Non-invasive, real-time imaging of early wound re-epithelialization *in situ*

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Abstract and Introduction

Non-healing wounds remain an increasing problem with care costs and mortality rates exceeding many cancers. Despite our deep understanding of the factors controlling healing, comprehensive wound diagnostic technologies are lacking, resulting in wound care failing to deliver consistent or favorable outcomes. To these ends, we have built and deployed a hand-held, non-invasive multispectral imager (MSI) capable of spatially capturing the presence or abundance of microbial pathogens while characterizing several key physical, chemical, and biological wound bed and edge features, including but not limited to inflammation, granulation, and epithelialization. Collectively, these wound metrics should enable practitioners with an unbiased, robust and real-time dataset offering opportunities for precise, personalized and, ultimately, superior wound care.

One key feature of healing, preceding observable, clinical signs (i.e., wound area reduction), is the onset of re-epithelialization, which we aimed to visualize via cellular metabolic activity with our MSI. We report on characterizing early epithelialization occurring in real-time using: (i) a pre-clinical ex-vivo human skin model of wound repair and ii) observational, IRB-approved studies evaluating host responses to microbial burden, and wound inflammatory, infective, reparative or healing responses. In pre-clinical studies, 8 mm by 3 mm wound explants were temporally imaged with our MSI, with re-epithelialization signatures validated in parallel by immunohistochemistry of early and late markers of epithelialization. With real-time imaging of ex-vivo tissue recovering from injury, we consistently visualized re-epithelialization invisible to the naked eye, correlating with the presence or absence of the markers of migration, proliferation and differentiation (e.g., K14, K6, K10, filaggrin). Additionally, real-time imaging of patients' non-healing wounds similarly revealed cellular metabolic activity corresponding with early epithelialization. Together, these data support the hypothesis that non-invasive, real-time characterization of non-healing wounds is achievable and will enable practitioners to treat non-vital or microbially-burden tissue while preserving wounds with early re-epithelialization, before observable clinical.

Methods

Antibodies and reagents: Antibodies and reagents for immunohistochemistry and immunofluorescence were Keratin 6a (1:100, BioLegend, #905701); Keratin 10 (1:500, BioLegend #905401); Keratin 14 (1:750, BioLegend, #905301); Filaggrin (1:200, Abcam, #ab3137); ProLong Gold Antifade Mounting Medium with DAPI (ThermoFisher Scientific; P36935); Image-iT FX signal enhancer (Molecular Probes, #I36933); Xylenes (Sigma, #108298).

Human ex vivo: Healthy human skin specimens were obtained as discarded tissue from reduction surgery procedures in accordance to institutional approvals. Specifically, protocol to obtain unidentified, discarded human skin specimens from reduction surgery was submitted to University of Miami Human Subject Research Office (HSRO). Upon review conducted by University of Miami Institutional Review Board (IRB) it was determined that such protocol does not constitute Human Subject Research. As such, this project was not subject to IRB review under 45 CFR46.101.2. Human skin specimens from reduction surgery were used to generate acute wounds as previously described [Castellanos et al., 2020; Jozic et al., 2019]. Briefly, a 3mm biopsy punch was used to create an acute wound within an 8mm biopsy (n=3 per time point) which were treated daily with media (DMEM supplemented with 10% FCS). Tissue was harvested at 0hr, 24hr and 72hrs post wounding, imaged using Precision Healing's Multispectral Imager (MSI), fixed in 10% formalin, processed and embedded in paraffin. Paraffin sections (5µm) were stained with hematoxylin and eosin for histological analysis, and immunohistochemistry experiments.

Immunohistochemistry: Formalin fixed/paraffin embedded tissue was cut at 5-7µm sections using a microtome. Slides containing sections were deparaffinized with xylene, and rehydrated in graded ethanol. Image-iT FX signal enhancer was then applied to rehydrated tissue specimens for 30min followed by incubation 5% bovine serum albumin in PBST, prior to incubation with appropriate antibody overnight. The following day, sections were then counterstained with AlexaFluor 488/594 coupled secondary antibodies for 1hr prior to mounting in ProLongTM Gold Antifade mounting medium with DAPI. Samples were imaged and analyzed using a Keyence BZ-X700 microscope.

Multi-spectral Imager (MSI): Real-time, live images were taken of breast explants recovering from injury ex vivo using the Precision Healing multispectral imager. The imager was exposed 7 times with an 8ms integration time.

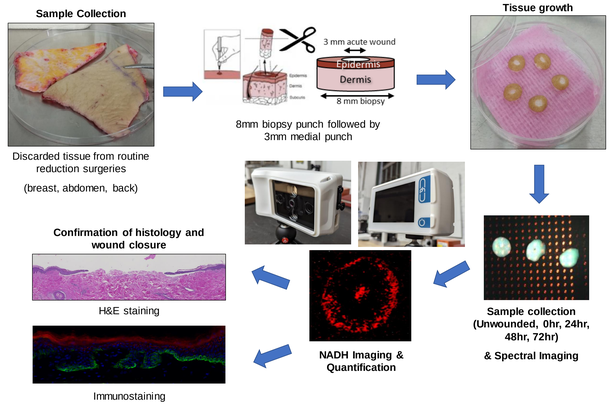
Image Processing: Captured images using Precision Healing imager were then processed for correction of field irregularities (Flat field correction) and for ambient lighting correction. This operation was performed on the image average created by the 7 exposures (see above, MSI) in synchronization with the illumination.

Jozic, I., et al., *Pharmacological and Genetic Inhibition of Caveolin-1 Promotes Epithelialization and Wound Closure.* Mol Ther, 2019. **27**(11): p. 1992-2004.

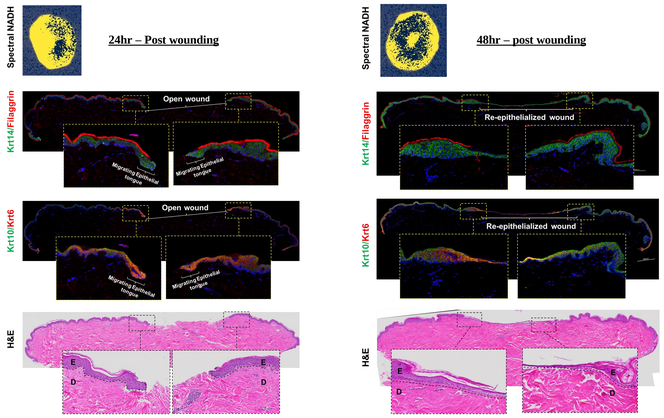
Castellanos, A., et al., *Multimodal, in Situ Imaging of Ex Vivo Human Skin Reveals Decrease of Cholesterol Sulfate in the Neoepithelium during Acute Wound Healing.* Anal Chem, 2020. **92**(1): p. 1386-1394.

Pre-clinical/human ex vivo data

Workflow depicting spectral imaging of human ex vivo wounds



Pre-clinical Results

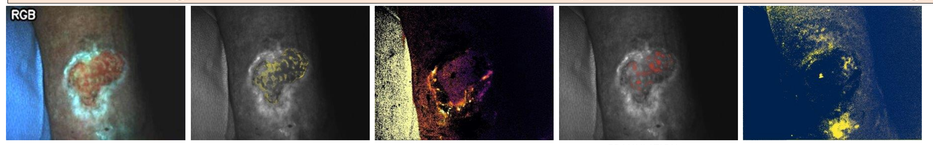


Observational IRB-approved Clinical Study

Patient presents at clinic with slough-covered VLU: sharp debridement to be performed

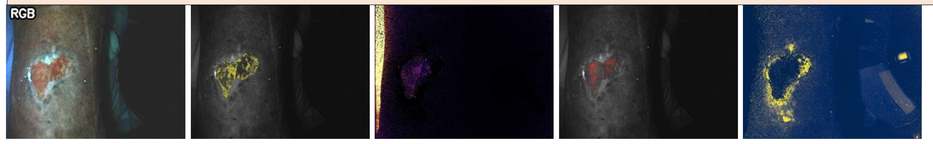
- High slough coverage in wound bed
- Red autofluorescing microbes detected (1+ coagulase-negative staphylococcus from swabbing)
- No granulation tissue detected
- Cellular metabolism signal present around full wound periphery
- ***Wound debrided following imaging without guidance by PH diagnostics guarding against/avoiding sharp debridement removal of early-epithelialization at wound edge.***

Non-guided sharp debridement removes early-epithelialization circumscribing wound bed/edge



- Slough coverage remains decreased 1 week following debridment
- Red auto fluorescing microbes detected at higher levels (2+ coagulase negative staphylococcus from swabbing)
- Granulation tissue present in wound increases
- ***Cellular metabolism signal at edge diminished***
- ***Previous week's sharp debridement removed early epithelializing tissue in addition to slough***

3 weeks post sharp debridement: Re-epithelialization circumscribing wound perimeter is captured by PH MSI and reflected in cellular metabolism



- Slough coverage detected
- below pre-debridement levels
- Red auto fluorescing microbes detected (3+ cutaneous flora from swabbing)
- Granulation tissue increasing in wound
- ***Cellular metabolism re-emerges at wound edge 3 weeks post sharp debridement***
- ***Increased cellular metabolism at wound edge coincides with reduced wound area***

Clinical Takeaways

- **PH MSI IS ABLE TO NON-INVASIVELY CAPTURE MICROBIAL BURDEN AND THE HOST RESPONSES TO INJURY, CHARACTERIZING**
  - **NON-VITAL TISSUES (SLOUGH AND ESCHAR)**
  - **VITAL TISSUES (GRANULATING and EPIITHELIALIZING WOUNDS)**
- **PH MSI IS ABLE TO SPATIALLY IDENTIFY WHERE MICROBIAL BURDEN EXISTS WITHIN THE WOUND BED OR PERI-WOUND REGIONS WHILE GIVING OPPORTUNITYIES FOR GUIDED DEBRIDEMENT**
- **PH MSI WILL ENABLE THE SWITCHING OF SHARP DEBRIIDEMENT TO ENZYMATIC DEBRIDEMENT, BEING ABLE TO RECOGNIZE WHERE OR WHETHER THE WOUND BED IS RE-EPITHELIALIZING OR HEALING, THUS PREVENTING 'RE-SETTING THE WOUND BED TO 'ZERO' EACH TIME SHARP DEBRIIDEMENT IS PERFORMED**