Published in final edited form as:

Methods Mol Biol. 2015 ; 1343: 35–51. doi:10.1007/978-1-4939-2963-4_4.

Experimental Approaches to Tissue Injury and Repair in Advanced Age

Aleah L. Brubaker, Stewart R. Carter, Elizabeth J. Kovacs

Abstract

Cutaneous wound healing is a complex physiological process. This process can be altered by multiple physiological and pathological factors. Multiple pathophysiological disturbances act to impair resolution of cutaneous wound injury, including obesity, diabetes, peripheral vascular disease, and advanced age. As our longevity increases without a concomitant increase in healthy living years, it is plausible to assume that problematic wound closure will continue to consume a large portion of our health care resources. Furthermore, advanced age is associated with numerous alterations in the innate and adaptive immune responses that complicate outcomes following cutaneous injury, trauma, or infection. Thus, models that examine the impact of advanced age on cutaneous wound repair will be of great benefit to the development of potential therapeutics that target age-related aberrancies in tissue repair. Herein, we detail two animal models of tissue injury, excisional wound injury and burn injury, that can be used to evaluate wound healing in the context of advanced age. We also describe modifications of these methods to examine wound infection following either excisional or burn injury. Lastly, we discuss methods of subsequent tissue analysis following injury. Models described below can be further adapted to genetically engineered murine strains to study the effects of aging and other co-morbidities on wound healing.

Keywords

Wounding healing; Animal model; Aging; Excisional biopsy; Burn injury; Infection

1 Introduction: Aging and Cutaneous Wound Healing

Cutaneous wound healing is a complex physiological process comprised of three primary phases: the inflammatory, proliferative, and remodeling phases [1, 2]. The inflammatory phase begins immediately following tissue injury. Platelets at the wound site act to aid in hemostasis and degranulate to release a host of pro-inflammatory mediators. Simultaneously, antimicrobial peptides released from cutaneous keratinocytes help to provide direct bactericidal activity following tissue injury. These mediators, alongside the cytokines and chemokines released from damaged keratinocytes and resident tissue leukocytes, aid in recruitment of innate immune cells to help prevent microorganism contamination and infection [3]. Early in the inflammatory phase, neutrophils predominate, helping phagocytose pathogens and debris [4]. Macrophages then enter the wound bed to aid in phagocytosis, eventually undergoing a phenotypic shift in order to facilitate the transition to the proliferative phase [5]. During the proliferative phase, keratinocytes aid in re-epithelialization by migrating over the open wound bed to restore the epidermis. Concurrent deposition of immature collagen by fibroblasts and angiogenesis help to restore the dermal

architecture and vascularity of the injured tissue, creating a provisional extracellular matrix [6, 7]. Over time, the proliferative phase gives way to the remodeling phase, where the initial type III collagen is replaced with type I collagen, which improves the tensile strength of the wound [2]. Additionally, the developing vasculature is pruned to form an efficient vascular network. Timely progression through these phases is required for efficient wound healing. Alterations in any of these phases of wound healing can impair wound closure, resulting in a range of clinical complications from infection to chronic wounds to excessive scar formation.

Currently, it is estimated the U.S. health care system spends 25 billion dollars treating chronic wounds and related complications [8]. Multiple pathophysiological disturbances act to impair resolution of cutaneous wound injury, including obesity, diabetes, peripheral vascular disease, and advanced age [8–10]. Of note, the average human lifespan continues to lengthen, with a growing number of individuals greater than 65. Moreover, the aforementioned co-morbidities are on the rise, compromising healthy living years in this aging population [11]. As our longevity increases without a concomitant increase in healthy living years, it is plausible to assume that problematic wound closure will continue to consume a large portion of our health care resources. Furthermore, advanced age is associated with numerous alterations in the innate and adaptive immune responses that complicate outcomes following cutaneous injury, trauma or infection [12, 13]. Thus, models that examine the impact of advanced age on cutaneous wound repair will be of great benefit to the development of potential therapeutics that target age-related aberrancies in tissue repair.

Herein, we detail two animal models of tissue injury, excisional wound injury and burn injury, that can be used to evaluate wound healing in the context of advanced age. These models were selected based on clinical reports of impaired wound healing, increased wound dehiscence and worse outcomes following burn trauma in elderly patients [8, 9, 14, 15]. We also describe modifications of these methods to examine wound infection following either excisional or burn injury. Lastly, we discuss methods of subsequent tissue analysis following injury. Models described below can be further adapted to genetically engineered murine strains to study the effects of aging and other co-morbidities on wound healing.

1.1 Excisional Wound Healing

Excisional wound injury models allow for evaluation of the phases of wound healing outlined above. In these models, a full thickness, cutaneous injury results in removal of the murine epidermis, dermis, hypodermis and the smooth muscle layer known as the panniculus carnosus. Depending on the interest of the researchers, the number and size of wounds can be varied using a standard dermal punch biopsy. Briefly, young and aged mice of the desired strains and age ranges are anesthetized and the fur is removed either by shaving with an electric animal clipper, hair removal cream or plucking. The skin is then lightly cleansed with ethanol, and the wounds are induced by lifting the murine skin in the dorsal midline and folding it over a hockey puck. Then 2–6 wounds, from 3 to 8 mm in diameter, can be created using dermal punch biopsies, allowing for symmetrical and identical sized wounds on either side of the dorsal midline (Fig. 1). This method also allows

for full excision of the epidermis, dermis, hypodermis, and panniculus carnosus as mentioned above. Mice can then be sacrificed at early (3 h–3 days), mid (5–10 days), or late (14 days onward) time points to evaluate the different phases of wound repair described above [1, 2]. At sacrifice, wounds are removed with a larger punch biopsy than used to injure the animal to allow for consistent removal of intact skin around the wound margin (Fig. 2). During dissection of the pelt of the mouse, it is important to carefully remove the tissue around the wound site to ensure removal of the entire wound matrix. This requires special attention during the early phases of wound repair when the granulation tissue is easily disrupted. After removal of the tissue, the wounds can be analyzed as detailed later in the chapter.

1.2 Burn Wound Healing

Similar to perturbations in excisional or incisional cutaneous injury with aging, advanced age is also associated with worse outcomes following burn trauma. Though improvements in clinical management over the past few decades have improved the mortality following burn injury in elderly patients over 65, clinical outcomes are still mediocre compared to young counterparts [14, 16]. In addition to direct cutaneous insult, burn injury also results in significant systemic inflammation and subsequent pulmonary complications, such as pneumonia and acute respiratory distress syndrome, which are particularly devastating in the elderly patient [17–19]. Thus, the model described within not only allows for direct comparison of burn wound healing with age, but also allows for evaluation of the systemic complications associated with burn trauma and aging. Briefly, young and aged mice of the age ranges delineated previously are anesthetized and the fur is removed as mentioned above. The mice are weighed and then placed in a burn template (Fig. 3) that represents ~12–15 % of their total body surface area (TBSA). The mice are then immersed in a 90–92 °C water bath to produce a ~12–15 % TBSA full thickness, scald injury (Fig. 4). Using this method, the damage encompasses the epidermis, dermis, hypodermis, and the murine panniculus carnosus. Mice can be sacrificed at similar time points as those used for excisional wound healing to evaluate the wound closure, as well as systemic inflammation and complications associated with burn trauma. It is important to note that in the absence of excisional debridement and grafting, the wound will heal significantly by contracture and fibrosis. At sacrifice, wounds are removed with an 8 mm punch biopsy which encompasses half of the burn wound tissue and half of the uninjured tissue just proximal to the burn site.

2 Materials

2.1 Prototypical Animal Strains in Aging Studies

1. BALB/c: Young 10–16 weeks, Aged 18–22 months [20, 21].
2. C57BL/6: Young 8–14 weeks, Aged 25–32 months [22].
3. CBA: Young 8–14 weeks, Aged 25–27 months [23].

2.2 Excisional Wound Injury and Isolation of Wound Tissue

1. Anesthesia solution: Ketamine (100 mg/mL solution), xylazine (100 mg/mL) and sterile saline. Prior to injury, combine 1 mL of stock ketamine, 0.2 mL of stock

xylazine and 3.8 mL of sterile, 0.9 % normal saline in a sterile 15 mL polypropylene conical tube. Mix by inverting the tube.

2. Sterile, 0.9 % normal saline, warmed to 37 °C.
3. Sterile alcohol prep pads.
4. Electric animal hair clippers with #40 blade.
5. Single-use, sterile, 25 G, 1 mL tuberculin syringes.
6. 3 mm punch biopsy for wounding (Acuderm Inc., Fort Lauderdale, FL).
7. Hockey puck.
8. Scissors and forceps.
9. 5 mm punch biopsy for removal of wounds and adjacent tissue from dorsal skin pelt (Acuderm Inc., Fort Lauderdale, FL).
10. Analytical balance and weigh boats.
11. Single use, straight edge razors (Personna America Safety Razor Co., Verona, VA).
12. Paper towels.
13. Examination gloves.
14. Heating pads.

2.3 Burn Wound Injury and Isolation of Wound Tissue

1. Anesthesia solution: Ketamine (100 mg/mL solution), xylazine (100 mg/mL) and sterile saline. Prior to injury, combine 1 mL of stock ketamine, 0.2 mL of stock xylazine and 3.8 mL of sterile, 0.9 % normal saline in a 15 mL polypropylene conical tube. Mix by inverting the tube.
2. Sterile 0.9 % normal saline, warmed to 37 °C (Hospira Inc., Lake Forest, IL).
3. Electric animal hair clippers (*see* Subheading 2.2).
4. Hot plate.
5. Thermometer.
6. Two Metal pans with lid (1 quart, ~21.5 × 11.5 cm pans, Fisher Scientific, Pittsburgh, PA).
7. Deionized water.
8. Single-use, sterile, 1 mL tuberculin syringes with 25 G needles Burn injury template (Fig. 3).
9. 8 mm punch biopsy for removal of burn tissue and adjacent tissue from dorsal skin pelt (Acuderm Inc., Fort Lauderdale, FL).
10. Analytical balance and weigh boats.

11. Single use straight edge razors (Personna America Safety Razor Co., Verona, VA).
12. Paper towels.
13. Examination gloves.
14. Stop watch or timer.
15. Heating pads.
16. Blunt dissecting scissors.
17. Forceps.

2.4 Subsequent Tissue Analysis: Excisional Wounds and Burn Wounds

Bacterial colonization

1. 5 mL polypropylene, round bottom tubes.
2. Sterile PBS.
3. Rotor-stator homogenizer.
4. Agar plates for desired bacteria (i.e., MSA for *S. aureus* and centrimide for *P. aeruginosa*).
5. Sterile plate spreaders.
6. Incubator.

2.5 Preparation for Measurement of Wound Closure by Pixels

1. Digital Canon EOS SLR Camera.
2. Camera stand.
3. Metric ruler.
4. Hockey puck.
5. Adobe Photoshop Version 7.0 (Adobe Systems Inc., San Jose, CA).

2.6 Preparation for Wound Cell Isolation for Flow Cytometry

1. 24-well tissue culture dishes.
2. Dispase Solution: 5 mL of RPMI 1640 culture media containing 10 % fetal bovine serum (FBS), 2 mM L-glutamine, 1 % penicillin/streptomycin, 3 mL of dispase II at 1 mg/mL (Roche Diagnostics, Indianapolis, IN), 2 mL of gentamicin sulfate at 10 mg/mL.
3. Plate shaker at 4 °C.

2.7 RNA Isolation from Wound Tissue

1. Liquid nitrogen.

2. 1.7 mL Eppendorf tubes.
3. RNA Easy Kit (Qiagen, Valencia, CA).

2.8 Protein Isolation from Wound Tissue

1. Liquid nitrogen.
2. 1.7 mL Eppendorf tubes.
3. 5 mL polypropylene tubes (BD Falcon, Bedford, MA).
4. Acrodisc filter, 1.2 mm, 32 mm (Utech Products, Schenectady, NY).
5. 1 mL sterile tuberculin syringes with 25 G needle.
6. BioRad Lysis Buffer (Lysis Buffer, Factor 1 and Factor 2; BioRad, Hercules, CA).
7. Ice and dry ice.

2.9 OCT Embedding of Wound Tissue

1. Tissue-Tek O.C.T compound (Sakura Finetek, Torrance, CA).
2. Straight edge razor (Personna America Safety Razor Co., Verona, VA).
3. Disposable base molds, 15 mm × 15 mm × 5 mm (Fischer HealthCare, Houston, TX).

2.10 Formalin Fixation of Wound Tissue

1. 10 % formalin.
2. Tissue-Tek Uni-cassette (Sakura Finetek, Torrance, CA).
3. Straight Edge Razor (Personna America Safety Razor Co., Verona, VA).

2.11 Immunofluorescent Staining of Wound Tissue

1. Superfrosted PLUS slides (Fischer Scientific, Pittsburgh, PA).
2. PAP pen (Sigma-Aldrich, St. Louis, MO).
3. 4 % paraformaldehyde (PFA) in sterile PBS, filtered, 37 °C Sterile PBS, filtered.
4. Normal Goat Serum (NGS, Jackson ImmunoResearch, West Grove, PA), or serum that is appropriate given the speciation of the secondary antibody in use.
5. Bovine Serum Albumin (BSA, Sigma-Aldrich, St. Louis, MO).
6. Primary and secondary antibodies of interest.
7. VectaShield Hard Set Mounting Media with DAPI (Vector Labs, Burlingame, CA).
8. Coverslips.

3 Methods

All animal procedures and protocols must be reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) in the investigator's home institution and researchers must be appropriately trained and qualified to carry out the procedures below.

3.1 Excisional Wound Injury and Isolation of Wound Tissue

1. Prepare anesthesia mixture fresh prior to use as described (*see* Subheading 2.2 above) and load into a single use syringe. Weigh the mice. For a 20–25 g mouse, 100 μ L of solution will result in 100 mg/kg ketamine/10 mg/kg xylazine as desired.
2. Grasp the mouse at the nape of the neck and tail. Tilt the head of the mouse downwards. Inject desired volume of anesthetic *intraperitoneally* (*i.p.*) in the left lower quadrant based on the weight of the animals to achieve 100 mg ketamine/10 mg xylazine as desired.
3. Following injection of the anesthesia, inject 1 mL of warmed, sterile saline *i.p.* in the left lower quadrant. This promotes distribution of the anesthetic and prevents dehydration as the full effects of anesthesia can last upwards of 3 h. Return the mouse to a clean cage and allow for the anesthesia to take effect, ~5 min.
4. Once the mouse no longer responds to firm pressure applied to the hind limb, shave the dorsum with animal clippers. Alternate methods of hair removal mentioned above can be used but will not be discussed here.
5. Cleanse the shaved area with an alcohol prep pad. Be sure to cleanse the entire area but do not douse the mouse in ethanol as excessive ethanol can perturb the epidermal barrier. Allow the ethanol to evaporate.
6. Lift the skin in the mid-dorsal line and fold over a hockey puck (Fig. 1a). Using a 3 mm (or size desired) dermal punch biopsy, push punch biopsy tool through the folded skin, such that a wound on each side of the dorsal midline is created (Fig. 1b). Repeat this procedure for the desired number of wounds (Fig. 1c). With 3–5 mm dorsal punch biopsies, we suggest creating 4–6 wounds per mouse. We recommend limiting larger wounds, 6 mm or greater, to two wounds per mouse dorsum.
7. Return the mice to clean cages on heating pads and allow for recovery from anesthesia (~3–4 h). Given that these studies involve the evaluation of the inflammatory stages of wound healing, no analgesics are given as they can interfere with the inflammatory immune process.
8. Sacrifice mice by CO₂ inhalation at desired time point to evaluate the various phases of wound healing: Inflammatory (3 h to 3 days), Proliferative (3–14 days), Remodeling (14 days and onwards). These time delineations will be variable to a certain degree depending on the initial size of the wound.
9. To harvest the wounds, carefully dissect the dorsal pelt from the mouse using scissors and forceps (Fig. 2a, b). Ensure that when removing tissue around the

wound site to include all granulation tissue. This requires patience particularly at early time points when the granulation tissue and wound matrix is still delicate.

10. Place the pelt on a hockey puck (Fig. 2c). Then, use a punch biopsy 2 mm larger than the size used to inflict the wounds to remove the wound tissue and surrounding wound margins (Fig. 2d, e). If evaluating wound size, photograph the pelt at a fixed distance with a metric ruler in the plane of the photo. Process the tissue as described below.

3.2 Burn Wound Injury and Isolation of Wound Tissue

1. Prepare anesthesia mixture prior to use on day of injury as described in Subheading 3.1 and load into single use syringe. Weigh the mice. For a 20–25 g mouse, 100 μ L of solution will result in 100 mg/kg ketamine/10 mg/kg xylazine as desired.
2. Grasp the mouse at the nape of the neck and tail. Tilt the head of the mouse downwards. Inject desired volume of anesthetic *i.p.* in the left lower quadrant based on the weight of the animals to achieve 100 mg ketamine/10 mg xylazine as desired.
3. Prepare the water bath by filling two metal containers with deionized water. Heat one container to 90–92 °C for scald burn injury. Keep the thermometer in the water bath at all times to ensure the temperature remains tightly controlled from mouse to mouse. The second container will be maintained at ambient temperature for sham injury (Fig. 3).
4. Once the mouse no longer responds to firm pressure applied to the hind limb, shave the dorsum with an electric clipper. Be sure to shave over the entire area of anticipated injury, from the nape of the neck to the tail. Shave beyond where the injury itself will be placed, as skin will contract after burn. Alternate methods of hair removal mentioned above can be used but will not be discussed here.
5. Based on the animal's weight, select the appropriate burn template to give a 12–15 % total body surface area (TBSA) burn (Fig. 3). When placing the mouse in the burn template, ensure that the dorsum of the animal is flush with the burn template by gently pushing on the abdomen. Ensure the tail remains out of the template and above the water.
6. Immerse the dorsum, template lined area of the mouse in the water bath for 8 s (Fig. 4). Watch the timer carefully to ensure the time in the water is a constant 8 s for each mouse. We recommend this temperature and timing to ensure a full thickness burn.
7. Immediately remove the mouse and blot the dorsal scald area with a paper towel to prevent continued scald injury.
8. Resuscitate the mouse by injecting 1 mL of warmed, sterile saline *i.p.* in the left lower quadrant.

9. Return the mouse to a clean cage and place the cages on heating pads for 3–4 h, or until the mice are aroused from anesthesia. Given that these studies involve evaluation the inflammatory stages of wound healing, no analgesics are given as they can interfere with the inflammatory immune process.
10. Sacrifice mice by CO₂ inhalation at desired time point to evaluate the various phases of wound healing as delineated above.
11. To harvest the wounds, carefully dissect the dorsal pelt from the mouse. Remove an extra 4 mm of tissue from the margin of the burn wound to include all granulation tissue.
12. Place the pelt on a hockey puck. Use a 5–8 mm punch biopsy to remove the burn tissue at the wound margin, such that half of the punch biopsy contained burn wound and the remaining half contains intact skin from the wound margin.
13. Process the tissue as described below.

3.3 Models of Combined Wound Injury and Infection: Excisional Wound Injury and *Staphylococcus aureus* Infection

1. Follow the steps above under Subheading 3.1 through **step 6**.
2. Immediately after injury, pipette desired CFU/mL in 10 µL directly into each open wound bed. Be sure to change pipette tips between each wound on a given mouse.
 - a. *S. aureus* growth and inoculation: *S. aureus* of the desired strain can be grown overnight in tryptic soy broth (TSB) at 37 °C under constant agitation. The next day, 1 mL of *S. aureus* in TSB is resuspended in 2 mL fresh TSB and incubated at 37 °C for 2 h under constant agitation to ensure mid-logarithmic growth at the time of application to cutaneous wounds. Bacterial concentration (CFU/mL) is then determined by absorbance at 600 nm and the final inoculum confirmed by back-plating on mannitol salt agar (MSA; BD Diagnostics, Sparks, MD).
3. Resume the protocol under Subheading 3.1 at **step 7**. Evaluation of bacterial colonization at the time of sacrifice is described below.

3.4 Burn Wound Injury and Topical *Pseudomonas aeruginosa* Infection

1. Follow the steps above under Subheading 3.2 through **step 8**.
 - a. *P. aeruginosa* growth and inoculation: *P. aeruginosa* of the desired strain can be grown overnight on centrimide agar plates (BD Diagnostics, Sparks, MD) at 37 °C. The next day, inoculate sterile saline with one loop of *P. aeruginosa* from centrimide agar plate. Bacterial concentration (CFU/mL) is then determined by absorbance at 600 nm and the final inoculum confirmed by back-plating on centrimide agar plates (BD Diagnostics, Sparks, MD).

2. After towel drying the mouse to prevent further scald injury, slowly pipette desired bacteria concentration in CFU/mL in 100 μ L directly onto the burn wound surface. (*Note:* A larger volume is used in the burn injury protocol secondary to the larger surface area of injury as compared to the individual wounds in the cutaneous punch biopsy model). Be sure to change pipette tips between each wound on a given mouse.
3. Resume the protocol under Subheading 3.2 at **step 9**. Evaluation of bacterial colonization at the time of sacrifice is described below.

3.5 Subsequent Tissue Analysis: Excisional Wounds and Burn Wounds Bacterial Colonization

1. Prior to euthanizing animals, place 1 mL of sterile PBS in each tube, with one tube per animal. Place tube on ice.
2. After harvesting the wounds from individual animals, place one wound in a 1 mL saline-filled tube. Maintain the tube on ice until all harvesting is complete.
 - a. *Note:* You may choose to weigh each wound as to express your final data as CFU/gm of tissue. If you do not weigh your tissue, you will express your data as CFU/mL or CFU/wound.
3. Using the rotor-stator homogenizer, homogenize the wound in saline. Maintain the tube on ice during homogenization.
4. Directly plate 20 μ L of homogenate onto agar plates in duplicate. Spread with sterile plate spreaders.
5. Next create 1:10 serial dilutions of the homogenate. The number of serial dilutions created will depend on the initial bacterial inoculum and expected growth. We recommend creating 8 serial dilutions of 1:10 for your initial experiments.
6. Plate each serial dilution in duplicate.
7. Incubate plates for 24–48 h depending on the bacterial species utilized.
8. Following incubation, count the colonies on each agar plate. Be sure to multiply the colony count on a given plate by the dilution factor. Average these colony counts to obtain the CFU/20 μ L and then multiply this by a factor of 50 to obtain the CFU/mL (or per wound). If expressing the data as per gram of tissue, multiply by the gram weight of the wound measured (*see Note 1*).

3.6 Measurement of Wound Closure by Digital Photography and Image Analysis

1. Attach the digital camera onto the camera stand at a fixed distance above the height of the hockey puck. We recommend a fixed distance of 20 cm.
2. Remove the dorsal pelt as described above in Subheading 3.2.
3. Place the pelt flat on the hockey puck. Align a metric ruler with the wounds in the frame of the photograph.

4. Photograph the pelt including all wounds in a single image.
5. Using Adobe Photoshop 7.0 (Adobe Systems Inc., San Jose, CA) determine the number of pixels in the open wound area using the magic wand tool, with zoom at 100 % and a tolerance setting of 60.
6. As controls, a separate set of animals should be sacrificed immediately following wound injury and wound size determined to represent day 0.
7. Each wound area at each time point is then compared with average pixels of day 0 wounds such that: $[(\text{individual wound pixels at day greater than day 0}) / (\text{averaged pixels of day 0 wounds}) \times 100]$ is used to determine the percent open wound area at each time point.
8. The individual wounds of each animal are then averaged to give one value for the open wound area for the animal. Example: If an animal has six wound sites, the % open area of each wound would be calculated as above, and then the wounds from that animal would be calculated as the average of the % open area of all six wounds (sum of % open wound area of six wounds divided by 6). Thus, the average of six wounds is an *N* of one individual animal.

3.7 Wound Cell Isolation for Flow Cytometry

A detailed procedure for isolation and staining of wound cells can be found in our previously published manuscript [24]. The procedure below only details the initial processing for tissue for flow cytometry following wound excision. Details of subsequent processing and staining can be carried out as described [24].

1. Prepare “Dispase Solution.”
2. Remove dorsal pelt and excise wounds from mice at desired time-points following excisional cutaneous injury as described above. Be sure to not use any ethanol in washing of the pelt as ethanol may disrupt the cell membrane and promote lysis. If you wish to wash the pelt, do so in sterile PBS.
3. Collect 2–3 wounds per animal and determine weight of samples in grams.
4. Mince wounds into small pieces (<2 mm × 2 mm) with a straight razor.
5. Place wound pieces into a 24-well culture plate (1 well/animal) filled with 15.4 mL “Dispase Solution”/gram of tissue (*see* Note 2).
6. Incubate plate overnight at 4 °C on a rotating shaker at a gentle setting.
7. Follow remaining step as outlined [24].

3.8 RNA Isolation from Wound Tissue

1. Immediately following removal of the wound tissue, add 1–2 wounds to a 1.7 mL Eppendorf tube and snap freeze in liquid nitrogen.
2. Store at –80 °C until ready to process tissue for RNA.

3. Using the RNA Easy Kit (Qiagen), isolate RNA as per the manufacturer's instructions (see Note 3).

3.9 Protein Isolation from Wound Tissue

1. Prepare Lysis Buffer: 9.9 mL BioRad Lysis Buffer, 40 μ L BioRad Factor I, 20 μ L BioRad Factor 2, and 40 μ L PMSF.
2. Place frozen tissue samples on dry ice.
3. Add 1 mL of Lysis Buffer to each polypropylene tube and keep on wet ice.
4. Using forceps, remove tissue and place in polypropylene tube and homogenize for 30 s until no tissue bits are left. Return to ice bucket.
5. Spin the polypropylene tubes at $300 \times g$ at 4 °C for 5 min.
6. Transfer the supernatant to 1.7 mL Eppendorf tubes.
7. Sonicate samples at a 30 % setting for 10 s.
8. Centrifuge at 4 °C for 10 min at $2655 \times g$ (5000 rpm using an Eppendorf centrifuge 5417 R, Eppendorf, Hamburg, Germany).
9. Remove the supernatant, avoiding the pellet, with a 1 mL single-use syringe.
10. Filter the supernatant through a 25 μ M single use syringe filter into a clean 1.7 mL Eppendorf tube. Maintain tube on ice.
11. Aliquot sample into fresh 1.7 mL Eppendorf tubes.
12. Snap freeze in liquid nitrogen and store at -80 °C until use.

3.10 OCT Embedding of Wound Tissue

1. Place a small amount of OCT compound into the Tissue Tray. Allow to lightly cover the bottom of the cassette.
2. Using the straight razor, cut the outer 1/3 of wound.
3. Place the wound so the straight edge is flush with edge of tissue cassette.
4. Cover the tissue with OCT, avoiding bubbles.
5. Freeze immediately by placing cassette on dry ice.
6. Store at -80 °C until use.

3.11 Formalin Fixation of Wound Tissue

1. Fill a plastic container with 10 % formalin.
2. Using the straight razor, cut the outer 1/3 of the wound.
3. Place the wound in the tissue cassette.
4. Place the tissue cassette in the formalin bath.
5. Store at room temperature until use.

3.12 Immunofluorescent Staining of Wound Tissue

1. Remove stored frozen tissue in OCT.
2. Section wound tissue at 3–8 μm onto Superfrost Plus slides. We recommend sectioning tissue at 5 μm .
3. Place sections in a humidified chamber at room temperature for 2 h.
4. Encircle sections with PAP pen.
5. Fix tissue sections in 4 % PFA (filtered, 37 °C) for 15 min. Tap off PFA.
6. Wash with sterile PBS three times for 5 min, tapping off PBS between washes (*see* Note 4).
7. Block with 10 % BSA and 3 % Normal Goat Serum (or normal serum of the species of the secondary antibody) in sterile PBS for 1 h at room temperature.
8. Tip slides to remove blocking solution. Do not wash the tissue sections after removal of the blocking solution.
9. Gently add ~100 μL of the primary antibody diluted in PBS to each tissue section. Note it will be important to titrate the primary antibody to an appropriate concentration (*see* Note 5). Incubate the slide in the primary antibody overnight at 4 °C. Be sure to include appropriate positive and negative controls.
10. Tap off the primary antibody. Wash the tissue sections three times with sterile PBS for 5 min each, tapping off PBS between each wash.
11. Incubate with secondary antibody diluted in PBS (titrated to the desired concentration) for 1 h at room temperature (*see* Note 6). Protect the slides from direct light.
12. Tap off the secondary antibody. Wash the slides three times for 5 min each in sterile PBS, tapping off PBS between washes.
13. Let slides air dry and then place 2–4 drops of VectaShield Mounting Media with DAPI nuclear stain on the slide and cover slip. Avoid air bubbles. Seal cover slip to slide with clear nail polish. Once completely dried, store samples at 4 °C.
14. Visualize tissue with fluorescent microscope of choice.

4 Notes

1. This calculation can be confusing without additional explanation. The wound is homogenized in 1 mL of sterile saline. 20 μL of this solution is then plated following 1:10 serial dilutions. As an example, say 40 colonies grew on the 10^3 serial dilution—thus, 40×1000 would be 4×10^4 CFU/20 μL . Multiplying this value by 50 will transform this in CFU/mL or 2×10^6 CFU/mL.
2. Again this calculation can be confusing without further explanation. The average 3 mm wound with a 5 mm excision perimeter weight from 0.015 to 0.025 g per wound. As an example, assume all wounds weigh 0.02 g and multiple this value

by the number of wounds that are used per isolation, for example three wounds, and this yields 0.06 g of tissue. Extending the calculation, if using the concentrations given above, then 15.4 mL of the dispase solution per gram of tissue is 15.4×0.06 results in 924 μ L.

3. We recommend using two wounds for RNA analysis to ensure enough RNA is isolated for subsequent PCR analysis. We also recommend homogenizing the tissue with a rotor-stator homogenizer, taking care to rinse the homogenizer between each sample with ethanol.
4. We recommend performing all the washes with PBS with the slides horizontal position, such that you gently pipette the PBS on and gently tap it off, rather than in a vertical position as you would if they were to wash the slides in Coplin jars. We found that this results in better retention of tissue on the Superfrosted plus slides.
5. Antibody titration will be dependent of the antibody and manufacturer. However, in our experience, the recommended concentration by the manufacturer tends to result in significant non-specific binding and high background. We recommend starting at half the concentration recommended by the manufacturer and then performing a 1:2 serial dilution.
6. Of note, it is important to have three control slide: (a) tissue that was blocked but received no antibody, (b) tissue that was blocked and stained with the primary antibody only, and (c) tissue that was blocked and stained with the secondary antibody only. The first two serve as negative controls as no fluorescent signal should be detected, the third allows you to determine the specificity of the secondary antibody for the primary antibody and the amount of background staining that is not specific. Significant signal on the third slide suggests: (a) poor blocking by the blocking solution, (b) poor affinity of the secondary antibody for the primary antibody, or (c) too high of a concentration of the secondary antibody.

Acknowledgements

The authors thank Jessica Palmer for her critical review and discussion of this manuscript. This work was supported by NIH R21 AI073987 (E.J.K.), R01 AG018859 (E.J.K.), T32 AG031780 (P.L.W.), T32 GM008750 (R.L.G.) and by the Dr. Ralph and Marian C. Falk Medical Research Trust (E.J.K.).

References

1. Singer AJ, Clark RA (1999) Cutaneous wound healing. *N Engl J Med* 341:738–746 [PubMed: 10471461]
2. Reinke JM, Sorg H (2012) Wound repair and regeneration. *Eur Surg Res* 49:35–43 [PubMed: 22797712]
3. Gillitzer R, Goebeler M (2001) Chemokines in cutaneous wound healing. *J Leukoc Biol* 69:513–521 [PubMed: 11310836]
4. Kim MH, Liu W, Borjesson DL et al. (2008) Dynamics of neutrophil infiltration during cutaneous wound healing and infection using fluorescence imaging. *J Invest Dermatol* 128:1812–1820 [PubMed: 18185533]

5. Daley JM, Brancato SK, Thomay AA et al. (2010) The phenotype of murine wound macrophages. *J Leukoc Biol* 87:59–67 [PubMed: 20052800]
6. Nissen NN, Polverini PJ, Koch AE et al. (1998) Vascular endothelial growth factor mediates angiogenic activity during the proliferative phase of wound healing. *Am J Pathol* 152:1445–1552 [PubMed: 9626049]
7. Swift ME, Kleinman HK, DiPietro LA (1999) Impaired wound repair and delayed angiogenesis in aged mice. *Lab Invest* 79:1479–1487 [PubMed: 10616199]
8. Sen CK, Gordillo GM, Roy S et al. (2009) Human skin wounds: a major and snowballing threat to public health and the economy. *Wound Repair Regen* 17:763–771 [PubMed: 19903300]
9. Kaye KS, Anderson DJ, Sloane R et al. (2009) The effect of surgical site infection on older operative patients. *J Am Geriatr Soc* 57:46–54 [PubMed: 19054183]
10. Vowden KR, Vowden P (2009) The prevalence, management and outcome for patients with lower limb ulceration identified in a wound care survey within one English health care district. *J Tissue Viability* 18:13–19 [PubMed: 19097793]
11. Christensen K, Doblhammer G, Rau R et al. (2009) Ageing populations: the challenges ahead. *Lancet* 374:1196–1208 [PubMed: 19801098]
12. Brubaker AL, Palmer JL, Kovacs EJ (2011) Age-related dysregulation of inflammation and innate immunity: lessons learned from rodent models. *Aging Dis* 2:346–360 [PubMed: 22396887]
13. Panda A, Arjona A, Sapey E et al. (2009) Human innate immunosenescence: causes and consequences for immunity in old age. *Trends Immunol* 30:325–333 [PubMed: 19541535]
14. Slater H, Gaisford JC (1981) Burns in older patients. *J Am Geriatr Soc* 29:74–76 [PubMed: 7462545]
15. Linn BS (1980) Age differences in the severity and outcome of burns. *J Am Geriatr Soc* 28: 118–123 [PubMed: 7354204]
16. Clayton MC, Solem LD, Ahrenholz DH (1995) Pulmonary failure in geriatric patients with burns: the need for a diagnosis-related group modifier. *J Burn Care Rehabil* 16: 451–454 [PubMed: 8582927]
17. Ely EW, Wheeler AP, Thompson BT et al. (2002) Recovery rate and prognosis in older persons who develop acute lung injury and the acute respiratory distress syndrome. *Ann Intern Med* 136:25–36 [PubMed: 11777361]
18. Dancey DR, Hayes J, Gomez M et al. (1999) ARDS in patients with thermal injury. *Intensive Care Med* 25:1231–1236 [PubMed: 10654206]
19. Le HQ, Zamboni W, Eriksson E et al. (1986) Burns in patients under 2 and over 70 years of age. *Ann Plast Surg* 17:39–44 [PubMed: 3273076]
20. Nomellini V, Brubaker AL, Mahbub S et al. (2012) Dysregulation of neutrophil CXCR2 and pulmonary endothelial ICAM-1 promotes age-related pulmonary inflammation. *Aging Dis* 3:234–247 [PubMed: 22724082]
21. Birjandi SZ, Ippolito JA, Ramadorai AK et al. (2011) Alterations in marginal zone macrophages and marginal zone B cells in old mice. *J Immunol* 186:3441–3451 [PubMed: 21307289]
22. Zook EC, Krishack PA, Zhang S et al. (2011) Overexpression of Foxn1 attenuates age-associated thymic involution and prevents the expansion of peripheral CD4 memory T cells. *Blood* 118:5723–5731 [PubMed: 21908422]
23. Bhattacharyya TK, Thomas JR (2004) Histomorphologic changes in aging skin: observations in the CBA mouse model. *Arch Facial Plast Surg* 6:21–25 [PubMed: 14732640]
24. Brubaker AL, Schneider DF, Palmer JL, Faunce DS, Kovacs EJ (2011) Wound cell isolation for flow cytometric and functional analysis neutrophils and natural killer T cells as negative regulators of wound healing. *J Immunol Methods* 373:161–166 [PubMed: 21889511]

Fig. 1.

Excisional wound by punch biopsy. Lift the skin in the mid-dorsal line and fold over a hockey puck (**a**). Push the punch biopsy tool through the folded skin, such that the puck is visible on the other side. This will create a wound on each side of the dorsal midline (**b**). Repeat this procedure for the desired number of wounds (**c**)

Fig. 2.

Wound harvesting. Carefully dissect the dorsal pelt from the mouse using scissors and forceps (**a**). Using scissors, cut around the wound site to ensure inclusion of all granulation tissue. Using forceps, provide counter traction while cutting directly on top of the muscular layer to include epidermis, dermis, hypodermis, and panniculus carnosus (**b**). Place the excised specimen on a hockey puck (**c**). Using a punch biopsy 2 mm larger than the size used to inflict the wounds, remove the wound tissue and surrounding wound margins (**d-e**)

Fig. 3. Scald burn apparatus and burn template. The apparatus consists of a metal container for water, heating plate, templates, and paper towels for drying mice after burn injury. The template is selected based on mouse weight in order to achieve a 12–15 % total body surface area burn. The inferior surface of the template has a portion removed that corresponds to the needed size. Hardware cloth is used to reinforce the bottom of the template

Fig. 4. Scald burn administration. Immerse the dorsum, template lined area of the mouse in the water bath for 8 s. Push gently on the abdomen to ensure the dorsum of the animal is flush with the burn template. The tail should remain out of the template and above the water