the midpoint of the intensity image stacks. All lifetime images were collected with a nonresonant galvanometric scanning system at pixel dwell times of $2\text{ }\mu\text{s}$ and integration times of 2 min. Time-resolved data were analyzed on SPCImage 6.4 (Becker & Hickl GmbH, Berlin, Germany). An instrument response func-

tion (IRF) for the system was measured by using second harmonic signal from urea crystal standards, and was found to have a full width at half maximum of 0.25 ns. For analysis, an incomplete multiexponential model was used, and lifetime images were spatially binned twice to get pixel photon counts to a minimum of 10,000 in the epithelium. Fits were generated using the measured IRF and a biexponential decay model (Equation 1) to separate the long (A2) and short (A1) lifetime components of bound and free NADH, respectively.^{12,16}

$$I(t) = I(0) \left[A1 * e^{-\frac{t}{\tau_1}} + A2 * e^{-\frac{t}{\tau_2}} \right] \quad (1)$$

Images with χ^2 values of <1.5 were considered to be appropriately fit. Regions of interest were manually traced to spatially isolate the TPEF signal from keratinocytes in an identical manner as the redox ratio calculations from the intensity stacks. The mean A1/A2 ratio of the keratinocytes was calculated by averaging the A1/A2 ratios of all pixels falling in the traced epithelial mask.

Ex vivo wound tissue processing

An area of $\sim 1 \text{ cm}^2$ around the wound site was excised from the mice following euthanasia after day 10 imaging and flash frozen in optimal cutting temperature compound at -80°C as described above. The frozen tissue blocks were sectioned to a thickness of $30 \mu\text{m}$ in a Leica CM1860 cryostat (Wetzlar, Germany) before being transferred to glass slides and stored at -80°C . Slides created from adjacent sections were fixed in paraformaldehyde and fluorescently labeled with polyclonal Ki67 Rabbit IgG at $1.25 \mu\text{g/mL}$ and Alexa 488 tagged Goat Anti-Rabbit IgG (ThermoFisher Scientific, Waltham, MA). Antibody-tagged samples were counterstained

with 300 nM DAPI (ThermoFisher Scientific). A Ki67 proliferation index was computed using a custom MATLAB function that created a threshold for the fluorescently labeled nuclei using Otsu's method. This function provided masks of Ki67 and DAPI, which when overlaid were able to quantify the number of cells where Ki67 and DAPI were colocalized ($\text{Ki67} \cap \text{DAPI}$). The final proliferation index was normalized to the total count of DAPI expressing nuclei using a ratio of $[(\text{Ki67} \cap \text{DAPI})/\text{DAPI}]$.

Statistical analysis

Changes in the *in vivo* redox ratio, A1/A2 ratio, and proliferation index were assessed using multi-factor ANOVAs with interactions to test for significant differences. The ANOVA design considered individual image locations as random effects nested with each mouse. Tukey's HSD tests were used for all *post hoc* analysis. Comparisons with $p < 0.05$ were considered to be statistically significant. For correlations between measurements, significance was determined assuming a null hypothesis that $R = 0$. Standard deviation (shown as error bars in all figures) was calculated from among mean values computed for individual animals. The mean values for individual mice are represented by white dots overlaid on all bar charts. All statistical analysis was completed using JMP[®] Pro 13.

RESULTS

Skin wound closure is delayed in aged mice

Wound size was measured before each imaging session and revealed differences between young and aged mice. Both the aged and young mice displayed a consistent decrease in wound size at each subsequent day until day 10 ($p < 0.0001$) (Fig. 1A). Aged mice demonstrated a delay in wound closure

Figure 1. Wound closure in young and aged mice was calculated by measuring wound area relative to the initial day 0 measurements. **(A)** The wound size of both aged and young groups decreased significantly between each subsequent day ($p < 0.001$). In addition, the aged mice displayed a higher average wound size compared to the young group ($p < 0.0001$). **(B)** The three-factor ANOVA of normalized wound size data revealed only postwound time point and age were significant effects. Error bars shown are standard deviation. Asterisks indicate significant differences ($p < 0.05$) between groups. Color images are available online.

with significantly larger wound sizes than young mice ($p < 0.0001$, Fig. 1B). The effects of sex on wound size during healing were not significant ($p = 0.179$), and no significant interactions among sex, age, and postwound day were detected (Fig. 1B).

MPM can monitor wounds noninvasively and longitudinally *in vivo*

In vivo MPM z-stacks taken with a $20\times$ objective at the superior, inferior, and right lateral edges of full-thickness excisional wounds in mice (Fig. 2A, B) enabled visualization of the epidermis, dermis, and wound bed with a resolution capable of discerning individual cells and collagen fibers (Fig. 2C, D). Keratinocytes were visible in the epidermis at the wound edge that primarily produced an emission at 460 and 755 nm excitation corresponding to NADH fluorescence (Fig. 2D; green).

At the wound-facing edge of the epithelium, a thin layer of keratin comprising the stratum corneum fluoresced at 525 nm emission at 900 nm excitation (Fig. 2D; blue). Under the epithelium, the collagen-containing dermis was visible from the strong second harmonic signal it produced in the 460 nm channel at 900 nm excitation (Fig. 2D; red). Hair follicles were also present in the dermis leaving a void in SHG signal and displaying strong NADH fluorescence. Hair contained in the follicles fluoresced strongly in both the NADH and FAD channels (Fig. 2). While the z-stacks collected at the wound edge consistently displayed strong cellular autofluorescence and collagen SHG from the remaining intact dermis at all time points (Fig. 2C, D), the site of excision did not produce any detectable autofluorescence until granulation tissue began to form around day 5. At the later time points of

Figure 2. Noninvasive, *in vivo* multiphoton imaging at the wound edge. **(A)** Anesthetized mice were imaged using a $20\times$ immersion objective and piezo motor to acquire multiple *in vivo* image z-stacks of endogenous optical signals. **(B)** Intensity image z-stacks were acquired at 3 locations at the edge of a 6mm excisional wound from each mouse at each time point as highlighted by the green, blue, and red squares. **(C)** 3D *in vivo* z-stack images at the wound edge are capable of collecting signal from NADH (755 nm ex./460 nm em., green), FAD and keratin (900 nm ex./525 nm em., blue), as well as collagen SHG (900 nm ex./460 nm em., red). By capturing the 3D topography of the wound edge using this endogenous optical contrast, regions such as the epithelium, intact dermis, and wound excision site can be spatially delineated (scale bar = $100\ \mu\text{m}$). **(D)** Representative en face, depth-resolved optical sections taken $125\ \mu\text{m}$ deep from the middle of the z-stacks collected at the wound edge demonstrate the ability of multiphoton microscopy to longitudinally follow the wound edge *in vivo* across time points in aged and young mice (scale bar = $100\ \mu\text{m}$). FAD, flavin adenine dinucleotide; NADH, nicotinamide adenine dinucleotide; SHG, second harmonic generation. Color images are available online.

days 7 and 10, a weak collagen SHG signal was visible in the granulation tissue in the wound, but had a normalized SHG intensity more than an order of magnitude lower than the collagen in the uninjured, adjacent dermis that is visible in the representative images (Fig. 2D).

Epithelial redox ratio is sensitive to differences between young and aged healing

Using fluorescence intensity to discriminate between specific wound regions (Fig. 2D), the epithelial edge was spatially isolated through manual segmentation and an optical redox ratio of FAD/(NADH+FAD) was computed without interference from keratin or collagen present in the surrounding tissue⁴ (Fig. 3A). The aged mice were consistently observed to have a higher average epithelial redox ratio than their young counterparts across all days ($p=0.0002$). Both the aged and young mice experienced a decrease in optical redox ratio within the epithelium between day 1 and days 3 & 5 ($p<0.0001$) (Fig. 3B). By day 10, however, the redox ratio had increased from the lowest point on day 3 for both groups ($p\leq 0.0018$). Differences in the epithelial redox ratio between sexes became visually apparent as early as day 3 (Figs. 3B and 4A). When the epithelial redox ratios were disaggregated by sex and age, aged male mice had a significantly higher redox ratio than young males ($p=0.0388$), and aged females had a higher average redox ratio ($p\leq 0.0322$) than young female and male mice (Fig. 4B).

Optical redox ratio detects sex-related differences in proliferation during healing

To explore potential causes for the changes in epithelial metabolism observed in the *in vivo* optical

Figure 4. Epithelial redox ratio is dependent on age and sex. (A) Representative images of keratinocytes in the epithelial edge of mice at day 3 indicate that redox ratio is dependent on age and sex (scale bar = 100 μm). (B) When averaged over all imaging time points, aged male mice had a higher redox ratio than young males ($p=0.0388$), and aged females had a higher epithelial redox ratio than both young males and young females ($p\leq 0.0322$). Error bars indicate standard deviation. Asterisks indicate significant differences ($p<0.05$) between groups. Color images are available online.

Figure 3. Longitudinal changes in epithelial metabolism during healing can be observed with an optical redox ratio. (A) An optical redox ratio of FAD/(NADH+FAD) fluorescence was generated for each z-stack and pseudo-colored using a jet color map. Representative en face optical sections from the z-stacks at the wound edge are displayed above, $\sim 125 \mu\text{m}$ from the surface of the tissue (scale bar = 100 μm). The epithelium (white dashed lines) was manually segmented in corresponding intensity images and used to calculate an average redox ratio for each mouse. (B) The epithelial redox ratio of aged and young mice initially decreased from day 1 to days 3 and 5 ($p<0.0001$). By day 10, however, the redox ratio had increased for both groups ($p\leq 0.0018$). Overall, the aged mice displayed a higher redox ratio than the young cohort ($p=0.0002$). Error bars indicate standard deviation. Asterisks indicate significant differences ($p<0.05$) between groups. Color images are available online.

redox ratio, the entirety of the wound edge epithelium was imaged from unstained wound sections harvested at the terminal day-10 time point using MPM. The *ex vivo* wound sections were imaged and normalized using the same acquisition parameters as the *in vivo* z-stacks (Fig. 5A). The epithelium was once again recognizable from strong emission in the NADH channel and was characterized by a lower optical redox ratio than the surrounding dermis and wound (Fig. 5A). However, the spatial distribution of the optical redox ratio was not uniform within the epithelium. The redox ratio was lower (~ 0.68 ; blue/

teal) in the keratinocytes at the edge of the initial wound and higher in the cells comprising the leading tip migrating across the wound (~ 0.8 ; yellow/orange) (Fig. 5B). The spatial distribution of the redox ratio in the wound tissue sections was consistent with the patterns observed as keratinocytes initially become more proliferative at the wound edge and then transition to be more migratory as they approach the center of the wound.³³

To elucidate the relationship between keratinocyte proliferation and optical redox ratio in the wound epithelium, a sample of adjacent wound

Figure 5. Optical redox ratio patterns in the wound epithelium coincide with markers of keratinocyte proliferation. **(A)** Epithelial keratinocytes in unstained wound sections are able to be distinguished from collagen SHG in the dermis and fibrin clot in the center of the wound site (scale bar = 250 μ m). **(B)** A spatial gradient in the redox ratio was observed in the wound edge epithelium, in which keratinocytes at the tip of the epithelial tongue migrating toward the wound center had a higher redox ratio than the basal cells (scale bar = 100 μ m). **(C)** Ki67 immunostaining revealed that the basal keratinocytes were highly proliferative, corresponding to regions with a lower redox ratio, while proliferation was not observed in the migrating tip (scale bar = 100 μ m). **(D)** A quantitative proliferation index measuring the ratio of DAPI-stained cells expressing Ki67 revealed differences in proliferation based on age and sex. The proliferation index increased for all mice 10 days postwounding ($p < 0.0001$). However, on day 10, aged mice had a lower proliferation index than the young mice ($p = 0.0106$), and aged females had a lower proliferation index than all other groups ($p \leq 0.0475$). **(E)** When the proliferation index was compared to the optical redox ratio in adjacent unstained tissue sections, a strong negative correlation was observed ($R = -0.7042$, $p = 0.0023$). Asterisks indicate significant differences ($p < 0.05$) between groups. Color images are available online.

tissue sections ($n=16$) was immunolabeled with the proliferative marker Ki67 and counterstained with DAPI. Ki67 expression had a similar spatial distribution to the optical redox ratios observed in the *ex vivo* sections with basal keratinocytes closest to the dermis expressing Ki67, while the tip of the epithelium lacked any Ki67 fluorescence (Fig. 5C). The DAPI counterstain allowed for a proliferation index to be calculated from within the *ex vivo* epithelial tongue by comparing the ratio of cells colocalizing Ki67 and DAPI ($\text{Ki67} \cap \text{DAPI}$) to the total number of DAPI expressing cells. This proliferation index of ($\text{Ki67} \cap \text{DAPI}$)/DAPI was quantified for each group of mice with respect to age and sex in the wound biopsies from day 0 and 10 wound tissue sections (Fig. 5D). Proliferation was markedly increased in the epithelium between day 0 and 10 after healing had begun ($p<0.0001$). On day 10, the aged mice displayed lower proliferation index values than the young mice ($p=0.0106$), and aged females displayed a significantly lower average proliferation index value than all other groups ($p\leq 0.0475$). A correlation was then calculated between the proliferation index and *ex vivo* epithelial redox ratio in the adjacent unstained tissue sections. A strong, negative correlation was observed ($R=-0.7042$; $p=0.0023$) with a high proliferation index value correlating to lower average optical redox ratios in the wound epithelium (Fig. 5E). The data presented provide quantitative evidence of a relationship between keratinocyte proliferation and the optical redox ratio in the wound epithelium consistent with what has been previously observed in diabetic wounds.³⁰

NADH fluorescence lifetime also identifies sex-related differences in epithelial metabolism of aged wounds

NADH fluorescence lifetime images provided added insight into the dynamic metabolic changes in the wound epithelium when biexponential fitting was used to discriminate free (A1) and protein-bound (A2) NADH (Fig. 6A). Across postwound time points and age groups, the epithelium displayed consistent lifetime values for the short ($\tau_1=564\pm 47$ ps) and long ($\tau_2=3,988\pm 252$ ps) lifetime components that are similar to those previously reported for free and bound NADH, respectively.⁸ However, significant differences in the relative amount of free (A1) and bound (A2) NADH were observed. The 24-month-old mice displayed a lower A1/A2 ratio than the 4-month-old mice ($p=0.0104$), and aged females displayed a lower A1/A2 ratio than all other groups ($p\leq 0.0441$) (Fig. 6B).

Figure 6. NADH fluorescence lifetime is sensitive to metabolic differences in age and sex during healing. **(A)** NADH fluorescence lifetime imaging microscopy was acquired en face at the center of each wound edge intensity z-stack, and the ratio of free (A1) to protein-bound (A2) NADH was computed. Keratinocytes can be discerned in the wound edge epithelium, and all pixels within the epithelium (white dashed lines) were used to calculate an average A1/A2 ratio for each mouse on each day (scale bar = 100 μm). **(B)** Across the 10 days postwounding, aged mice had a lower A1/A2 ratio than the young mice ($p=0.0104$) with aged females having a lower A1/A2 ratio than any other group ($p\leq 0.0441$). Error bars indicate standard deviation. Asterisks indicate significant differences ($p<0.05$) between groups. Color images are available online.

NADH fluorescence lifetime correlates to optical redox ratio during keratinocyte proliferation

Acquisition of NADH fluorescence lifetime images at the center of each TPEF intensity z-stack location for each mouse on days 1, 3, 5, 7, and 10 enabled the evaluation of the relationship between the optical redox ratio and A1/A2 ratio (Fig. 7). Overall, there was a significant, negative correlation ($R=-0.2083$; $p=0.0305$) between redox ratio and A1/A2 ratio in the epithelial edge. However, when this correlation was disaggregated by day, negative correlations were only observed on day 3 ($R=-0.5167$; $p=0.0165$) and day 5 ($R=-0.5908$;

Figure 7. Keratinocyte NADH lifetime and optical redox ratio correlate during periods of proliferation. **(A)** Direct comparisons between the redox ratio and NADH lifetime could be made from the keratinocytes (within white dashed lines) at individual wound locations as demonstrated in the representative images. **(B)** Across all days, there was a significant, slightly negative correlation between optical redox ratio and NADH lifetime. When split by day, however, a strong negative correlation was only seen on days 3 ($R = -0.5167$; $p = 0.0165$) and 5 ($R = -0.5908$; $p = 0.0048$), highlighted by pink-colored data points when keratinocytes are primarily proliferating. The time points of days 1, 7, and 10 were not significantly correlated and are represented by white data points. Color images are available online.

$p = 0.0048$). The strong, negative correlations observed on days 3 and 5 overlap with the onset of keratinocyte proliferation,^{30,34} while days 7 and 10 are associated with the transition to keratinocyte migration.³³

DISCUSSION

As nonhealing wounds disproportionately affect the elderly, this study sought to utilize the natural fluorescence of NADH and FAD to monitor dynamic changes in wound metabolism of aged mice and elucidate the effects intrinsic aging has on these endogenous optical biomarkers. Using MPM, epithelial keratinocytes were longitudinally tracked *in vivo* in mice at 4 and 24 months of age by acquiring 3D z-stack images at the wound edge (Fig. 2). Isolating epithelial keratinocytes made quantification of an optical redox ratio possible in the wound edge, where aged mice consistently displayed a higher redox ratio than young mice starting as early as days 3 and 5 (Fig. 3). These metabolic shifts in wound healing with age are in contrast to the lack of a difference in the optical redox ratio between diabetic and healthy mice at these same postwound time points.³⁰ While advanced age resulted in an elevated redox ratio during the early stages of healing, diabetes drives the redox ratio down at the later stages.³⁰ This study indicates that the spatial and temporal dy-

namics of the epithelial redox ratio are strongly associated with changes in keratinocyte proliferation (Fig. 5). Fluorescence lifetime measurements indicated a decrease in the ratio of free/bound NADH in aged mice, and aged females in particular, which is consistent with decreased proliferation (Fig. 6). In contrast, diabetic wounds did not have significantly different NADH fluorescence lifetime outcomes compared to healthy wounds in previous work.³⁰ Collectively, our findings suggest that both the optical redox ratio and NADH lifetime might be useful metrics in quantitatively discerning metabolic changes in wound healing related to age and sex from the influence of comorbidities such as diabetes.

The early decrease in epithelial redox ratio observed in both the aged and young groups between days 3 and 5 postwounding (Fig. 3) is consistent with the onset of keratinocyte proliferation we have previously reported.³⁰ Briefly, this is due to the biosynthetic demands of proliferation that require a significant amount of carbon to create new organelles, structural molecules, and phospholipid membranes before cell division.^{35,36} Cells increase glucose uptake to satisfy these demands and divert glycolytic metabolites toward macromolecule synthesis rather than further catabolism and efficient ATP production.³⁷ Without a proportional increase in ATP consumption, increased glycolysis causes the concentration of free NADH to increase, lead-

ing to a lower redox ratio of FAD/(NADH+FAD) and an increase in the A1/A2 ratio of free/bound NADH in proliferating keratinocytes. Similar trends indicating an increase in free NADH have been reported in the redox ratios and NADH lifetime of highly proliferative epithelial cancers.^{4,38,39} Despite the similarity in temporal redox ratio shifts between aged and young mice, we also observed that aged wounds consistently displayed a higher optical redox ratio (Figs. 3 and 4), lower A1/A2 ratio (Fig. 6), and were less proliferative (Fig. 5) than young wounds. This overall decrease in proliferative capacity may be the result of increased senescent keratinocyte populations in aged skin^{40,41} where fewer proliferating keratinocytes would directly increase the average optical redox ratio at the wound edge.

In addition to the optical redox ratio, NADH fluorescence lifetime images were able to discriminate metabolic changes occurring in the keratinocytes of aged and young mice. Previous studies have reported that an A1/A2 ratio of NADH lifetime species increases with glycolysis during the proliferation of epithelial cells.⁴² Highly proliferative cells such as stem cells^{42,43} and aggressive carcinomas¹⁶ also display an accumulation of free NADH and lower mean fluorescence lifetimes. These reports are consistent with the higher optical redox ratio and decreased A1/A2 ratio measured in the less proliferative aged females in our study (Fig. 6), as well as the significant correlation between the A1/A2 ratio and optical redox ratio we observed on days 3 and 5 (Fig. 7). Interestingly, when correlations between the epithelial redox ratio and A1/A2 ratio were made with respect to day, only days 3 and 5 had strong negative correlations (Fig. 7) overlapping with peak keratinocyte proliferation in the wound edge epithelium.³⁰ While the optical redox ratio alone requires longitudinal monitoring of wounds to assess the stage of wound healing, a combination of optical redox ratio and A1/A2 ratio images acquired at the same wound site may serve to detect proliferation in a single snapshot in time based on the strength of the correlation coefficient. This correlation between metrics may be particularly helpful in evaluating wound status in clinical settings where longitudinal optical monitoring may not be feasible.

The optical redox ratio (Fig. 4), proliferation index (Fig. 5), and NADH fluorescence lifetime (Fig. 6) all strongly implicate sex as an important factor in wound metabolism. Aged females displayed a lower average proliferation index, higher optical redox ratio, and decreased A1/A2 ratio than

any other group. Sex-linked hormones such as estrogen have been observed to play significant roles in wound healing and skin aging, particularly in postmenopausal women.^{44,45} As a topical treatment, estrogen has been found to accelerate wound healing in the elderly,⁴⁶ with postmenopausal women displaying increased wound closure rates after being given hormone replacement therapy.⁴⁷ Estrogen can further prevent excessive neutrophil accumulation⁴⁶ and has been found to protect against chronic wound formation in older women postmenopause.^{48,49} Estrogen can even help prevent age-related senescence in a variety of cell types.^{50–52} As a signaling molecule, estrogen interacts directly with receptors on the outer membranes of the nucleus and mitochondria to promote the expression of transcription factors like NRF1 responsible for upregulating proteins crucial to cellular respiration and mitochondrial biogenesis.^{53,54} In addition, estrogen that interacts with mitochondrial estrogen receptors activates signaling cascades that limit reactive oxygen species production and provide protection from oxidative damage that accumulates with age.⁵³ When a wound is sustained, estrogen encourages cell growth, mitigates damage and inflammation, and prevents senescence. The decrease in circulating estrogen following menopause can influence a variety of wound healing factors that inhibit cell proliferation and result in higher *in vivo* optical redox ratios and lower A1/A2 ratios that were observed *in vivo*.

Despite differences in keratinocyte metabolism between male and female mice, significant differences in wound size were not as detectable between the sexes ($p=0.179$). Although aged females displayed overall larger wounds compared to young females ($p=0.0003$), wound size measurements were too underpowered to detect differences between age and sex within any single postwound time point. We observed higher mouse-mouse variability in wound closure compared to the relatively low variability between *in vivo* redox ratios and NADH lifetimes. Although age-dependent differences in wound size and cell metabolism were observed, future work is needed to understand the role of sex-dependent differences in cell metabolism and their potential relationship with wound closure.

We have demonstrated that *in vivo* MPM is capable of providing longitudinal, quantitative measurements of cell metabolism and is sensitive to changes in skin wound healing associated with advanced age. Collection of an *in vivo* optical redox ratio has been traditionally proven difficult

in complex tissues like the skin due to interference from other endogenous chromophores, but high-resolution TPEF imaging enabled the spatial isolation of keratinocyte metabolism in the epithelium. NADH fluorescence lifetime has seen use in assessing *in vivo* tissue,^{55–59} but its relationship to the optical redox ratio during skin repair has not been previously evaluated. In this study, we show that both the optical redox ratio and NADH lifetime are sensitive to the metabolic demands of keratinocyte proliferation during wound healing, which varies across biological factors such as age and sex in mice (Figs. 4–6). Future work will explore the underlying mechanisms of these age- and sex-related differences in keratinocyte function and the interplay between sex hormones, the inflammatory response, and reepithelialization. Due to differences in the aging process between mice and humans, additional work is necessary to investigate whether the findings in this study can be extended to humans. Collagen SHG data may also provide insight into differences in the structure and composition of the wound and adjacent skin between aged and young mice. By applying MPM to *in vivo* models of wound healing, this work demonstrates that an optical redox ratio and NADH fluorescence lifetime can serve as quantitative biomarkers capable of monitoring changes in wound metabolism that result from intrinsic aging. These surrogate biomarkers for wound healing may supplement traditional histological and immunostaining techniques in wound healing research, and can provide a noninvasive means to detect different causes of delayed wound healing in the clinic to provide patient-specific treatment.

INNOVATION

This study demonstrates that *in vivo* MPM can provide multiple noninvasive, quantitative biomarkers of wound healing sensitive to intrinsic aging. By taking advantage of the intrinsic fluorescence of NADH and FAD, no stains or dyes are needed to quantify wound metabolism. The advent of clinical multiphoton systems such as the MPTFlex⁶⁰ for skin cancer detection and the development of rapid optical redox imaging utilizing wavelength mixing⁶¹ provide clear directions for the clinical translation of *in vivo* MPM to assist in elderly wound care.

KEY FINDINGS

- *In vivo* MPM can be used to noninvasively assess wound organization and metabolism based on the natural fluorescence of NADH and FAD.
- Keratinocyte metabolism exhibited age- and sex-dependent differences during healing, which correlated with a reduction in cell proliferation in aged female mice.
- The changes in wound metabolism associated with intrinsic aging differed from those changes observed in previous diabetic wound healing studies.
- Cellular autofluorescence may serve as a noninvasive surrogate biomarker of wound healing, which could be used in the future to help guide patient-specific treatment options.

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Abbreviations and Acronyms

FAD = flavin adenine dinucleotide
 FLIM = fluorescence lifetime
 imaging microscopy
 IRF = instrument response function
 MPM = multiphoton microscopy
 NADH = nicotinamide adenine dinucleotide
 SHG = second harmonic generation
 TPEF = two-photon excited fluorescence