## Human Ex Vivo Wound Model and Whole-Mount Staining Approach to Accurately Evaluate Skin Repair.
### --Manuscript Draft--

| | |
|---|---|
| Article Type: | Invited Results Article - JoVE Produced Video |
| Manuscript Number: | JoVE62326R1 |
| Full Title: | Human Ex Vivo Wound Model and Whole-Mount Staining Approach to Accurately Evaluate Skin Repair. |
| Corresponding Author: | Holly Wilkinson<br>Hull York Medical School<br>Hull, East Riding of Yorkshire and the Humber UNITED KINGDOM |
| Corresponding Author's Institution: | Hull York Medical School |
| Corresponding Author E-Mail: | h.n.wilkinson@hull.ac.uk |
| Order of Authors: | Holly Wilkinson |
| | Alexandria Kidd |
| | Elizabeth Roberts |
| | Matthew Hardman |
| Additional Information: | |
| Question | Response |
| Please specify the section of the submitted manuscript. | Developmental Biology |
| Please indicate whether this article will be Standard Access or Open Access. | Open Access (US$4,200) |
| Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations. | Hull, United Kingdom |
| Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below: | I agree to the UK Author License Agreement (for UK authors only) |
| Please provide any comments to the journal here. | |

**TITLE:**

Human Ex Vivo Wound Model and Whole-Mount Staining Approach to Accurately Evaluate Skin Repair

**AUTHORS AND AFFILIATIONS:**

Holly Nicola Wilkinson[1], Alexandria Sarah Kidd[1], Elizabeth Rose Roberts[1] and Matthew James Hardman[1]

[1]Centre for Atherothrombosis and Metabolic Disease, Hull York Medical School, University of Hull, HU6 7RX.

**CORRESPONDING AUTHOR:**

Holly N Wilkinson          (h.n.wilkinson@hull.ac.uk)

**EMAIL ADDRESSES:**

Holly Nicola Wilkinson          (h.n.wilkinson@hull.ac.uk)

Alexandria Sarah Kidd          (Alex.Kidd@hull.ac.uk)

Elizabeth Rose Roberts          (e.roberts@hull.ac.uk)

Matthew James Hardman          (m.hardman@hull.ac.uk)

**KEYWORDS:**

wound healing, skin, human, ex vivo, whole-mount, staining, diabetes, aging, chronic wound

**SUMMARY:**

Here we demonstrate an optimized technique for assessing wound repair using ex vivo human skin combined with a whole-mount staining approach. This methodology provides a pre-clinical platform for the evaluation of potential wound therapies.

**ABSTRACT:**

Chronic non-healing wounds, which primarily affect the elderly and diabetic, are a significant area of clinical unmet need. Unfortunately, current chronic wound treatments are inadequate, while available pre-clinical models poorly predict the clinical efficacy of new therapies. Here we describe a high throughput, pre-clinical model to assess multiple aspects of the human skin repair response. Partial thickness wounds were created in human ex vivo skin and cultured across a healing time course. Skin wound biopsies were collected in fixative for the whole-mount staining procedure. Fixed samples were blocked and incubated in primary antibody, with detection achieved via fluorescently conjugated secondary antibody. Wounds were counterstained and imaged via confocal microscopy before calculating percentage wound closure (re-epithelialization) in each biopsy. Applying this protocol, we reveal that 2 mm excisional wounds created in healthy donor skin are fully re-epithelialized by day 4-5 post-wounding. On the contrary, closure rates of diabetic skin wounds are significantly reduced, accompanied by perturbed barrier reformation. Combining human skin wounding with a novel whole-mount staining approach allows a rapid and reproducible method to quantify ex vivo wound repair. Collectively, this protocol provides a valuable human platform to evaluate the effectiveness of

45    potential wound therapies, transforming pre-clinical testing and validation.
46
47    **INTRODUCTION:**
48    Chronic, non-healing wounds, which are highly prevalent in the elderly and diabetic, are majorly
49    unappreciated area of clinical unmet need. These wounds present a major physical and
50    psychological burden to patients and cost healthcare providers billions each year to treat[1].
51    Despite improved understanding of wound biology and advancements in technology, up to 40%
52    of chronic wounds still fail to heal following best standard care[2]. Thus, 14-26% of patients with
53    diabetic foot ulcers subsequently require amputation[3], while 5-year post-amputation mortality
54    rate stands at approximately 70%[4]. As a result, there is an urgent requirement to develop
55    efficacious new therapies to improve patient quality of life while reducing the substantial
56    healthcare burden imposed by poor healing wounds. Poorly predictive pre-clinical models remain
57    a significant hurdle to the development of effective new therapies.
58
59    Wound repair is a dynamic and multifaceted process involving a diverse range of cell types,
60    countless levels of communication and a tissue environment that is temporally remodeled. Skin
61    healing is underpinned by four major reparative stages: hemostasis, inflammation, proliferation,
62    and matrix remodeling. These stages ultimately act to prevent blood loss and infection, close the
63    wound surface (a process termed re-epithelialization) and return the skin to an uninjured state[5].
64    Chronic wounds are associated with diverse etiology and widespread perturbation to healing
65    processes[6], further complicating the identification of therapeutic targets. Nevertheless, a broad
66    range of models have been developed to both elucidate the molecular and cellular drivers of
67    wound pathology and test new therapeutic approaches[7].
68
69    The most used wound repair model is acute wounding in the mouse. Mice are highly tractable
70    for mechanistic studies and provide validated models of ageing and diabetes[8]. Despite the
71    general similarities shown amongst mouse and human healing, between-species differences in
72    skin structure and healing dynamics remain. This means most murine wound research does not
73    easily translate to the clinic[9]. Consequently, there has been a push towards human in vitro and
74    ex vivo systems with high applicability and translatability[10,11].
75
76    Here we provide an in-depth protocol for performing partial thickness excisional wounds in ex
77    vivo human skin. We, also, outline our whole-mount staining approach as a highly reproducible
78    method of evaluating ex vivo human skin healing. We show the trajectory of epidermal repair
79    (re-epithelialization) and subsequent barrier formation, evaluating the rate of wound closure in
80    healthy versus diabetic human skin. Finally, we demonstrate how whole-mount staining can be
81    adapted for use with a range of antibodies to assess various aspects of the healing response.
82
83    **PROTOCOL:**
84    Human skin was obtained from patients undergoing reconstructive surgery at Castle Hill Hospital
85    and Hull Royal Infirmary (Hull, UK) under full informed, written patient consent, institutional
86    guidelines, and ethical approval (LRECs: 17/SC/0220 and 19/NE/0150). Non-diabetic skin was
87    collected from patients undergoing routine surgery (mean age = 68). Diabetic skin was selected
88    from donors who had established type II diabetes and a history of ulceration (mean age = 81).

89  Samples from surgery were transported in holding media and processed immediately upon
90  arrival at the laboratory. All experimental steps using unfixed human tissue were performed at
91  Biosafety Level-2 (BSL-2) in a class II laminar flow biosafety cabinet.
92
93  **1.      Preparation of skin culture media and staining reagents**
94
95  NOTE: All reagent and consumable details are provided in the **Table of Materials**. Ensure all
96  reagents and equipment used for the processing and culture of human tissue are sterile. Sterilize
97  instruments prior to the use and decontaminate with disinfectant following contact with the
98  tissue. Decontaminate waste products in 1% disinfectant before disposal.
99
100 1.1.     Holding media: Supplement high glucose Dulbecco's Modified Eagle Medium (DMEM)
101 with 2 mM L-glutamine and 4% (v/v) antibiotic-antimycotic solution.
102
103 1.2.     Hank's balanced salt solution (HBSS) with antibiotics: Add 4% (v/v) antibiotic-antimycotic
104 solution to HBSS. Store at 4 °C until use.
105
106 1.3.     Dulbecco's phosphate buffered saline (DPBS): Prepare DPBS by dissolving 9.6 g of DPBS
107 powder per liter of distilled water (dH$_2$O). Autoclave to sterilize and store at 4 °C until use.
108
109 1.4.     Human skin growth media: Supplement high glucose DMEM with 2 mM L-glutamine, 1%
110 (v/v) antibiotic-antimycotic solution and 10% (v/v) fetal bovine serum. Store at 4 °C until use.
111
112 1.5.     Skin fixative: To 450 mL of dH$_2$O, add 40 mL of formaldehyde solution, 10 mL of glacial
113 acetic acid, 4.5 g of sodium chloride and 0.25 g of alkyltrimethylammonium bromide. Store at
114 room temperature (RT) and use within a few days.
115
116 CAUTION: Fixative is hazardous (irritant and flammable). Handle with care and dispose of via an
117 appropriate route.
118
119 1.6.     Phosphate buffered saline (PBS): Prepare PBS for whole mount staining by adding 6 g of
120 sodium chloride to 100 mL of phosphate buffer solution and 900 mL of dH$_2$O.
121
122 1.7.     Staining wash buffer: Dissolve 0.5% (v/v) Triton X-100 in PBS.
123
124 1.8.     Blocking buffer: Add 0.2% (w/v) sodium azide and 2% (v/v) animal serum to staining wash
125 buffer. Store at 4 °C for up to two weeks.
126
127 NOTE: Block in the serum of the secondary antibody host species. Sodium azide will prevent
128 bacterial growth during incubation.
129
130 1.9.     DAPI working solution: Prepare a 5 mg/mL stock of 4',6-diamidino-2-phenylindole (DAPI)
131 in dimethyl sulfoxide. Dilute the stock 1:1,000 in staining wash buffer to give a 5 µg/mL DAPI
132 working solution.

133

134    1.10.    Peroxidase block: Add 0.3% (v/v) hydrogen peroxide to staining wash buffer. Store at 4 °C
135    until use. Keep in the dark to prevent decomposition.

136

137    1.11.    ABC-HRP Kit:

138

139    1.11.1. HRP-conjugated secondary antibody: 1 drop of biotinylated rabbit anti-goat IgG in 5 mL
140    of staining buffer. Store at 4 °C for up to two weeks.

141

142    NOTE: The kit/secondary used will depend on the host species of the primary antibody.

143

144    1.11.2. Avidin-biotin complex (ABC) reagent: 2 drops of reagent A and 2 drops of reagent B in 5
145    mL of staining wash buffer. ABC reagent should be prepared at least 30 min prior to use. Store at
146    4 °C for up to two weeks.

147

148    1.12.    Peroxidase substrate: 3 drops of reagent 1, 2 drops of reagent 2, 2 drops of reagent 3 and
149    2 drops of hydrogen peroxide in 5 mL of $dH_2O$. Peroxidase substrate should be freshly prepared
150    immediately before use and cannot be stored.

151

152    **2.    Preparation of skin for wounding**

153

154    NOTE: These steps should be performed in a class II laminar flow biosafety cabinet.

155

156    2.1.    Collect the skin in holding media and transport to the BSL-2 cabinet.

157

158    2.2.    Place the skin dermis side down within a 90 mm sterile Petri dish and remove adipose
159    tissue with sterile scissors.

160

161    2.3.    Place the skin in a 50 mL tube containing 25 mL of HBSS with antibiotics for 10 min at RT.
162    Shake intermittently to remove any residual blood and adipose tissue.

163

164    2.4.    Repeat step 2.3 using a new 50 mL tube.

165

166    2.5.    Place the skin in a fresh 50 mL tube containing 25 mL of HBSS, this time without antibiotics
167    for 10 min at RT. Shake as in step 2.3.

168

169    2.6.    Perform a final skin rinse by placing skin in a new tube with 25 mL of DPBS. The skin is
170    now ready to wound.

171

172    **3.    Creating ex vivo human skin wounds**

173

174    NOTE: These steps should be performed in a class II laminar flow biosafety cabinet.

175

176    3.1.    Prepare the skin culture dishes prior to wounding. In a 60 mm Petri dish, stack two sterile

177 absorbent pads and add 4 mL of human skin media via the side of the dish. Place a sterile nylon
178 filter membrane onto the absorbent pad stack.

179
180 NOTE: Skin media can be altered depending on the required treatment conditions. Up to three
181 wound explants may be cultured on each stack.

182
183 3.2.    Dry the dermal side of the skin on sterile gauze in a 90 mm Petri dish to remove residual
184 DPBS.

185
186 NOTE: This prevents the skin from sliding around when wounding.

187
188 3.3.    Place the skin dermis side down on a clean 90 mm Petri dish lid and dab the epidermis
189 dry with fresh sterile gauze.

190
191 NOTE: It is easier to wound the skin in a Petri dish lid than the base. Subsequent work should be
192 carried out quickly to prevent the skin drying out.

193
194 3.4.    Holding the skin taut, press a 2 mm biopsy punch against the skin and twist gently. Do not
195 punch entirely through the skin.

196
197 NOTE: Partial thickness wounds are designed to punch through the epidermis and partially into
198 the dermis. There may be donor-to-donor and site-to-site variability in the force required to
199 create the partial thickness wound.

200
201 3.5.    Use curved toothed tissue forceps to pick up each side of the 2 mm wound and hook
202 curved iris scissors under the 2 mm wound to cut it out uniformly.

203
204 3.6.    Biopsy around the central 2 mm wound using a 6 mm biopsy punch to create a 6 mm
205 explant with a partial thickness 2 mm wound in the center.

206
207 NOTE: A 6 mm biopsy punch may be used to score the skin to mark out where each 2 mm wound
208 should be. Take care not to pierce through the tissue entirely. Create wound explants in a
209 honeycomb pattern to reduce wastage.

210
211 3.7.    Place wound explants epidermis side up on the nylon filter membrane stack (prepared in
212 step 3.1).

213
214 NOTE: When handling wound explants, be careful not to damage the central wound. Use small
215 forceps and pick up each explant at opposite sides.

216
217 3.8.    Incubate wounds at 32-37 °C and 5% $CO_2$ in a humidified atmosphere (90-95%) for 1-7
218 days. Replace the media every 2-3 days.

219
220 **4.    Whole mount staining of ex vivo wounds**

221

NOTE: This section describes immunofluorescence and immunoperoxidase staining methods. Mix all reagents well before use.

4.1.    Fluorescent staining method

4.1.1.  Collect wound explants in 1.5 mL microcentrifuge tubes containing 500 µL of skin fixative and incubate at 4 °C overnight.

NOTE: The fixative used in this protocol works well for the described antibodies. Optimization will be required for other antibodies. Tissue fixation longer than 24 h may lead to over-fixation.

4.1.2.  The following day remove the fixative and replace with 1 mL of staining wash buffer. Biopsies can be stored in staining wash buffer at 4 °C up to 2 weeks prior to staining.

NOTE: For all wash buffer steps, use a serological pipette or pipette tip, taking care not to damage the wound.

4.1.3.  Aspirate the staining wash buffer and perform one more rinse with 1 mL of staining wash buffer.

4.1.4.  Calculate the amount of blocking buffer required for steps 4.1.5-4.1.6 (number of samples x 300 µL = amount of blocking buffer in µL). Make extra buffer if required.

4.1.5.  Add 150 µL of blocking buffer to each sample and incubate for 1 h at RT. For all staining steps, ensure each sample is sufficiently covered and that there are no bubbles covering the biopsy wound surface.

NOTE: This step onwards can be performed in 1.5 mL microcentrifuge tubes or in a 48 well plate. If using a 48 well plate, incubate the wounds face down in each well.

4.1.6.  Dilute the primary antibody in the remaining blocking buffer.

NOTE: Anti-mouse keratin 14 (K14) diluted 1:1,000 in blocking buffer works well. Optimize this step for use with other antibodies or multiple probes.

4.1.7.  Aspirate the blocking buffer and add 150 µL primary antibody per well/microcentrifuge tube. Incubate wound explants in primary antibody at 4 °C overnight.

4.1.8.  The next day, aspirate the primary antibody and rinse in staining wash buffer containing 0.2% sodium azide for 1 h at RT (500 µL per sample).

4.1.9.  Perform three more rinsing steps using staining wash buffer (30 min per wash, 500 µL per sample).

265

266 4.1.10. Dilute the fluorescently conjugated secondary antibody in staining wash buffer (e.g., goat
267 anti-mouse 488 at 1:400 dilution).

268

269 4.1.11. Calculate the required amount of secondary antibody (number of samples x 150 µL =
270 amount in µL).

271

272 4.1.12. Add 150 µL of secondary antibody to each well/microcentrifuge tube. Incubate for 1 h at
273 RT. Perform incubation steps 4.1.10 – 4.1.16 in the dark as the secondary antibody is light
274 sensitive.

275

276 NOTE: This step can be performed at 4 °C overnight if required. Optimize the concentration of
277 secondary antibody required for adequate signal and limited background staining.

278

279 4.1.13. Remove the secondary antibody and perform 3 x 30 min rinses with staining wash buffer
280 (500 µL per sample).

281

282 4.1.14. Discard the leftover wash buffer and calculate the amount of DAPI working solution
283 required (as per step 4.1.11).

284

285 4.1.15. Counterstain each explant with 150 µL of DAPI working solution for 10 min at RT.

286

287 NOTE: DAPI will stain cell nuclei blue. Hoechst dye can be used as an alternative to DAPI.

288

289 4.1.16. Perform two final 30 min washes with staining wash buffer (500 µL per sample). Biopsies
290 can be stored in staining wash buffer at 4 °C in the dark up to two weeks prior to imaging.

291

292 4.2.     Brightfield staining method.

293

294 4.2.1.  Perform steps 4.1.1 – 4.1.3.

295

296 4.2.2.  Quench endogenous peroxidase activity with peroxidase block at 4 °C overnight.

297

298 NOTE: This step is important when using an HRP-conjugated antibody to reduce non-specific
299 background staining from the tissue. Highly vascularized tissue will contain more endogenous
300 peroxidase activity.

301

302 4.2.3.  Discard the peroxidase block and rinse twice for 30 min in staining wash buffer.

303

304 4.2.4.  Perform steps 4.1.4 – 4.1.8.

305

306 NOTE: Washes after step 4.1.7 are particularly important to remove sodium azide from the
307 samples. If sodium azide is not adequately removed, it will inactivate the HRP and interfere with
308 staining detection.

309

310  4.2.5.  Add 150 µL HRP-conjugated secondary antibody to each well/microcentrifuge tube and
311  incubate overnight at 4 °C or 1 h at RT.

312

313  4.2.6.  Remove the secondary antibody and perform 3 x 30 min washes in staining wash buffer.

314

315  4.2.7.  Add 150 µL ABC reagent to each well/microcentrifuge tube and incubate overnight at 4
316  °C or 1 hour at RT.

317

318  4.2.8.  Aspirate the ABC reagent and perform 3 x 30 min washes in staining wash buffer.

319

320  4.2.9.  Add 150 µL peroxidase substrate to one explant and determine the time required to
321  detect a noticeable color change.

322

323  NOTE: Choose a sample where strong staining is expected. In this case, a red ring to show the
324  migrating epidermis (K14). 3,3'-diaminobenzidine-4, or any other appropriate chromogenic
325  substrate, may be used as a replacement for this peroxidase substrate.

326

327  4.2.10. Once a color change is observed, remove the peroxidase substrate, and replace with 1 mL
328  of dH$_2$O.

329

330  4.2.11. Repeat the peroxidase substrate detection for the other explants, incubating for the time
331  determined in step 4.2.11.

332

333  4.2.12. Rinse all explants with 1 mL of dH$_2$O to remove residual peroxidase substrate. Although
334  explants may be stored up to one week at 4 °C prior to imaging, it is better to image them as soon
335  as possible to prevent leaching of the peroxidase substrate into the dH$_2$O over time.

336

337  **5.      Imaging and quantification**

338

339  5.1.    Fluorescent imaging

340

341  NOTE: Fluorescent imaging is performed using a confocal laser-scanning microscope. However,
342  an inverted fluorescent microscope may be sufficient for acquiring 2D images to quantify wound
343  closure rates. When selecting secondary antibodies, ensure that the chosen fluorochromes are
344  compatible with the excitation and emission spectra of the microscopy equipment available.

345

346  5.1.1.  Use a confocal laser-scanning microscope equipped with a 2.5x, 10x and 20x objective, x-
347  y-z motorized stage, digital camera, and acquisition software. Switch on the transmitted light
348  detector (TPMT) to allow easy visualization of each biopsy and to enable measurement of total
349  wound closure. Alternatively, measure each wound via brightfield microscopy following
350  fluorescence imaging.

351

352  5.1.2.  Place a 60 mm Petri dish base on the imaging platform and add a thin layer (around 1 mL)

353  of DPBS.

354

355  NOTE: If too much DPBS is used, the biopsy will move around during imaging. Alternatively use a
356  48 well plate if a plate holder is available.

357

358  5.1.3.   Use small tissue forceps to transfer wound explants from wells/microcentrifuge tubes to
359  the Petri dish containing DPBS. Place the wound side down in the Petri dish.

360

361  5.1.4.   Use the eyepiece and fluorescent lamp to locate and focus on the wound. If bubbles are
362  trapped under the sample in the field of view, pick up the wound with tissue forceps and
363  reposition.

364

365  5.1.5.   Set up the imaging software, ensuring equal pinhole size between channels for optimum
366  confocality. For this, check the value of one airy unit for each channel and select the largest value.
367  Select scan speed, image quality and averaging.

368

369  NOTE: The fluorochromes of the conjugated secondary antibodies and the chosen counterstain
370  (e.g., DAPI) will dictate the channels required.

371

372  5.1.6.   Switch on the live acquisition software and adjust the laser power and gain of each
373  channel to the levels required to visualize staining. Reduce background noise by increasing the
374  digital offset.

375

376  5.1.7.   Position the wound in the center of the imaging plane.

377

378  NOTE: If the wound does not fill the entire image due to using a smaller objective or creating a
379  larger wound, take a panel of images and stitch them together (manually or with a tiling function
380  in the relevant imaging software).

381

382  5.1.8.   Acquire images of the wound biopsies. Use the same imaging settings between explants.

383

384  NOTE: Higher power images will allow assessment of tissue structures and cellular marker
385  expression and location.

386

387  5.1.9.   Collect serial Z stacks through the wound, especially where the tissue is not completely
388  flat against the Petri dish. Use analysis software to collapse the Z stack into a single maximum
389  intensity projection image.

390

391  5.2.     Brightfield imaging

392

393  NOTE: Brightfield imaging of immunoperoxidase stained biopsies can be performed in multiple
394  ways.

395

396  5.2.1.   Inverted microscope imaging: Prepare wound explants for imaging by placing them in a

397 Petri dish/well as described in steps 5.1.2-5.1.3. Acquire digital images under brightfield
398 illumination on an inverted microscope equipped with a digital camera. Stitch together multiple
399 images if required.
400
401 5.2.2. Wireless digital microscope imaging: Use a wireless digital microscope connected to a
402 phone or laptop to obtain high quality images in a cost-effective manner. Place explants wound
403 side up onto some tissue and remove any residual dH$_2$O (or staining wash buffer) from sample
404 storage. Position the wound explant in the center of the microscope field of view. Acquire images
405 using the connected camera.
406
407 5.3.    Quantification
408
409 NOTE: Percentage wound closure can be quantified in any software that allows freehand shapes
410 to be drawn and measured. ImageJ can be used to perform quantification as follows:
411
412 5.3.1.  Open the image to be quantified in ImageJ software.
413
414 5.3.2.  Use the freehand shape tool to draw around the outside of the re-epithelialized wound
415 where it meets the normal skin. Press **M** (or **Analyze | Measure**) to acquire an 'outer' area
416 measurement.
417
418 NOTE: The re-epithelialized wound tissue texture differs from normal skin. The images do not
419 need to be scaled prior to this type of analysis.
420
421 5.3.3.  Use the freehand shape tool to draw around the open wound area. This is where the open
422 wound meets the inside edge of the re-epithelializing tissue. Press **M** (or **Analyze | Measure**) to
423 acquire an 'inner' area measurement.
424
425 5.3.4.  Use the following equation to deduce percentage wound re-epithelialization/closure:
426
427
428   $\% \, Closure \, = \, (Outer \, wound \, area - Inner \, wound \, Area)/(Outer \, wound \, Area \,) \; x \, 100$
429
430 NOTE: Percentage area coverage of antibodies can be deduced in the same way (e.g., K14) or as
431 a percentage of the total wound area. Percentage intensity can also provide semi-quantitative
432 information about tissue level expression of markers of interest, while high power imaging
433 presents expression data at the cellular level.
434
435 **RESULTS:**
436 In this report, we present a novel ex vivo skin wounding and whole-mount staining approach to
437 assess factors that influence the human skin repair response. **Figure 1A** shows a schematic of the
438 procedural pipeline, which can be performed in 3-10 days, depending on wound incubation
439 times. The partial thickness wounds are cultured on membrane stacks at the air : membrane
440 interface and can be collected for whole-mount staining, embedded in paraffin or OCT medium

441 for general histology, or frozen in liquid nitrogen for biochemical analysis (**Figure 1B**). We
442 generally create 2 mm partial thickness wounds within the center of 6 mm explants. However,
443 the size of the wound and surrounding explant may be altered depending on requirements. The
444 whole-mount procedure has been successfully adapted for both immunoperoxidase and
445 immunofluorescence staining methods (**Figure 1C**).
446
447 Immunofluorescence allows for the probing of tissue with multiple antibodies. For this, we advise
448 using primary antibodies raised in different species, and species-matched fluorescently
449 conjugated secondary antibodies to limit cross-species reactivity. Antibody concentrations and
450 incubation times will need to be optimized. If background staining is observed, reduce antibody
451 concentrations, increase wash steps, and add blocking buffer to the secondary antibody. Fresh
452 tissue viability can be directly assessed with commercial viability dyes (see **Table of Materials**).
453 We also show that tissue may be fixed post viability staining and successfully imaged when it is
454 practically suitable (**Figure 1D**).
455
456 [Place **Figure 1** here]
457
458 The most widely applicable use for whole mount staining of wounds is to determine wound
459 closure rate in a more reproducible manner than can be provided via histological sectioning.
460 Percentage closure was quantified as percentage re-epithelialization of the wound surface, as
461 demonstrated in **Figure 2A**. Percentage area coverage of specific markers can be measured from
462 the total wound area or as a percentage of the re-epithelialized wound. We characterized healing
463 in healthy (non-diabetic) versus diabetic skin across a time course of seven days, collecting
464 wounds at each day post-wounding (representative images, **Figure 2B**). Healthy skin wounds
465 closed over time as expected, with full closure observed in most samples by day 4-5. On the
466 contrary, diabetic skin wounds failed to close fully within the seven-day analysis period (**Figure
467 2C**). A significant delay in wound closure was observed between healthy and diabetic skin wounds
468 when comparing healing rates at each time-point post-injury ($P < 0.001$ to day 6, $P < 0.05$ at day
469 6 and $P < 0.05$ to $P < 0.001$ at day 7).
470
471 Following assessment of overall wound closure rates; we measured the percentage of the entire
472 wound area (outer area in **Figure 2A**) where K14 positive cells could be visualized (green staining
473 in **Figure 2B**). Interestingly, we observed that in healthy ex vivo skin wounds, K14 staining peaked
474 at day 2 and then rapidly declined (significance at each time-point versus the day 2 peak, **Figure
475 2D**). This is likely reflecting re-formation of the early epidermal barrier, excluding K14 antibody
476 penetration through differentiated epidermal layers (see **Figure 2E** schematic). During the re-
477 epithelialization process, basal layer (K14+ve) keratinocytes migrate inwards over the open
478 wound such that the epidermis closer to the outer wound edge forms earlier than the epidermis
479 closer to the inner wound edge (migrating front). While the front edge of the newly formed
480 epidermis continues to migrate to close the remaining open wound, the outer edge epidermis
481 begins to differentiate to reform the other epidermal layers. In early healing, we would thus
482 expect to see most of the re-epithelialized area consists of basal (K14+ve) cells, while in later
483 repair K14 staining is lost as the epidermis differentiates from the outside inwards (see whole-
484 mount images in **Figure 2E**). Therefore, the decline in K14 staining shown in **Figure 2D** (downward

485  arrows) correlates with increased epidermal differentiation. Interestingly, visible K14 staining
486  peaked earlier in healthy (day 2) versus diabetic (day 4) wounds, further demonstrating that re-
487  epithelialization and subsequent epidermal differentiation are delayed in diabetic skin wounds.
488
489  [Place **Figure 2** here]
490
491  We next used whole-mount staining to explore tissue expression and localization of other
492  wound-relevant markers in non-diabetic skin (**Figure 3**). All antibodies used and their working
493  concentrations are provided in the **Table of Materials**. Blood vessels in the open wound stained
494  positively with alpha smooth muscle actin (a-SMA) antibody, used in combination with K14 to
495  delineate the epidermal edges in lower power images (**Figure 3A**). The dermal matrix was stained
496  with antibodies against collagen type I (COL 1) and fibronectin (Fn). Here collagen was observed
497  as abundant thick fibers while fibronectin fibers were sparse, wavy, and thin (**Figure 3A**). Our
498  whole-mount staining approach is also able to provide cell level resolution of staining, as
499  demonstrated for K14-positive keratinocytes (**Figure 3B**).
500
501  Finally, we show that human ex vivo wounds possess resident immune cells, with Langerhans
502  cells detected around newly formed epidermis at day 3 post-wounding (**Figure 3C**). Indeed, these
503  results suggest that whole-mount staining may be used to investigate key features of the healing
504  response including inflammation, proliferation, and the extracellular matrix (**Figure 4A**). Taken
505  together, our data reveal that the combined ex vivo skin wounding and whole-mount staining
506  procedure is a valid method to assess various aspects of healthy and diabetic (pathological)
507  human skin repair.
508
509  [Place **Figure 3** and **Figure 4** here]
510
511  **FIGURE AND TABLE LEGENDS:**
512  **Figure 1: The human ex vivo wounding and whole-mount staining approach**. (**A**) Pipeline
513  depicting the procedural workflow from collecting skin and performing *ex vivo* wounding, to
514  staining tissue and analyzing data. (**B**) Diagram demonstrating the human *ex vivo* skin wound
515  culture system with analyses routinely performed on the tissue. (**C**) Whole-mount staining can
516  be employed using both immunoperoxidase and immunofluorescence techniques. K14 = keratin
517  14. (**D**) Live tissue may be stained with commercial viability dyes and imaged successfully post
518  fixation. Bar = 100 µm. This staining was performed in non-diabetic skin.
519
520  **Figure 2: Whole-mount staining reveals perturbed healing rates in diabetic versus healthy skin.**
521  (**A**) The method used to quantify wound closure from outer and inner wound measurements.
522  Brightfield images show keratin 14 (K14) staining in red. Bar = 300 µm. (**B**) Representative images
523  of healing over time (day post-wounding) in healthy and diabetic skin. Bar = 500 µm. K14 = green.
524  DAPI = blue nuclei. (**C**) Quantification of wound closure rates (percentage re-epithelialization)
525  showing that ex vivo wounds from healthy skin close significantly faster than ex vivo wounds
526  from diabetic skin. H = healthy. Db = diabetic. (**D**) Percentage K14 staining peaks earlier in healthy
527  versus diabetic skin and then declines in line with increased epidermal differentiation (down
528  arrows). (**E**) K14 (basal epidermal cell) staining is lost as the epidermis differentiates. D =

529   differentiated. ND = not differentiated. White dotted lines depict inner and outer wound edges.
530   White arrows = direction of migration. n = 6 wounds per donor, per time point. Mean +/- SEM. *
531   = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$. Healthy and diabetic compared at each healing time
532   point in **C** (*P* value for least significant comparison). Temporal change in K14 staining compared
533   to peak for each donor in **D**.

534

535   **Figure 3: Optimization of the whole-mount staining approach for use with other antibodies.** (**A**)
536   Blood vessels were stained with alpha smooth muscle actin (α-SMA, green) and keratin 14 (K14,
537   red), while matrix fibers were stained with collagen I (COL 1, red) and fibronectin (Fn, green). (**B**)
538   The whole-mount procedure provides up to cell level resolution of localization (K14, green; K1,
539   red). (**C**) CD1a+ve Langerhans cells (green) observed in newly formed epidermis. DAPI = blue
540   nuclei. Bar = 100 μm. White dotted lines show inner and outer wound edges and separate wound
541   from epidermis. This staining was performed in non-diabetic skin.

542

543   **Figure 4: Validity of the whole-mount staining procedure for assessing wound healing.** (**A**)
544   Illustration depicting how the whole-mount staining technique can evaluate wound-relevant
545   processes. Antibodies used = red text. K14 = keratin 14. COL 1 = collagen 1. Fn = fibronectin. (**B**)
546   The whole-mount staining procedure (blue arrows) introduces less variability to wound closure
547   measurements than standard histological analysis (red arrows). S1 = section 1. WE = wound edge.
548   Bar = 300 μm. This staining was performed in non-diabetic skin.

549

550   **DISCUSSION:**
551   In this experimental protocol, we describe an optimized method for evaluating wound closure in
552   human ex vivo skin using whole-mount tissue staining. This is an important resource to allow
553   critical evaluation of potential wound treatments, and to provide better understanding of the
554   human wound repair response. We have published healing assessment in ex vivo skin wounds
555   previously[12,13], yet in these reports the whole-mount staining approach was not used to measure
556   wound closure. Whole-mount staining is far easier and requires less technical experience than
557   standard histology, which involves paraffin or OCT embedding and sectioning of samples. The
558   whole-mount procedure also reduces experimental variability, allowing quantification of the
559   entire wound and not just a single transverse section at a defined position within the tissue (see
560   **Figure 4B** for comparative illustration). We fully support the importance of quantifying healing
561   of the entire non-symmetrical wound structure, as clearly outlined by Rhea and Dunnwald for
562   murine acute wounds[14]. These authors showed the importance of serially sectioning in vivo
563   excisional wounds for reproducible and precise measurements of wound morphology. Serial
564   sectioning could equally be applied to human ex vivo wounds; however, for accurate
565   quantification of wound closure and re-epithelialization, high throughput whole-mount staining
566   should be the preferred method. We note that this whole-mount staining protocol should also
567   be compatible with subsequent processing (wax or OCT) for traditional histological analysis.

568

569   Whole-mount staining is not without disadvantages. While it affords higher reproducibility in
570   wound healing experiments, it does require the use of more tissue for analysis than standard
571   histological techniques. This may be an issue where tissue access is limited, particularly where
572   multiple antibodies need to be assessed. An alternative approach would be to employ an

573  incisional wounding method where wound width is relatively uniform and variability is reduced
574  (as shown in mouse and human wounds[15,16]). However, excisional wounds remain more
575  applicable to most pathological wound types[17].

577  In this study, 2 mm partial thickness wounds were created within the center of 6 mm skin
578  explants. This method may be optimized for alternative excisional wound and explant sizes at
579  different skin depths[18]. In addition, the force required to generate wounds will vary between
580  donors, where aged skin will require less force to biopsy. We would also avoid using skin
581  displaying prominent stretch marks or other structural alterations. We have validated a range of
582  antibodies to consider different aspects of the ex vivo healing response. This protocol may also
583  be used with other skin-relevant antibodies, where antibody concentrations and incubation
584  times will need to be optimized. Nevertheless, we believe our protocol is most suited to absolute
585  quantification of total wound closure, followed by spatial assessment of specific proteins of
586  interest. While whole mount provides reduced resolution of immunolocalization versus standard
587  histological analysis of tissue sections, it provides additional 3D information that is missing from
588  standard 2D histology.

590  One caveat of assessing healing in ex vivo skin versus in vivo models is that it lacks a systemic
591  response. An important aspect of wound repair is inflammation and subsequent tissue
592  granulation, which is caused by an influx of inflammatory cells and endothelial cells from the
593  vasculature[19]. Despite this limitation, ex vivo skin still provides a better recapitulation of clinical
594  healing than cell-based wound assays. In vitro experiments in general involve single cell type
595  monolayers or co-cultures grown on tissue culture plastic, whereas ex vivo skin provides a native
596  environment to explore cell behavior. More recently, a number of skin equivalent systems have
597  emerged, where skin is grown in a laboratory setting from artificial matrix and isolated skin
598  cells[20,21]. Although these models mimic human skin better than most in vitro approaches, they
599  still do not fully simulate the native tissue environment and are generally too fragile to injure
600  reproducibly. Additionally, we (and others) have demonstrated that ex vivo human skin tissue
601  retains resident immune cells, which will no doubt contribute to repair[22,23]. Future work should
602  now focus on extending the viability and immunocompetency of the *ex vivo* model for late-stage
603  healing assessment[24]. One option is further advancement of promising organ-on-a-chip
604  technologies capable of prolonging tissue viability and maintaining native skin architecture for
605  up to two weeks in culture[25]. Ex vivo models have also begun to consider the importance of the
606  skin inflammatory response by successfully incorporating immune cells, such as neutrophils, into
607  the host tissue[26] or injecting host tissue with antibodies to elicit an immune reaction[27]. We expect
608  that these findings will pave the way for development of more refined and translatable methods
609  in the future.

611  A major benefit of using ex vivo skin to measure wound closure is the ability to compare healing
612  rates in healthy (e.g., non-diabetic) versus pathological (e.g., diabetic or aged) tissue. Here we
613  showed that re-epithelialization and barrier formation are indeed impaired in diabetic versus
614  healthy ex vivo wounds. Indeed, this provides a route for pre-clinical assessment of pathological
615  repair, where ageing and diabetes are major risk factors for developing chronic wounds[1]. While
616  in vitro pathological models exist, such as cells isolated from aged and diabetic tissue, or cells

617 cultured in high glucose to mimic hyperglycemia[28,29], these cells can quickly lose their phenotype
618 once removed from the in vivo microenvironment. An important component of the extrinsic
619 pathological healing environment is the dermal matrix, which is altered in both ageing and
620 diabetes[30]. Indeed, this perturbed matrix affects the behavior of resident and naïve
621 fibroblasts[31,32]. Thus, the importance of studying cells in their host tissue environment cannot be
622 underestimated.
623
624 In summary, our protocol provides an important platform to quantify human wound re-
625 epithelialization, explore regulatory factors and to test the validity and efficacy of potential
626 therapeutics[12,13]. While pre-clinical testing does still require in vivo approaches, a combined
627 strategy using ex vivo human tissue and in vivo murine wounding should refine the pre-clinical
628 pathway, reducing animal use while increasing cross-species translatability.
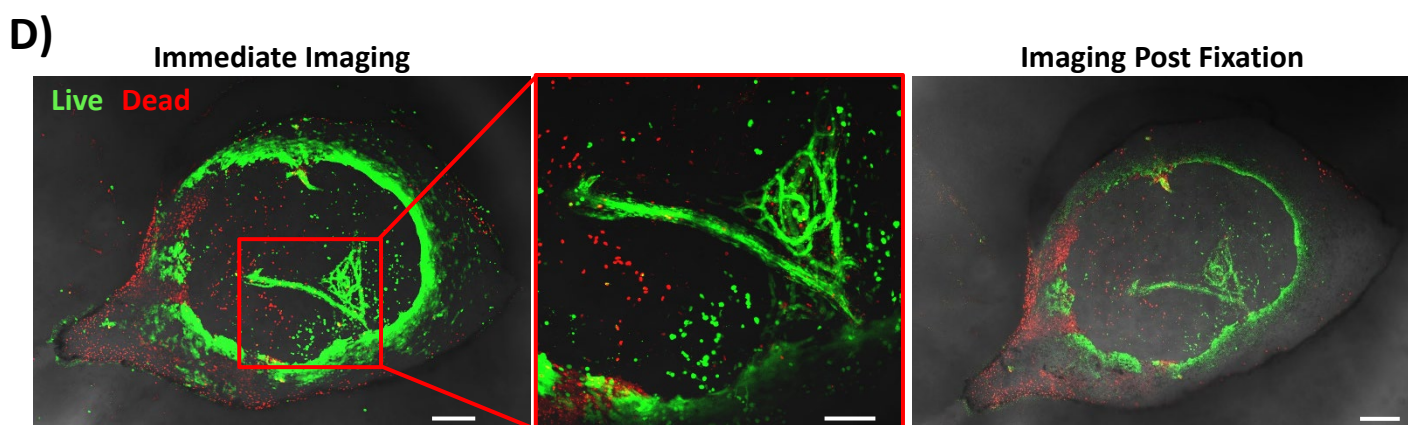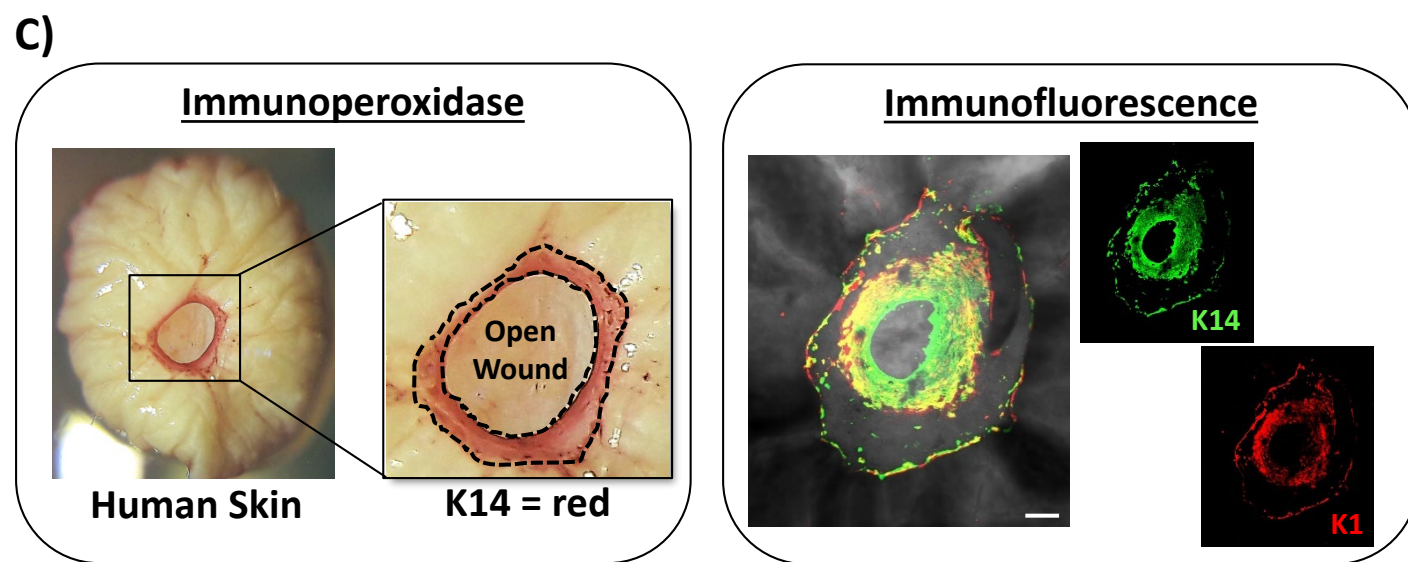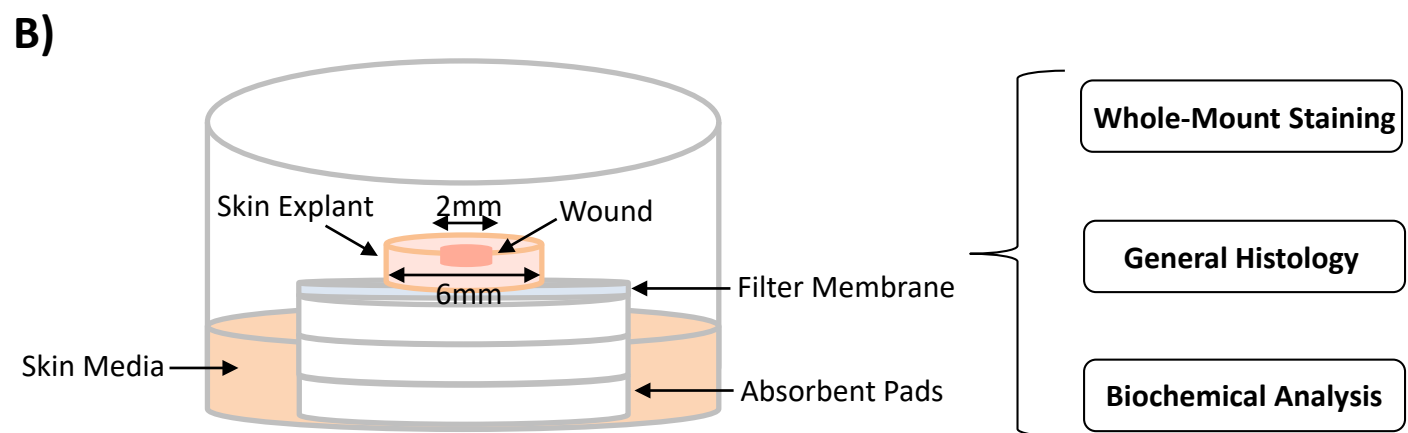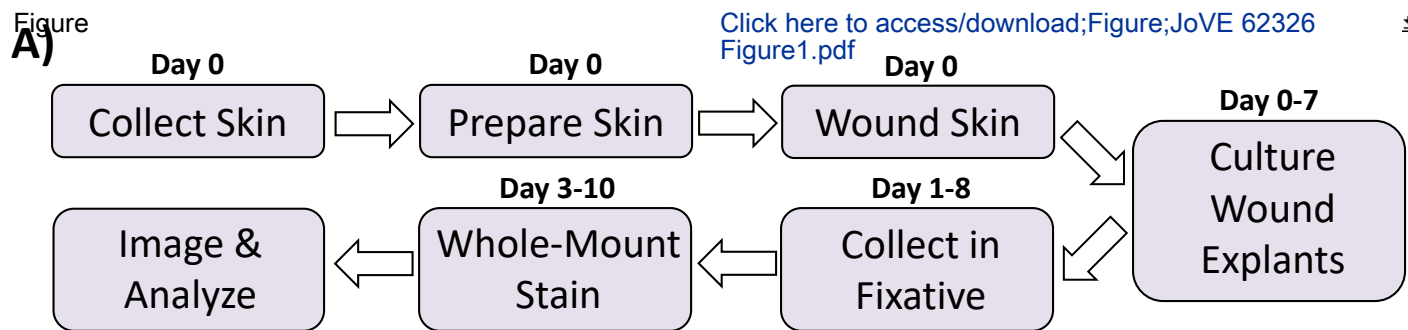629

634

635 **DISCLOSURES:**
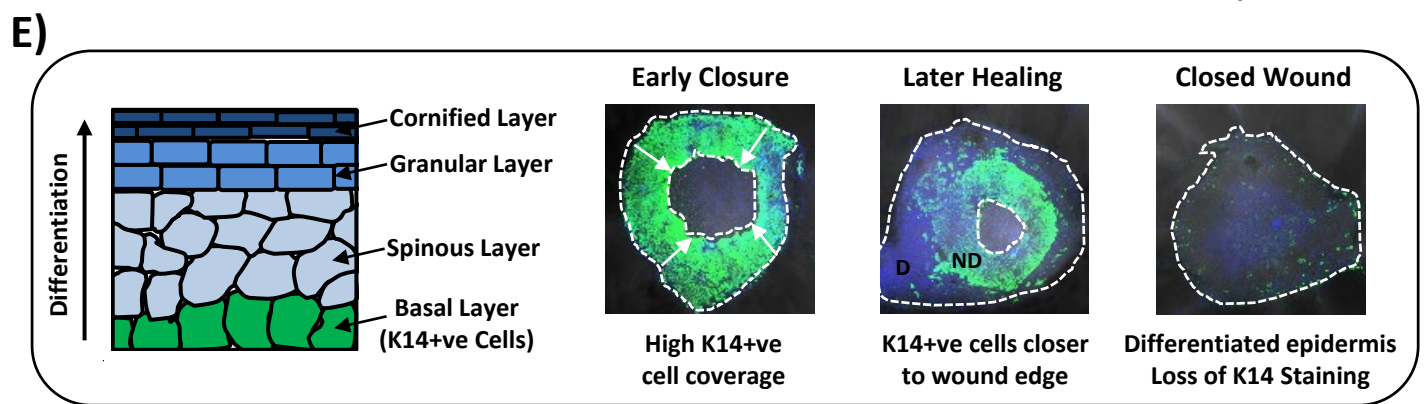636 The authors declare no conflicts of interest.
637

638 **REFERENCES:**
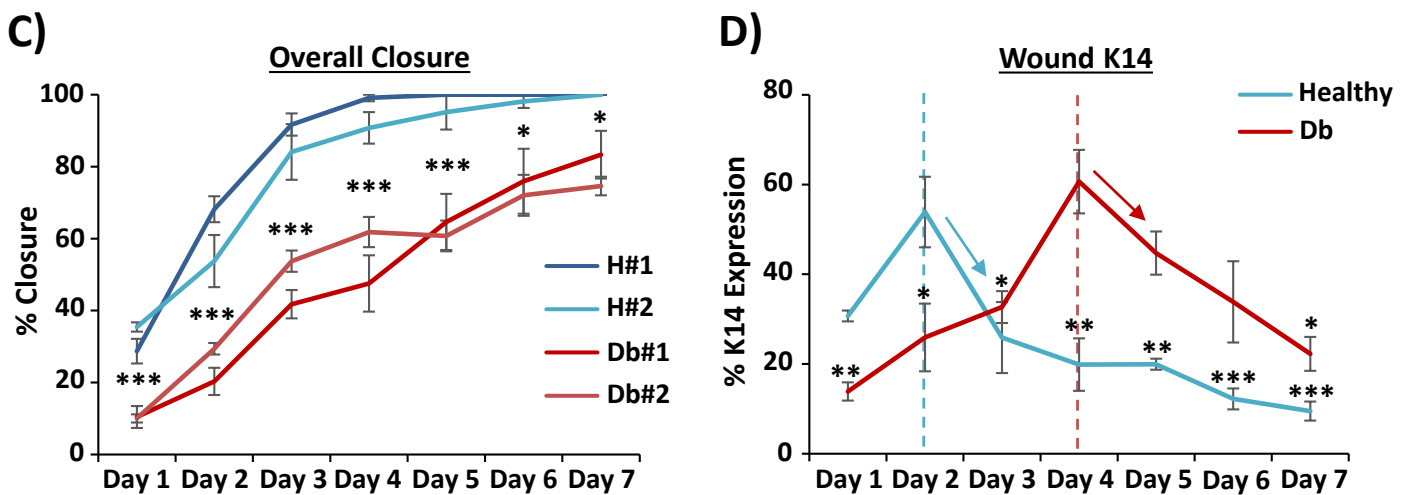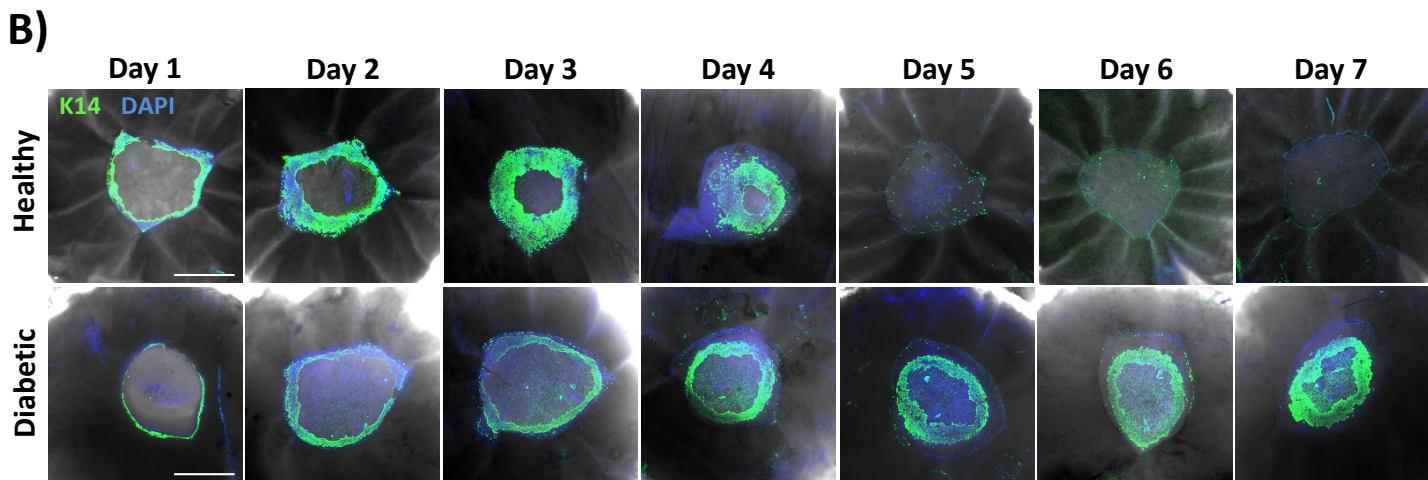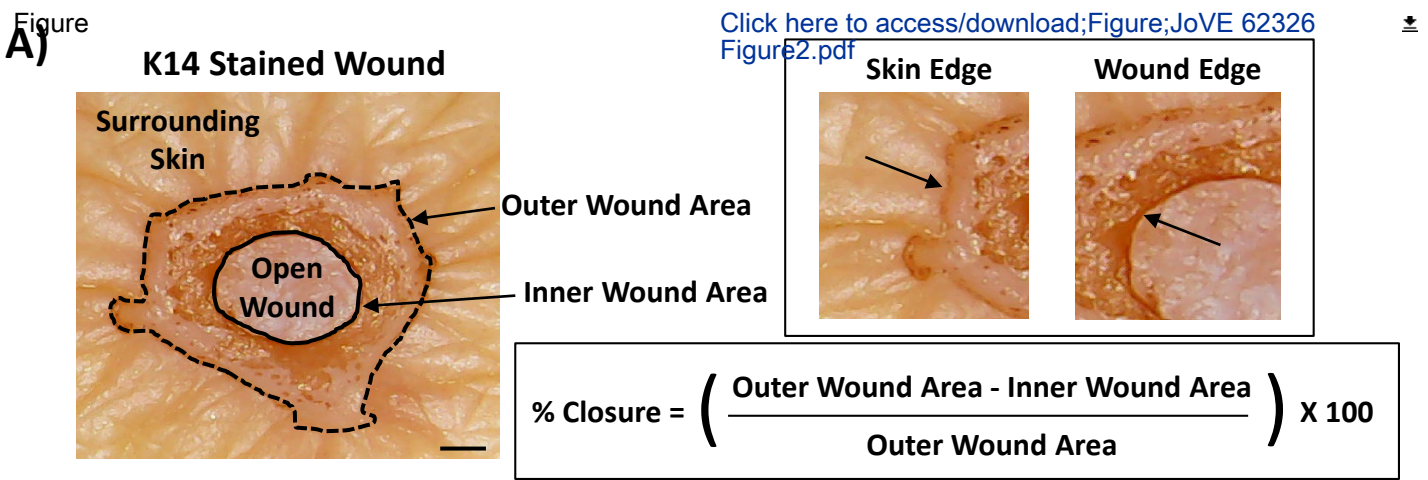639 1.     Lindholm, C., Searle, R. Wound management for the 21st century: combining
640 effectiveness and efficiency. *International Wound Journal*. **13**, 5-15 (2016).
641 2.     Guest, J. F. et al. Health economic burden that different wound types impose on the UK's
642 National Health Service. *International Wound Journal*. **14** (2), 322-330 (2017).
643 3.     Guest, J. F., Fuller, G. W., Vowden, P. Diabetic foot ulcer management in clinical practice
644 in the UK: costs and outcomes. *International Wound Journal*. **15** (1), 43-52 (2018).
645 4.     López-Valverde, M. E., et al. Perioperative and long-term all-cause mortality in patients
646 with diabetes who underwent a lower extremity amputation. *Diabetes Research and Clinical
647 Practice*. **141**, 175-180 (2018).
648 5.     Wilkinson, H. N., Hardman, M. J. The role of estrogen in cutaneous ageing and
649 repair. *Maturitas*. **103**, 60-64 (2017).
650 6.     Frykberg, R. G., Banks, J. Challenges in the treatment of chronic wounds. *Advances in
651 Wound Care*. **4** (9), 560-582 (2015).
652 7.     Wilkinson, H. N., Hardman, M. J. Wound healing: cellular mechanisms and pathological
653 outcomes. *Open Biology*. **10** (9), 200223 (2020).
654 8.     Ansell, D. M., Holden, K. A., Hardman, M. J. Animal models of wound repair: Are they
655 cutting it? *Experimental Dermatology*. **21** (8), 581-585 (2012).
656 9.     Elliot, S., Wikramanayake, T. C., Jozic, I., Tomic-Canic, M. A modeling conundrum: murine
657 models for cutaneous wound healing. *Journal of Investigative Dermatology*. **138** (4), 736-740
658 (2018).
659 10.    Mazio, C. et al. Pre-vascularized dermis model for fast and functional anastomosis with
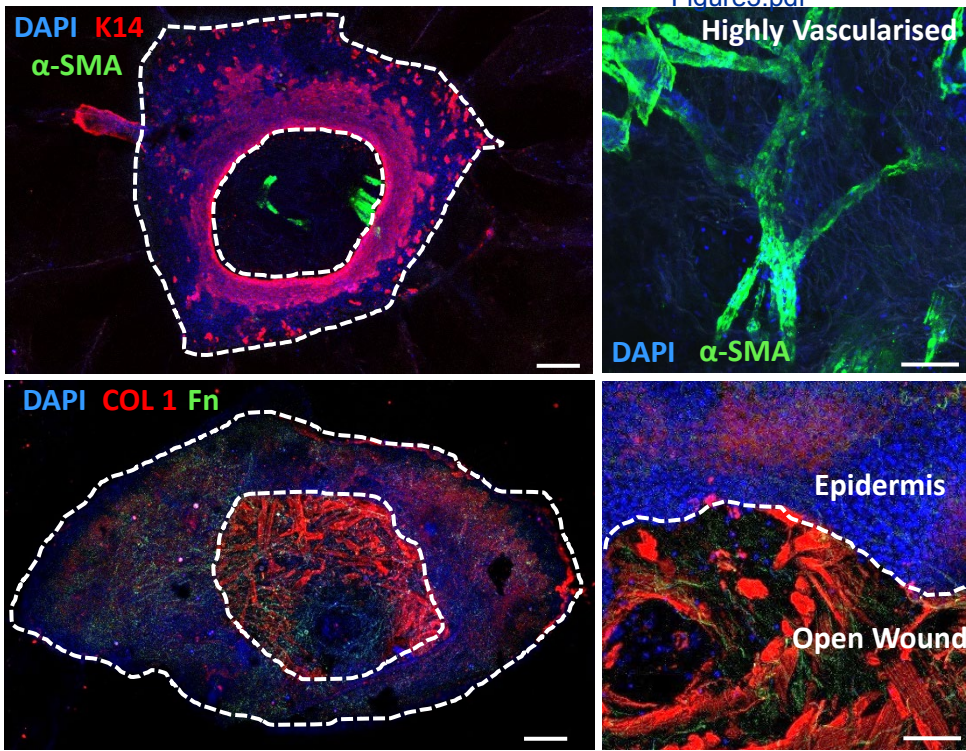660 host vasculature. *Biomaterials*. **192**, 159-170 (2019).

661    11.    Wilkinson, H. N., Iveson, S., Catherall, P., Hardman, M.J. A novel silver bioactive glass
662    elicits antimicrobial efficacy against Pseudomonas aeruginosa and Staphylococcus aureus in an
663    ex vivo skin wound biofilm model. *Frontiers in Microbiology*. **9**, 1450 (2018).

664    12.    Wilkinson, H. N. et al. Elevated local senescence in diabetic wound healing is linked to
665    pathological repair via CXCR2. *Journal of Investigative Dermatology*. **139** (5), 1171-1181 (2019).

666    13.    Wilkinson, H. N. et al. Tissue iron promotes wound repair via M2 macrophage polarization
667    and the chemokine (CC motif) ligands 17 and 22. *The American Journal of Pathology*. **189** (11),
668    2196-2208 (2019).

669    14.    Rhea, L., Dunnwald, M. Murine excisional wound healing model and histological
670    morphometric wound analysis. *Journal of Visualized Experiments.* **162**, e61616 (2020).

671    15.    Ansell, D. M., Campbell, L., Thomason, H. A., Brass, A., Hardman, M.J. A statistical analysis
672    of murine incisional and excisional acute wound models. *Wound Repair and Regeneration*. **22** (2),
673    281-287 (2014).

674    16.    Rizzo, A. E., Beckett, L. A., Baier, B. S., Isseroff, R. R. The linear excisional wound: an
675    improved model for human ex vivo wound epithelialization studies. *Skin Research and*
676    *Technology.* **18** (1), 125-132 (2012).

677    17.    Olsson, M., et al. The humanistic and economic burden of chronic wounds: a systematic
678    review. *Wound Repair and Regeneration*. **27** (1), 114-125 (2019).

679    18.    Mendoza-Garcia, J., Sebastian, A., Alonso-Rasgado, T., Bayat, A. Optimization of an ex vivo
680    wound healing model in the adult human skin: Functional evaluation using photodynamic
681    therapy. *Wound Repair and Regeneration*. **23** (5), 685-702 (2015).

682    19.    Brownhill, V. R., et al. Pre-clinical assessment of single-use negative pressure wound
683    therapy during in vivo porcine wound healing. *Advances in Wound Care*. "In Press" 2020.

684    20.    Diekmann, J., et al. A three-dimensional skin equivalent reflecting some aspects of in vivo
685    aged skin. *Experimental Dermatology*. **25** (1), 56-61 (2016).

686    21.    Vidal Yucha, S. E., Tamamoto, K. A., Nguyen, H., Cairns, D. M., Kaplan, D. L. Human skin
687    equivalents demonstrate need for neuro-immuno-cutaneous system. *Advanced Biosystems*. **3**
688    (1), 1800283 (2019).

689    22.    Dijkgraaf, F. E. et al. Tissue patrol by resident memory CD8+ T cells in human skin. *Nature*
690    *Immunology*. **20** (6), 756-764 (2019).

691    23.    He, X., de Oliveira, V. L., Keijsers, R., Joosten, I., Koenen, H. J. Lymphocyte isolation from
692    human skin for phenotypic analysis and ex vivo cell culture. *Journal of Visualized Experiments*.
693    (110), e52564 (2016).

694    24.    Pupovac, A. et al. Toward immunocompetent 3D skin models. *Advanced Healthcare*
695    *Materials*. **7** (12), 1701405 (2018).

696    25.    Ataç, B. et al. Skin and hair on-a-chip: in vitro skin models versus ex vivo tissue
697    maintenance with dynamic perfusion. *Lab on a Chip*. **13** (18), 3555-3561 (2013).

698    26.    Kim, J. J. et al. A microscale, full-thickness, human skin on a chip assay simulating
699    neutrophil responses to skin infection and antibiotic treatments. *Lab on a Chip*. **19** (18), 3094-
700    3103 (2019).

701    27.    Jardet, C. et al. Development and characterization of a human Th17-driven ex vivo skin
702    inflammation model. *Experimental Dermatology*. **29** (10), 993-1003 (2020).

703    28.    Chen, J. L. et al. Metformin attenuates diabetes-induced tau hyperphosphorylation in
704    vitro and in vivo by enhancing autophagic clearance. *Experimental Neurology*. **311**, 44-56 (2019).

705    29.    Demirovic, D., Rattan, S. I. Curcumin induces stress response and hormetically modulates
706    wound healing ability of human skin fibroblasts undergoing ageing in vitro. *Biogerontology*. **12**
707    (5), 437-444 (2011).
708    30.    Wilkinson, H. N., Hardman, M. J. Wound senescence: A functional link between diabetes
709    and ageing? *Experimental Dermatology*. **30** (1), 68-73 (2020).
710    31.    Fisher, G. J. et al. Collagen fragmentation promotes oxidative stress and elevates matrix
711    metalloproteinase-1 in fibroblasts in aged human skin. *The American Journal of Pathology*. **174**
712    (1), 101-114 (2009).
713    32.    Quan, T., Little, E., Quan, H., Voorhees, J. J., Fisher, G. J. Elevated matrix
714    metalloproteinases and collagen fragmentation in photodamaged human skin: impact of altered
715    extracellular matrix microenvironment on dermal fibroblast function. *Journal of Investigative*
716    *Dermatology*. **133** (5), 1362 (2013).
717

**A)**

Day 0 — Collect Skin → Day 0 — Prepare Skin → Day 0 — Wound Skin → Day 0-7 — Culture Wound Explants

Day 3-10 — Whole-Mount Stain ← Day 1-8 — Collect in Fixative ← Culture Wound Explants

Image & Analyze ← Whole-Mount Stain

**B)**

Skin Explant, 2mm, Wound, 6mm, Filter Membrane, Skin Media, Absorbent Pads

Whole-Mount Staining
General Histology
Biochemical Analysis

**C)**

Immunoperoxidase — Human Skin — Open Wound — K14 = red

Immunofluorescence — K14, K1

**D)**

Immediate Imaging — Live Dead

Imaging Post Fixation

# A) K14 Stained Wound

Surrounding Skin

Outer Wound Area

Open Wound

Inner Wound Area

**Skin Edge**

**Wound Edge**

$$\% \text{ Closure} = \left( \frac{\text{Outer Wound Area} - \text{Inner Wound Area}}{\text{Outer Wound Area}} \right) \times 100$$

## B)

| Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 6 | Day 7 |

K14  DAPI

Healthy

Diabetic

## C) Overall Closure

% Closure

Day 1  Day 2  Day 3  Day 4  Day 5  Day 6  Day 7

*** *** *** *** *** * *

— H#1
— H#2
— Db#1
— Db#2

## D) Wound K14

% K14 Expression

— Healthy
— Db

Day 1  Day 2  Day 3  Day 4  Day 5  Day 6  Day 7

** * * ** ** *** *

## E)

Differentiation

Cornified Layer

Granular Layer

Spinous Layer

Basal Layer (K14+ve Cells)

**Early Closure**

High K14+ve cell coverage

**Later Healing**

D   ND

K14+ve cells closer to wound edge

**Closed Wound**

Differentiated epidermis Loss of K14 Staining

Figure

**A)**

DAPI K14 α-SMA

Highly Vascularised

DAPI α-SMA

DAPI COL 1 Fn

Epidermis

Open Wound

**B)**

DAPI K14 K1

Cell Level Resolution

**C)**

K14 CD1a

CD1a – Langerhans

**A)**

**Inflammation**

Langerhans
**CD1a**

**Proliferation**

Migrating Epidermis · **K14, K1**

Blood
Vessels
**α-SMA**

**Extracellular Matrix**

**COL I**
**Fn**

**B)**

**Whole-Mount Staining versus Histological Staining**

Open
Wound

Measures Entire Wound Area

**Section 1 WEs**

S1

S2

**Section 2 WEs**

Section 1 = 40% closure
Section 2 = 70% closure

**Transverse Sections Through Wounds**

Epidermis
Wound
**WE**     **WE**
Dermis

Measures Specific Wound Region
Highly Variable

| Name of Material/ Equipment | Company |
|---|---|
| 50 mL Falcon Tubes | Falcon |
| 1.5 ml TubeOne Microcentrifuge Tubes, Natural (Sterile) | Starlab |
| 48-Well CytoOne Plate, TC-Treated | Starlab |
| Acetic Acid Glacial | Fisher Chemical |
| Alkyltrimethylammonium Bromide | Sigma-Aldrich |
| Anti-Alpha Smooth Muscle Actin Antibody [1A4] | Abcam |
| Anti-Collagen I Antibody | Abcam |
| Anti-Cytokeratin 14 Antibody [LL002] | Abcam |
| CD1A Antibody (CTB6) | Santa Cruz Biotechnology |
| DAPI (4',6-diamidino-2-phenylindole, dihydrochloride) | Thermo Fisher Scientific |
| Falcon 60mm Petri dishes | Falcon |
| Fibronectin Antibody (EP5) | Santa Cruz Biotechnology |
| Formaldehyde, Extra Pure, Solution 37-41%, SLR | Fisher Chemical |
| Gauze Swabs | Medisave |
| Gibco™ Antibiotic-Antimycotic Solution | Thermo Fisher Scientific |
| Gibco DMEM, high glucose, no glutamine | Thermo Fisher Scientific |
| Gibco Fetal Bovine Serum | Thermo Fisher Scientific |
| Gibco HBSS, no calcium, no magnesium | Thermo Fisher Scientific |
| Gibco L-Glutamine (200 mM) | Thermo Fisher Scientific |
| Hydrogen Peroxide | Sigma-Aldrich |
| ImageJ Software | National Institutes of Health |
| Invitrogen IgG (H+L) Cross-Adsorbed Goat anti-Mouse, Alexa Fluor 488 | Thermo Fisher Scientific |
| Invitrogen IgG (H+L) Cross-Adsorbed Goat anti-Rabbit, Alexa Fluor 594 | Thermo Fisher Scientific |
| Invitrogen LIVE/DEAD Viability/Cytotoxicity Kit, for mammalian cells | Thermo Fisher Scientific |
| Iris Forceps, 10 cm, Curved, 1x2 teeth | World Precision Instruments |
| Iris Scissors, 11 cm, Curved, SuperCut, Tungsten Carbide | World Precision Instruments |
| Iris Scissors, 11 cm, Straight, SuperCut, Tungsten Carbide | World Precision Instruments |
| Keratin 1 Polyclonal Antibody, Purified | Biolegend |
| Keratin 14 Polyclonal Antibody, Purified | Biolegend |
| LSM 710 Confocal Laser Scanning Microscope | Carl Zeiss |
| Merck Millipore Absorbent pads | Merck Millipore |
| Merck Millipore Nylon Hydrophilic Membrane Filters | Merck Millipore |

| | |
|---|---|
| Normal Goat Serum Solution | Vector Laboratories |
| Phosphate Buffer Solution | Sigma-Aldrich |
| Sodium Azide | Sigma-Aldrich |
| Sodium Chloride | Fisher Bioreagents |
| Sterilisation Pouches | Medisave |
| Stiefel 2mm biopsy punches | Medisave |
| Stiefel 6mm biopsy punches | Medisave |
| Thermo Scientific Sterilin Standard 90mm Petri Dishes | Thermo Fisher Scientific |
| Triton X-100 | Fisher Chemical |
| VECTASTAIN Elite ABC-HRP Kit, Peroxidase (Rabbit IgG) | Vector Laboratories |
| Vector NovaRED Substrate Kit, Peroxidase (HRP) | Vector Laboratories |
| Wireless Digital Microscope | Jiusion |

| Catalog Number | Comments/Description |
|---|---|
| 352070 | *For skin washing* |
| S1615-5510 | *For whole-mount staining* |
| CC7682-7548 | *For whole-mount staining* |
| A/0400/PB15 | *Part of fixative* |
| M7635 | *Part of fixative* |
| ab7817 | *Stains blood vessels* |
| ab34710 | *Stains collagen* |
| ab7800 | *Stains epidermis* |
| sc-5265 | *Stains Langerhans cells* |
| 62247 | *Counterstain for cell nuclei* |
| 353004 | *Human ex vivo culture* |
| sc-8422 | *Stains fibronectin* |
| F/1501/PB17 | *Part of fixative* |
| CS1650 | *To clean skin* |
| *15240062* | *Human ex vivo culture* |
| 11960044 | *Human ex vivo culture* |
| 10500064 | *Human ex vivo culture* |
| 14170088 | *Human ex vivo culture* |
| 25030081 | *Human ex vivo culture* |
| H1009-100ML | *For immunoperoxidase staining* |
| N/A | *For image analysis* |
| A11001 | *Secondary antibody used depends on required fluorochromes and primary antibody* |
| A11012 | *Secondary antibody used depends on required fluorochromes and primary antibody* |
| L3224 | *For viability assessment of tissue* |
| 15917 | *To create wounds* |
| 501264 | *To create wounds* |
| 501263 | *To remove adipose tissue* |
| 905201 | *Stains epidermis* |
| 905301 | *Stains epidermis* |
| Discontinued | *For fluorescent imaging* |
| AP10045S0 | *Human ex vivo culture* |
| HNWP04700 | *Human ex vivo culture* |

| | |
|---|---|
| S-1000-20 | *Animal serum used depends on secondary antibody* |
| P3619 | *For wash buffer* |
| S2002 | *For blocking buffer* |
| BP358-212 | *Part of fixative* |
| SH3710 | *To sterilise instruments* |
| BI0500 | *For partial thickness wound* |
| BI2000 | *For outer explant* |
| 101VR20 | *To prepare skin* |
| T/3751/08 | *For wash buffer* |
| PK-6101 | *For immunoperoxidase staining; HRP kit used depends on primary antibody* |
| SK-4800 | *For immunoperoxidase staining* |
| N/A | *For brightfield imaging* |

| Name of Material / Equipment | Company | Catalog Number | ents/Description |
|---|---|---|---|

Centre for Atherothrombosis and
Metabolic Disease

Hull York Medical School

**Hull**
University of Hull
Hull, HU6 7RX, UK

**York**
University of York
York, YO10 5DD, UK

www.hyms.ac.uk

4th December 2020

Dear Editor,

We are delighted that our enclosed manuscript '*Human Ex Vivo Wound Model and Whole-Mount Staining Approach to Accurately Evaluate Skin Repair*' has been favourably reviewed for publication in *The Journal of Visualized Experiments*. Please find below our rebuttal to the editorial and peer review comments.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

2. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s). Please ensure that citations follow an ascending chronology E.g. Line 534.

3. Line 103: Please specify the chemical(s) used.

4. Check lines 150-153. Are a total of 3 rinsing steps involved?

5. Line 186: Specify the humidity level.

6. Line 324: "Collect Z stacks.." instead of "Z stacks could be.."

7. Please include a single space between the quantity and its unit E.g. Line 377, 511: "3 mm" instead of "3mm".

8. Please do not use personal pronouns such as "we", "our" etc. in the protocol section.

9. Please include a single line spacing between the protocol steps. Furthermore, any text that cannot be written in the imperative tense may be added as a "Note." E.g.: Line 322-323

10. 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. E.g. NovaRed, Alexa Fluor, Zeiss etc.

**Response: We have now made the requested editorial changes to the manuscript. For point 4 we have also amended the text for clarification that there are 4 rinsing steps (2 x HBSS plus Abs, 1 x HBSS without antibiotics and 1 x DPBS).**

---

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The submitted manuscript by Wilkinson et al., describes the development of an ex vivo 3D human skin wound healing model and whole mount staining approach, for the quantification of wound healing responses in skin. The validation and potential usefulness of this model were confirmed by the impaired 'wound' re-epithelialization observed in skin derived from diabetic patients compared to normal skin, akin to non-healing apparent in chronic wounds such as diabetic foot ulcers, which are a major issue for healthcare providers worldwide. Consequently, by using human skin samples, this model not only supports the 3Rs remit in biomedical research, but could further be utilised in future for the routine screening of new therapeutic entities, in terms of identifying their respective wound healing efficacies in promoting diabetic skin wound re-epithelialisation and their mechanisms of action. As a considerable limitation to current chronic wound therapy development involves the inadequate nature of existing 3D organotypic and ex vivo models, the current model described here may aid the successful translation of novel therapeutics into approved clinical use in future. Overall, this is an interesting and well-performed study, which builds on previous ex vivo wound healing model work published by the Authors. Thus, the manuscript is concise and generally well-written, whilst providing sufficient and straightforward to follow details on the methods and techniques used, in order to allow this experimental model to be reproduced in other laboratories. Consequently, I only have relatively limited number of minor comments for the Authors to address:-

1. Protocol (page 4). It would be useful if further details were included on the patients from which the non-diabetic and diabetic skin biopsies were collected? This is particularly true for the diabetic skin and how these patients were selected?

**Response: We thank the reviewer for raising this point. Non-diabetic skin was collected from patients undergoing routine surgery (mean age = 68). Diabetic skin was selected from donors who had established type II diabetes and a history of ulceration (mean age = 81). We have updated the text to include this information.**

2. Related to this, it appears that the Human Skin Growth Media used was high glucose DMEM (presumably at 25 mM concentrations)? Could the Authors clarify whether both non-diabetic and diabetic ex vivo cultures were maintained in high glucose DMEM, as I would assume that non-diabetic cultures were maintained in more normal glucose concentrations (i.e. 5.5 mM glucose-containing DMEM)? If this is the case, details of the normal glucose medium needs to be included here. if not, could the Authors justify why both non-diabetic and diabetic ex vivo cultures were maintained in high glucose DMEM?

**Response: All cultures were maintained in high glucose DMEM. The rationale for this is to allow evaluation of intrinsic differences in cellular behaviour rather than differences that may in fact be due to culturing each group in different glucose levels. Indeed, the fact that we see a difference between non-diabetic and diabetic skin when both are cultured in the same media conditions demonstrates that there are intrinsic cellular differences in the diabetic skin that contribute to delayed healing. It would be interesting to explore the role of these intrinsic versus extrinsic factors in skin culture in the future.**

3. Protocol (page 6). Could the Authors please provide details on to what extent 'wound' formation by punch biopsy can vary between patient donors and skin sites used? i.e. Could this lead to potential problems with variability in wound healing responses within experimental groups and between different experimental groups?

**Response: The reviewer raises an important point. In our extensive experience, we do observe donor differences at the point of wounding. For example, aged skin generally requires less force to generate wounds and skin with significant stretch marks can be**

**challenging to use. However, in general, most skin is suitable for ex vivo wounding. We have not directly compared the healing potential of ex vivo skin derived from different body sites, but we have successfully used skin from different body sites for ex vivo wound healing studies.**

**As with all human research, there is inherent variability between donors. There are also many external factors that can influence ex vivo healing, a significant one being the time from surgical tissue collection to ex vivo wound set up. For this very reason, we never split treatment groups across multiple donors/sites. We have updated the discussion text to include some of this information.**

4. Results (page 11). The Authors very nicely show enhanced wound closure and re-epithelialization in normal skin 'wounds' over 7 days in culture. However, have the Authors extended these cultures beyond 7 days and if so, does the model show any evidence of keratinocyte differentiation/stratification within this air/medium interface model, once wound repair is complete?

**Response: We have not extended ex vivo skin cultures beyond 7 days as we primarily use this model to evaluate wound re-epithelialisation. We agree that it would be interesting in the future to explore keratinisation/stratification of the re-epithelialised tissue.**

5. Blood vessel assessment (page 13 and Figure 3). Could the Authors please explain the choice of a-SMA as a blood vessel marker, over more established angiogenic markers, e.g. CD31?

**Response: We have optimised the α-SMA monoclonal from Abcam for both histological and whole-mount wound samples. We thank the reviewer for the suggestion of exploring CD31 and will do so in the future.**

6. Figures 3 and 4 (pages 13 and 14). Could the Authors please clarify which ex vivo skin samples were used to generate the data presented in Figures 3 and 4? I presume that these are derived from normal skin ex vivo cultures, but need to be confirmed within the text and the Figure legends. Related to this, could the Authors explain why data for both normal and diabetic skin were not included here, as characterisation of these additional parameters would provide added strength to the ex vivo model, in terms of the additional differences between normal and diabetic skin wound healing observed.

**Response: The data presented in Figure 3 and 4 pertains to normal (non-diabetic) skin. We have updated both text and Figure legends to clarify this. We agree that detailed characterisation of the ex vivo diabetic healing model is important, but we feel this is beyond the scope of this current methods paper. Our approach was to include diabetic skin data in Figure 2 to demonstrate the utility of the ex vivo model to show differences in overall wound closure. By contrast, the focus of Figure 3 was to illustrate the diversity of markers that can be evaluated. Nevertheless, we thank the reviewer for this suggestion and we plan to address it in the future.**

Reviewer #2:

Manuscript Summary:

This study describes a human ex vivo wound model and whole mount staining for the performance of high throughput re-epithelialization measurements. The manuscript is well written and touches on the main strength and weaknesses of the model.

Minor Concerns:

The authors did not elaborate on the possibilities to use these samples for traditional histological analyses that could complement their whole mount evaluation. Can this be done? If so, it may be interesting to indicate how. It would have been interesting to know if findings on the whole mount correlates with findings on sections.

**Response: It is absolutely possible to process (wax or OCT) and section whole-mount samples for histology. This allows direct comparison of whole-mount measured versus histological measured healing. We have not attempted to perform immunohistochemistry on samples that have been whole-mount stained, but in theory this should be possible. We have updated the manuscript discussion to emphasise the possibility of combining whole-mount staining with subsequent traditional histological analysis.**

What is the minimum size of human tissue required for these studies? Understanding that it will warry from one experimental design to another, it may be useful for the audience to have an appreciation of how much tissue is needed, considering that human samples may not be trivial to acquire.

**Response: We agree with the point the reviewer makes that access to human skin is not trivial. For a time course experiment as shown (using n=6 per group), a 5x5 cm piece of skin should suffice when using a biopsy diameter of 6mm.**

Figure 2, panel D, last panel on the right. It is indicated: Loss of K14. This is somewhat inaccurate, as K14 is not loss, but the visualization of K14 is impaired because of lack of penetration of the antibody. At a minimum, it should say Loss of K14 staining. To this point, careful wording is warranted when discussing this issue in the manuscript, to make sure that it is the loss of the staining, and not the loss of the protein that is occurring with healing.

**Response: We thank the reviewer for making this point. We have now carefully amended the figure and text for clarification.**

Figure 3, panels B and C are hard to interpret. What is the orientation? What are we looking at?

**Response: Both panels B and C are ex vivo wounds imaged in the same orientation as A (i.e. wounded epidermis side up). Panel B illustrates keratin localisation in migrating epidermis at the edge of the wound at high power. Panel C shows Langerhans cell staining (CD1a) in the epidermis of a fully re-epithelialised wound.**

Figure 4, panel A, extracellular panel: I would recommend using COL 1 and Fn .

**Response: We have amended the text as suggested.**

Figure 4, panel B. By itself, this panel is hard to understand. One suggestion would be to not separate the two parts of the panel, but rather having one common title (whole-mount staining versus histological staining), with two downward arrows to their respective staining. The "measures entire wound area" and "measures specific…" should be placed at the same level to highlight the contrast. "Measures specific wound region orientation and sectioning variability" is confusing, may be could say "Measures Specific Wound Region Highly Variable" .

**Response. We thank the reviewer for their insight and we have amended the panel as suggested.**

Minor details:

2mm and 6mm punches: throughout the manuscript, there is no space between the number and the unit. Typically, the scientific language would require a space. Is it common rule to omit the space in this case?

P4, line 95, the use of "theatre" is awkward

P5, line 134, igG should be capitalized
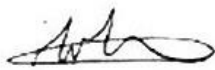
P10, line 333, "petri" should be capitalize

P12, line 408, "wound K14 levels" is awkward

P15, line 518, should say "reduced"

**Response: We thank the reviewer for highlighting these points and we have made amendments as suggested (red text where appropriate).**

Thank you again for considering our work.

Yours Sincerely,

**Dr Holly N Wilkinson**
**Lecturer in Wound Healing**
**Hull York Medical School**
**T: +44 (0)1482 461879**
**E: h.n.wilkinson@hull.ac.uk**