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Laser capture microdissection on surgical tissues to identify aberrant gene expression in impaired wound healing in Type 2 diabetes

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TITLE:

Laser capture microdissection on surgical tissues to identify aberrant gene expression in impaired wound healing in Type 2 diabetes

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KEYWORDS:

Diabetes, wound healing, chronic wounds, ex vivo, laser capture microdissection, microarray

SUMMARY:

This technique provides a guide to inflicting ex vivo wounds, performing laser capture microdissection and quantifying changes in gene expression related to poor wound healing processes in diabetes using clinically relevant human tissue.

ABSTRACT:

The global prevalence Type 2 diabetes mellitus (T2DM) is escalating at a rapid rate. Patients with T2DM suffer from a multitude of complications and one of these is impaired wound healing. This can lead to the development of non-healing sores or foot ulcers and ultimately to amputation. In healthy individuals, wound healing follows a controlled and overlapping sequence of events encompassing inflammation, proliferation, and remodelling. In T2DM, one or more of these steps becomes dysfunctional. Current models to study impaired wound healing in T2DM include in vitro scratch wound assays, skin equivalents, or animal models to examine molecular mechanisms underpinning wound healing and/or potential therapeutic options. However, these do not fully recapitulate the complex wound healing process in T2DM patients, and ex vivo human skin tests are problematic due to the ethics of taking punch

biopsies from patients where it is known they will heal poorly. Here, a technique is described whereby expression profiles of the specific cells involved in the (dys)functional wound healing response in T2DM patients can be examined using surplus tissue discarded following amputation or elective cosmetic surgery. In this protocol samples of donated skin are collected, wounded, cultured ex vivo in the air liquid interface, fixed at different time points and sectioned. Specific cell types involved in wound healing (e.g., epidermal keratinocytes, dermal fibroblasts (papillary and reticular), the vasculature) are isolated using laser capture microdissection and differences in gene expression analyzed by sequencing or microarray, with genes of interest further validated by qPCR. This protocol can be used to identify inherent differences in gene expression between both poorly healing and intact skin, in patients with or without diabetes, using tissue ordinarily discarded following surgery. It will yield greater understanding of the molecular mechanisms contributing to T2DM chronic wounds and lower limb loss.

INTRODUCTION:

The incidence of type 2 diabetes is growing globally, driven by an obesity epidemic and physical inactivity. Poor wound healing is common in these patients and up to 25% of patients will develop a chronic non-healing wound[1]. The mechanisms underpinning this are complex and incompletely understood, limiting the discovery rate for new therapeutics. One of the contributing factors to this is the lack of a suitable model for studying wound healing in type 2 diabetes patients. Thus, the purpose of this method is to provide a physiologically relevant ex vivo model for examining wound healing in those at risk of chronic wounds, allowing for transcriptomic analysis to progress the identification of new therapeutic targets.

There are multiple models currently available to study wound healing, and each have their strengths and weaknesses. In vivo animal models, such as the db/db mouse[2] or streptozotocin-induced diabetic rats[3] are widely used; however, wounds in rodent models heal via contraction, which is very different to the mechanism employed in the human body, and have limited success in translation to clinical trials[4,5]. The benefits of using human tissue are well recognized[4], but are complicated by the ethics of inflicting experimental wounds on individuals who are already known to sustain an impaired wound healing response. Consequently, studies on human subjects with diabetes is more commonly directed towards the inflammatory response rather than experimenting on excised tissue[6]. Organ-on-a-chip[7] and artificial skin models[8] are also available. These have the benefit of being able to analyze human cell contributions but give little indication of inter-patient variability. Thus, clinically relevant models to study the progress of wound healing in vulnerable patient populations could accelerate mechanistic understanding and drug discovery in this area.

The ex vivo wounding protocol described below is adapted from Stojadinovic and Tomic-Canic, 2013[9]. It is suitable for examining transcriptional data from controlled wounding of human tissue samples ex vivo and can be applied to clinical samples from patients with poor wound healing (e.g., type 2 diabetes, elderly individuals), in order to advance knowledge on how wound healing is impacted in these conditions and potentially how it can be restored.

PROTOCOL:

This protocol relies on the provision of human surgical tissue. Ethical approval and informed patient consent were obtained prior to experimentation, and the study conformed with the principles outlined in the Declaration of Helsinki.

1. Collection of tissue and ex vivo wounding

1.1. Collect surgical tissue following limb amputation/surgery into a sterile container containing Dulbecco's Modified Eagle Medium (DMEM) with 5% penicillin-streptomycin-fungizone, 2 mM L-glutamine and 10% fetal bovine serum (FBS; complete growth medium).

NOTE: Tissue collected in this manner can be stored at 4 °C for up to 24 h prior to experimentation. To ensure viability of tissue for ex vivo experiments, it is recommended to process the tissue as soon as possible or to maintain a fixed time gap between collection and wounding to standardize experiments across multiple tissue donations. If the tissue is to be stored for any amount of time, an RNA stabilization solution should be added to the sample to maintain RNA integrity.

1.2. Using sterile forceps, transfer the tissue to a 60 mm Petri dish filled with sterile phosphate buffered saline (PBS) supplemented with 5% penicillin-streptomycin-fungizone. Trim the fat off the dermis using a sterile scalpel and/or surgical scissors and discard. Transfer the tissue to a fresh petri dish filled with sterile PBS.

1.3. Attach two sterile blades together such that there is a 1 mm gap between the two blades. Score two parallel linear lines, ~1 mm apart, across the length of the epidermis and the upper part of the papillary dermis[10,11].

1.4. Remove the epidermis between the score lines using sterile forceps and surgical scissors to create a linear wound.

1.5. Use a scalpel to cut the tissue into small rectangles (maximum size of 1 cm^2) encompassing the wound with intact epidermis on either side.

NOTE: To maintain tissue and subsequent RNA integrity, steps 1.2-1.5 should be conducted as quickly as possible.

1.6. Take an image of the wounded tissue using a microdissection microscope at 4x magnification ensuring the field of view captures the entirety of the wound and the surrounding intact epidermal tissue.

1.7. Using sterile forceps, place the tissue rectangles on a tissue culture insert in a 6-well plate and gently pipette 2 mL of complete growth medium into the well. This will ensure that the sample is cultured at the liquid-air interface.

1.8. Incubate at 37 °C in 5% CO_2 in air for up to 120 h.

NOTE: The tissue should be imaged on a microdissection microscope at the same magnification as in step 1.6 at the end of the incubation. Images can also be taken every 24 h if necessary.

2. Tissue fixation and cryosectioning

2.1. Snap freeze the tissue in liquid nitrogen. Place a small amount of cryostat-compatible cutting medium onto a cryostat chuck and embed the tissue within it. Orient the tissue perpendicular to the chuck so that the cutting face will cut through all the layers of the skin including the wounded area. Store at -80 °C.

NOTE: The protocol can be paused here.

2.2. Clean the cryostat at room temperature with 70% ethanol and RNase decontamination spray. Set the cryostat temperature to -28 °C.

2.3. Check that the orientation of chuck and cryostat blade can produce sections that encompass the full thickness of the tissue including the wounded tissue and underlying dermis.

2.4. Using the cryostat, cut up to ten 7 µm sections from each wound and place on to RNA-free microdissection slides.

2.5. Store the slides in the original box that the microdissections slides were provided in at -80 °C to ensure an RNase-free environment.

NOTE: The protocol can be paused here but be aware RNA degradation will increase the longer the storage time is.

3. Laser capture microdissection

3.1. Place the microdissection slides with the tissue section in dry ice and go to the laser capture microdissection microscope room.

3.2. Air-dry the 1st slide and quickly proceed to stain the sections with hematoxylin using an RNase-free hematoxylin and eosin staining kit immediately prior to performing laser capture microdissection.

3.3. Visualize the wounded tissue using a laser capture microdissection microscope at 10x magnification and take a picture of the area of interest that will be laser captured.

3.4. Trace around that area (for example, epithelial tongue, granulation tissue, microvessels) and collect into microdissection 0.5 mL diffuser isolation caps using the software instructions

for the particular microscope that is being used.

3.5. Re-visualize and image the laser captured area of interest using the laser capture microdissection microscope at 10x magnification to demonstrate the location and complete excision of the dissected tissue.

NOTE: Perform laser capture for each sample (up to 10 sections) for a maximum time of 1 h to minimize RNA degradation.

3.6. Add RNA storage buffer to the tube containing the microdissected tissue according to manufacturer's instructions, and store in dry ice until it can be transferred to a -80 °C freezer.

NOTE: The protocol can be temporarily paused here.

4. Quantification of differential gene expression

4.1. Centrifuge the sample tubes at full speed for 1 min.

4.2. Isolate RNA from the microdissected tissue following the manufacturer's instructions.

4.3. Elute the RNA in a final volume of 12 µL of RNase free water and amplify the RNA using an RNA amplification kit and thermal cycler, according to manufacturer's instructions. Two rounds of amplification are recommended to ensure sufficient RNA for further analysis. Use the amplification conditions in **Table 1**.

[Table 1 here]

4.4. Quantify the concentration and purity of the amplified RNA. Absorbance ratios of A260/230 (purity) and A260/280 (contaminants) > 1.8 are suitable for further analysis.

NOTE: Purified amplified RNA can be used for focused gene expression studies (steps 4.5-4.6) or can be sent away for microarray analysis.

4.5. Synthesize cDNA using high-capacity cDNA reverse transcription kit according to manufacturer's instructions. Representative conditions are as follows:

- 25 °C for 10 min
- 37 °C for 2 h
- 85 °C for 5 min
- 4 °C hold

4.6. Perform quantitative PCR using 0.5 µL of cDNA and 1 µM primers (of the gene of interest) according to manufacturer's instructions. Representative conditions and primer sequences are as follows.

4.6.1. Use the following conditions for quantitative PCR conditions: 95 °C for 10 minutes; 40 cycles of 95 °C for 15 s and 62 °C for 1 min; 95 °C for 5 min; and 4 °C hold.

4.6.2. Use the following primer sequences:

GAPDH

Forward	5'-TATAAATTGAGCCCGCAGCC-3'
Reverse	5'-CGACCAAATCCGTTGACTCC-3'

KRT17

Forward	5'-AGGGAGAGGATGCCCACCTG-3'
Reverse	5'-GCGGGAGGAGATGACCTTGC-3'

5. Data interpretation

5.1. Calculate the rate of wound healing using the images of the ex vivo tissue generated in steps 1.6-1.8 using ImageJ. There are multiple recent publications highlighting different variations on how to automatically analyze wound areas in ImageJ[12-14].

5.2. Quantify gene expression by using the ΔC_T method, comparing the threshold cycles for the gene of interest compared to that of the housekeeper (e.g., glyceraldehyde-3-phosphate; GAPDH). To do this, note the C_T value for the housekeeper and for the gene of interest.

5.2.1. Calculate the difference between the two C_T values to normalize the amount of mRNA present and control for differences in RNA extraction concentrations between different isolations:

$\Delta C_T = C_T$ (gene of interest) – C_T (housekeeper)

5.2.2. Calculate the magnitude of difference between the C_T values to give the relative quantification of the gene of interest, expressed as a percentage of the housekeeper:

Relative quantification = $(2^{-\Delta CT}) \times 100$

5.2.3. Examine differences between different patient donors/disease states by comparing the relative quantifications of the genes of interest for each sample.

NOTE: A schematic of the entire technique can be found in **Figure 1**.

REPRESENTATIVE RESULTS:

Following the protocol, a 48 h timepoint was chosen to generate representative results. The creation of the initial wound in surplus tissue from elective cosmetic surgery can be seen in **Figure 2A** where the excised wound is clearly visible. Haematoxylin and eosin staining confirms that this has generated a full thickness wound (**Figure 2B**). After 48 h, partial closure of the wound is visible under the light microscope (**Figure 2C**). Histological staining reveals the epithelial tongue that is progressing to heal the wound (**Figure 2D**), demonstrating that the ex vivo wound healing model is a valid proxy for in vivo wound healing.

After sectioning and staining with haematoxylin and eosin, the healing wound was visualized on the laser capture microdissection system and the wound area selected (**Figure 3A**). This area was completely excised using this method as can be seen in **Figure 3B**. RNA quality and purity was reasonable (analyzed by RNA integrity number (RIN); **Figure 3C**) – poor quality amplified RNA would have many peaks and troughs indicating multiple degradation products. Isolation of small tissue sections may yield RNA that is of a very low concentration that makes valid interpretation of qPCR difficult. **Figure 3D** demonstrates the variation in RNA concentration to be expected using this technique, with a range of 2.00 to 6.15 ng/mL. Importantly, even dilute samples were able to give robust C_T values for both housekeeper (*GAPDH*) and skin-specific genes of interest (keratin 17; *KRT17*; Figure 3E), confirming the suitability of the technique for comparative transcriptomic studies.

FIGURE AND TABLE LEGENDS:

Figure 1: Flow diagram of the complete technique to perform gene expression analysis on laser microdissected tissue from wounded skin. Tissue is wounded, allowed to heal in a tissue incubator and imaged (steps 1-6) before being cut into 7 μm sections using a cryostat (step 7). The region of interest (e.g. epithelial tongue) is identified and collected using laser capture microdissection (steps 8-9) and RNA isolated, purified and gene expression determined (steps 10-12).

Figure 2: Ex vivo wound model. Human tissue was wounded by creating two parallel cuts and removing the tissue between to leave a uniform wounded area (**A**, light microscope, scale bar = 200 μm; **B**, haemoatoxylin and eosin staining, scale bar = 100 μm). The tissue was cultured in a standard tissue culture incubator at 37^{o}C in 5% CO_2 in air for 48 h (**C**, light microscope, scale bar = 100 μm; **D**, haematoxylin and eosin staining, scale bar = 200 μm). Arrow heads indicate epithelial tongue.

Figure 3: Laser capture microdissection and gene expression. A. The region of interest (in this case, healed tissue) was identified using haematoxylin staining and collected using laser capture microdissection. **B.** The same region imaged after microdissection. Scale bars = 50 μm. **C.** Representative electropherogram of RNA that was isolated, amplified and quantified from the laser microdissected tissue. **D.** RNA concentration from collected tissue (n=24 samples). **E.** Reproducible detection of *GAPDH* and *KRT17* expression using qPCR (n=13 samples, mean ± SEM).

Table 1: Amplification conditions.

DISCUSSION:

As the incidence of chronic disorders such as type 2 diabetes increases globally, the need for techniques that can facilitate pathophysiologically relevant studies becomes more urgent. The protocol described above provides a standardized method for examining transcriptomic data from ex vivo healing wounds utilizing human tissue.

This protocol is dependent on the provision of surplus clinical tissue for which ethical permission has been granted from the relevant authority, and from patients who have given informed consent. Commonly this will be from patients undergoing amputation or elective cosmetic surgeries. The clinical demographics of donors needs to be carefully considered, however one of the strengths of this method is the opportunity to perform studies on tissues from multiple patient donors from healthy control individuals (for example, those who are undergoing amputation following an accident or who are having elective cosmetic procedures) and those with chronic diseases such as type 2 diabetes. Furthermore, as the method can be paused at numerous points (see protocol), ex vivo wounds can be prepped as and when surgeries take place and then stored for side-by-side RNA analysis once enough tissue samples have been secured.

The most critical step in this protocol is laser capture microdissection (protocol section 3). Whilst there are many publications that utilize this technique for transcriptomic analysis, the current report is the first to provide a thorough experimental protocol to apply this to studies on human tissue from patients with an impaired wound healing response. It is essential that laser capture is performed as quickly as possible and for one hour as an absolute maximum. This is to minimize RNA degradation as the frozen sections return to room temperature and can be monitored during the amplified RNA quality check in protocol section 4.

While this technique adds value in allowing consideration of interpatient variability in human wound healing mechanisms, it does have some limitations. The impact of inflammatory cells cannot be assessed as the ex vivo model has no functioning circulation. If this is the focus of a study, then in vivo models (both animal[2,3] and human[6]) would be of more benefit. Furthermore, the provision of clinically relevant tissue can be a limiting factor. In these instances, artificial skin[8] or skin-on-a-chip[7] models may be more accessible. Regardless, one of the major strengths of the protocol is that it can study transcriptional level events from wounded human tissue, from multiple donors and from different patient groups. Ordinarily, studies of this kind would be conducted using in vitro models which, whilst providing valuable insight, do not replicate the complexities of wounded tissue[15]. Altogether, this technique is a useful adjunct to the currently existing suite of in vitro, in vivo and ex vivo models for wound healing in diabetes.

In summary, this workflow can be used to study wound healing mechanisms not only in type 2 diabetes but also other clinically vulnerable groups, for example the elderly or those with connective tissue disorders. The continued development of proteomic and metabolomic assays with enhanced sensitivity[16-18] will expand the applications of this technique even further.

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DISCLOSURES:

The authors have nothing to disclose.

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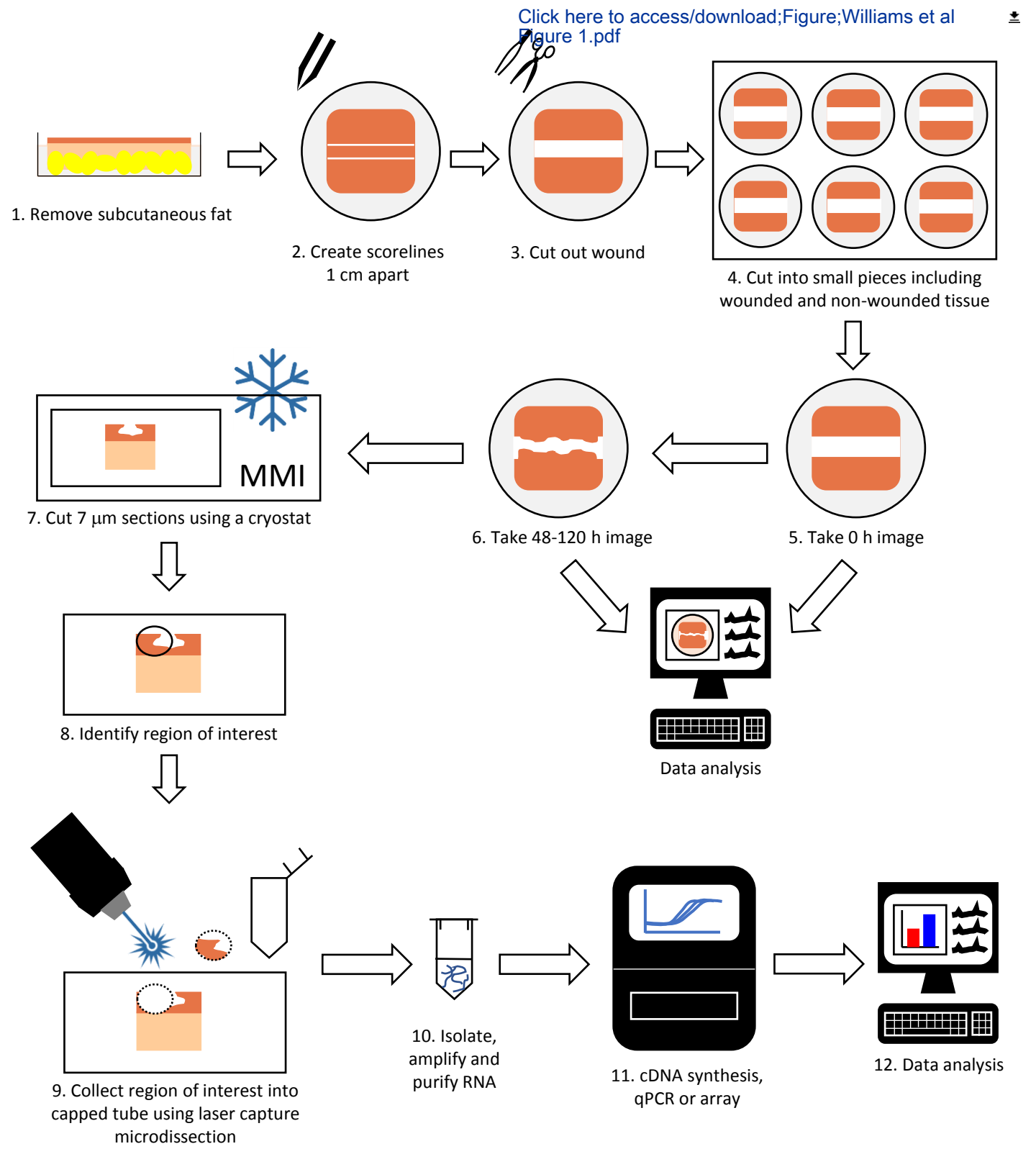

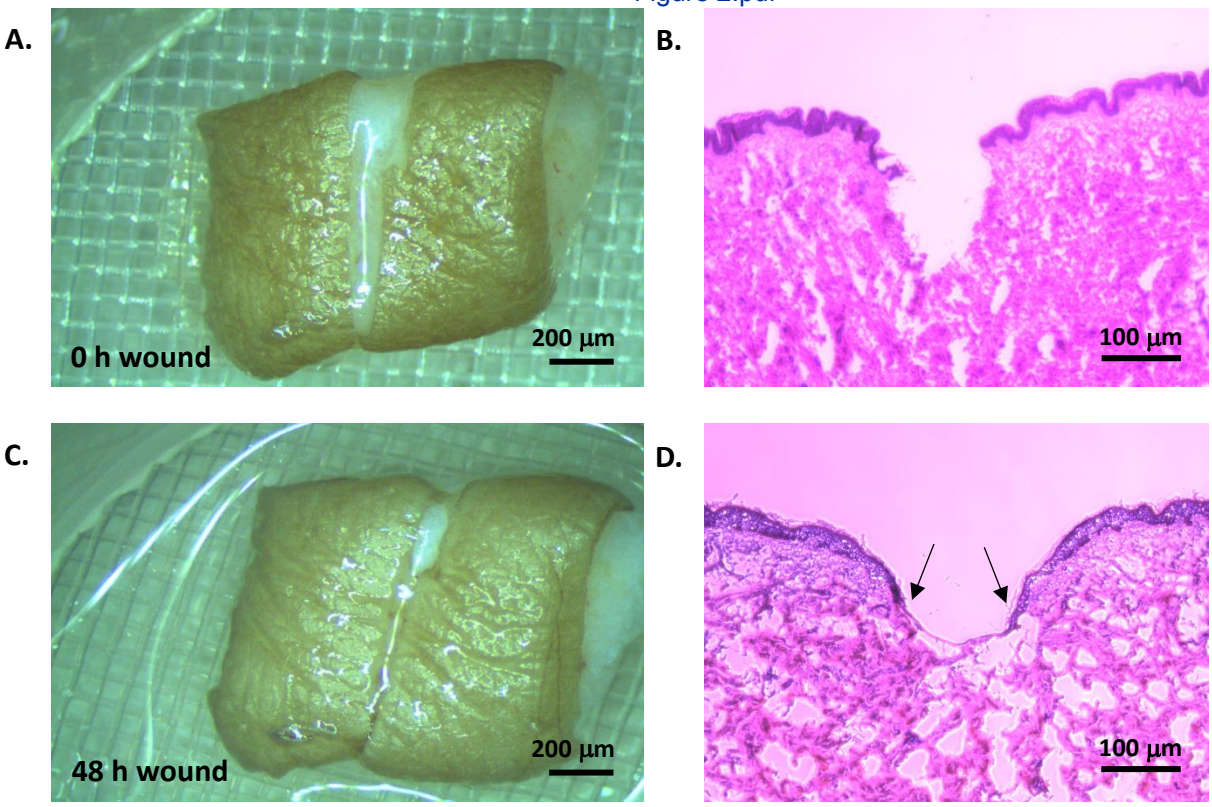

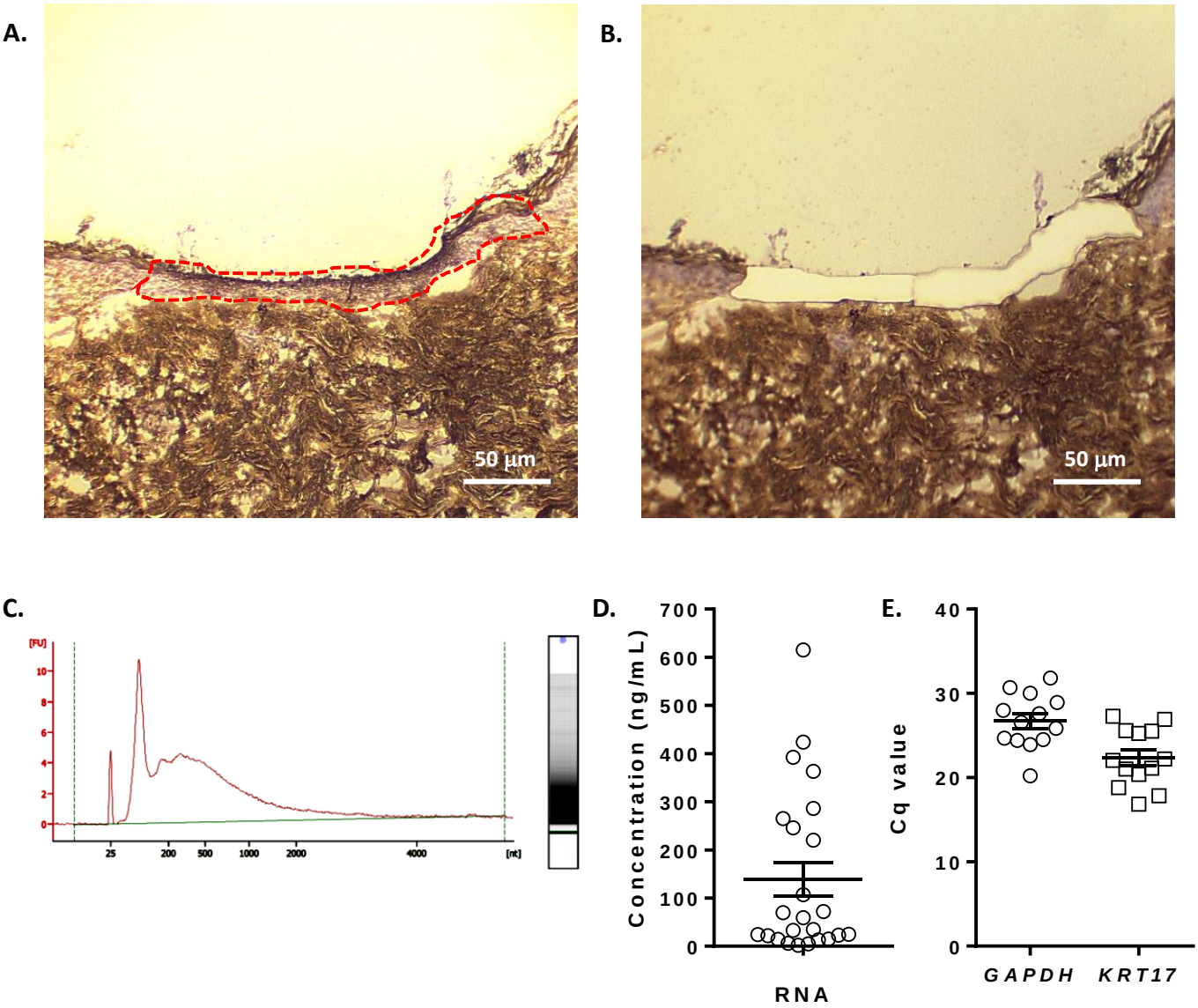

Table 1

First amplication round

First strand synthesis

Step	Temperature	Time
1	65 °C	5 min
2	4 °C	hold
3	42 °C	45 min
4	4 °C	hold
5	37 °C	20 min
6	95 °C	5 min
7	4 °C	hold

Second strand synthesis

Step	Temperature	Time
1	95 °C	2 min
2	4 °C	hold
3	25 °C	5 min
4	37 °C	10 min
5	70 °C	5 min
6	4 °C	hold

in vitro transcription

Step	Temperature	Time
1	42 °C	3 h
2	4 °C	hold
3	37 °C	15 min
4	4 °C	hold

Second amplication round

First strand synthesis

Step	Temperature	Time
1	65 °C	5 min
2	4 °C	hold
3	25 °C	10 min
4	37 °C	45 min
5	4 °C	hold

Second strand synthesis

Step	Temperature	Time
1	95 °C	2 min
2	4 °C	hold
3	37 °C	15
4	70 °C	5 min
5	4 °C	hold

in vitro transcription

Step	Temperature	Time
1	42 °C	6 h
2	4 °C	hold
3	37 °C	15 min
4	4 °C	hold

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Arcturus RiboAmp PLUS kit	ThermoFisher Scientific	KIT0521	RNA amplification kit
Diffuser Caps 0.5mL	MMI	K10028161	Laser capture microdissection caps; 50 pack
Dulbecco's Modified Eagle Medium (DMEM)	Sigma-Aldrich	D6046	With 1000 mg/L glucose, L-glutamine, and sodium bicarbonate, liquid, sterile-filtered, suitable for cell culture
Foetal Bovine Serum	Thermo Fisher Scientific	10270106	Cell culture supplement
H&E Staining Kit Plus	MMI	K10028305	Rnase-free haematoxylin and eosin staining kit
High capacity cDNA reverse transcription kit	Applied Biosystems	4368814	Reverse transcription kit
L-glutamine	Thermo Fisher Scientific	25030149	Cell culture supplement
MembraneSlides	MMI	K10028153	Laer capture microdissection slides; 5 per box
Netwell Mesh Insert	Corning	3479	Cell culture insert
Penicillin-Streptomycin-Fungizone	Thermo Fisher Scientific	15070-063 15290-026	Cell culture supplement
OCT	Tissue-Tek Sakura	4583	Cryostat-compatible cutting medium
PBS	Thermo Fisher Scientific	10209252	Five tablets per 100ml sterile water and then autoclaved for cell culture use
RNeasy Micro Kit	Qiagen	74004	RNA extraction kit
RNase Away	Sigma-Aldrich	83931	RNase spray
Sterile blades	Scientific Laboratory Supplies	INS4974	Tissue dissection implements
Support Slide	MMI	K10028159	Laser capture microdissection support slide, RNase-free
Surgical scissors	Scientific Laboratory Supplies	INS4860	Tissue dissection implements
Surgical forceps	Scientific Laboratory Supplies	INS2026	Tissue dissection implements
SYBR Green Supermix	Applied Biosystems	4344463	Quantitative PCR mastermix

JoVE62091 Revisions.

We thank the editor and reviewers for their constructive consideration of our manuscript. We have addressed each comment point-by-point below, and all amendments made to the manuscript are highlighted in track changes.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

We have proofread the manuscript and are confident there are no spelling or grammar issues. All abbreviations are defined at first use.

2. Please shorten your title to "Laser Capture Microdissection on Surgical Tissues to Identify Aberrant Gene Expression in Impaired Wound Healing in Type 2 Diabetes"

The title has been amended as requested.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Netwell insert; Nikon T2000; RNeasy Micro Kit (Qiagen); ArcturusTM RiboAmp1 PLUS kit (ThermoFisher); NanoDrop; IMPLEN NanoPhotometer; Agilent 2100 87 BioAnalyzer; Applied Biosystems

We have removed all reference to companies from the text, replaced them with generic terms, and amended the Table of Materials and Reagents to highlight which entries correspond to the generic descriptors in the text.

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have removed all personal pronouns.

5. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have added extra detail throughout the protocol, including in the data analysis section.

6. 4.3: What are the amplification conditions?

The conditions for RNA amplification and cDNA synthesis have now been added to the text.

7. 4.6: What are the qPCR conditions? Which primers did you use (for which genes)?

The qPCR conditions and primer sequences have now been added to the text.

8. Please do not abbreviate journal names in the reference list.

The reference list has been amended to remove any abbreviated names and replace them with the full journal titles.

9. Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend.

The scale bars were already present on the images. They have now been defined in the appropriate figure legends.

Reviewer #1:

Authors criticize other approach of modeling of wound healing process, but there was no description about probable benefits of their suggested method and their preferences compared to other methods. There were similar articles in the literature: (Methods Mol Biol 2018;1733:225-237. doi: 10.1007/978-1-4939-7601-0_19. Laser Capture Microdissection of Epithelium from a Wound Healing Model for MicroRNA Analysis Alyne Simões, Zujian Chen, Yan Zhao, Lin Chen, Virgilia Macias, Luisa A DiPietro, Xiaofeng Zhou; Methods Mol Biol 2015;1219:115-37. doi: 10.1007/978-1-4939-1661-0_10. Laser capture microdissection for gene expression analysis Mallikarjun Bidarimath, Andrew K Edwards, Chandrakant Tayade; Methods Mol Biol 2012;884:289-304. doi: 10.1007/978-1-61779-848-1_21. Use of laser capture microdissection for analysis of retinal mRNA/miRNA expression and DNA methylation. Laszlo Hackler Jr, Tomohiro Masuda, Verity F Oliver, Shannath L Merbs, Donald J Zack).

As the reviewer has highlighted, we appreciate that the technique of laser capture microdissection for gene analysis is not a new technique. However, the novel aspect to our method is the combination of this with clinically relevant surgical tissue, *ex vivo* wounding,

and the application of it to human disease states where *in vivo* wound healing assays (and indeed tissue sampling as a whole) are not possible due to ethical concerns regarding to the compromised wound healing processes evident in these individuals. Thus, this is an innovative application of existing techniques, which is within the scope of JoVE. We have added a sentence referring to this in paragraph 3 of the Discussion.

Protocol.
This protocol relies on the provision of human surgical tissue, so there was not any innovation regarding getting samples, and or an alternative method for other models of studying wound healing process. Moreover researchers could not rely of getting samples based on occasion during a unexpected time. Authors should focus on probable new items in this protocol and illustrate laser capture section.

This protocol has been designed to specifically analyse wound healing processes from *ex vivo* tissue, thus we did not find it appropriate to suggest alternative models within the technique. However, in the Introduction and Discussion we refer to multiple other wound healing models including *in vivo* animal studies, *in vitro* cell culture analyses, artificial skin and skin-on-a-chip models. We evaluate their strengths and weaknesses within the context of the strengths and weaknesses of our presented technique.

Provision of tissue can be problematic, particularly in the current Covid-19 climate when elective surgeries are not being performed. However, urgent operations involving amputations of limbs (e.g. chronic or gangrenous diabetic foot ulcers, crush injuries) are still continuing. Furthermore, one of the strengths of our protocol is that the method can be paused after the *ex vivo* wounding and before sectioning, with samples being stored at -80^{o}C until multiple patient tissues have been processed, so that laser capture microdissection and gene expression analysis can be carried out on multiple samples in parallel.

We have expanded the technical description of the laser capture section.

Ex vivo experiment was lower method compared with human biopsy and or animal model.

With respect, we do not agree that *ex vivo* analyses are inferior to *in vivo* human or animal studies. As we discuss in the Introduction and Discussion sections, each model has its own strengths and weaknesses. Undeniably, studying wound healing in human patients is the ideal scenario however when looking at patients with impaired wound healing, this is not an ethical approach. Furthermore, animal wounds heal via a different mechanism (contraction) to human wounds. Thus, our *ex vivo* model that uses clinically relevant surplus tissues from patients who have impaired wound healing (e.g. diabetes) as a biproduct of amputation maintains the strength of using full-thickness human skin from multiple patient donors without incurring any ethical constraints.

There were no information about nature of samples

The nature of the samples (surplus from elective cosmetic or amputation procedures) is alluded to at the start of the Methods section and in the Discussion. We have amended the text in the Results section to specify that the representative data was generated from elective cosmetic procedures.

There was no new idea in discussion. Conclusion should be report main and significant results

In keeping with the author guidelines from JoVE, we focussed our Discussion on the strengths and weaknesses of our technique in the context of alternative experimental models and scenarios. For example, the suggestions for alternative techniques if the aim of the experiments is to study the impact of the immune system on wound healing, and the suggestions that our protocol could be used to expand our understanding of wound healing processes in aged skin or in skin from patients with connective tissue disorders. The results are there as an illustration of the type of results that can be expected from this protocol, rather than as the focus of the article itself.

Reviewer #2:

Manuscript Summary:

The presented article has a good impact in the field of wound healing mechanism and it could be also useful in similar fields in which the role of skin needs to be studied and examined in depth. Title and abstract are appropriate for this method article which is clear, simple to follow an easily replicable by researchers in the field. Although the entire technique is composed by steps mainly existing in the literature, I think this new technique it is a resource for researchers learning. All materials and equipment needed are listed. Here below some comments that should be considered and implemented.

We thank the reviewer for their comments.

Major Concerns:

Authors explain the critical step of laser capture microdissection but I think another step should be considered as critical (not from a technical point of view but for the reliability of the results): although authors explain to process the tissue as soon as possible (lines 102-104), gene expression could be modified by medium and timing of storage. The addition of

a RNA stabilizer in the medium should be considered in the procedure. Moreover, if many samples are processed for an experiment, the storage period should be the same among different samples in order to correctly compare the data.

We agree with this suggestion and have amended the text in the Methods section accordingly.

A missing information is the use of "controls" in the protocol. Probably this information miss because of the difficulties of retrieve tissues from controls but I think it is fundamental to discuss this issue for a correct interpretation of the results. Of course, controls have to be contextualized in the field; probably, donor affected by diseases different from diabetes in which no impact on wound healing mechanism are known and in which surgery is necessary could be considered to retrieve biological materials on which perform the experiments.

As the reviewer states, the definition of 'control' will differ from study to study. We have added a sentence suggesting what might constitute a 'control' in the Discussion section.

Minor Concerns:

Regarding the quantification of the RNA, I suggest to add the QUBIT instrument for a more reliable evaluation of the not degraded RNA amount (lines 193-196).

As we have removed all reference to specific instruments and reagents throughout the text, we cannot add this instrument as a suggestion.

An additional useful information to include in the "representative results" section it is the modality by which the RNA degradation is considered by bioanalyzer instrument (RIN or DV200 values). Of course the degree of degradation and the quantity of material is strictly related to the requested input/performance of the kits used downstream.

We have added the information that we used the RIN modality to analyse RNA degradation in the representative results.

Another additional information that could be reported for the entire technique it is the requested time of processing which is fundamental to retrieve good quality RNA and final results.

We perceive that this will vary for each individual lab according to the particulars of the tissue they are using or their research question. Thus, we have included a Note in the protocol to suggest that processing of the tissue wound should be conducted as quickly as possible.