

Video Article

In Vivo Imaging of Reactive Oxygen Species in a Murine Wound Model

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Abstract

The generation of reactive oxygen species (ROS) is a hallmark of inflammatory processes, but in excess, oxidative stress is widely implicated in various pathologies such as cancer, atherosclerosis and diabetes. We have previously shown that dysfunction of the Nuclear factor (erythroid-derived 2)-like 2 (Nrf2)/ Kelch-like erythroid cell-derived protein 1 (Keap1) signaling pathway leads to extreme ROS imbalance during cutaneous wound healing in diabetes. Since ROS levels are an important indicator of progression of wound healing, specific and accurate quantification techniques are valuable. Several *in vitro* assays to measure ROS in cells and tissues have been described; however, they only provide a single cumulative measurement per sample. More recently, the development of protein-based indicators and imaging modalities have allowed for unique spatiotemporal analyses. L-012 (C₁₃H₈ClN₄NaO₂) is a luminol derivative that can be used for both *in vivo* and *in vitro* chemiluminescent detection of ROS generated by NADPH oxidase. L-012 emits a stronger signal than other fluorescent probes and has been shown to be both sensitive and reliable for detecting ROS. The time lapse applicability of L-012-facilitated imaging provides valuable information about inflammatory processes while reducing the need for sacrifice and overall reducing the number of study animals. Here, we describe a protocol utilizing L-012-facilitated *in vivo* imaging to quantify oxidative stress in a model of excisional wound healing using diabetic mice with locally dysfunctional Nrf2/Keap1.

Video Link

The video component of this article can be found at <https://www.jove.com/video/58450/>

Introduction

Oxygen metabolites generated through inflammatory processes contribute to various signaling cascades as well as destructive alteration of cellular components¹. Utilizing sensitive and specific techniques to measure ROS is critical for studying inflammatory processes and characterizing the effects of oxidative stress. *In vivo* imaging is valuable because of its ability to provide dynamic spatial and temporal data in living tissue. L-012 is a synthetic chemiluminescent probe that is highly sensitive for superoxide anions and produces a higher light intensity than other fluorescent probes in cells, tissues, and whole blood^{1,2,3,4}. It has been successfully employed for *in vivo* imaging in murine models to study several inflammatory diseases, including arthritis and colitis^{5,6}. It has yet to be employed in an established cutaneous wound healing model. Measurement of ROS generated is equally relevant to assess the progression of wound healing under different conditions. The sensitivity and noninvasive nature of this method makes it a promising technique for studying wound healing across murine models.

Nrf2 is a major driver of the antioxidant response and a transcriptional factor with specificity for the antioxidant response element (ARE) common to the promoter regions of several antioxidant enzymes⁸. In the absence of oxidative stress, Nrf2 is sequestered in the cytoplasm by Keap1, which subsequently causes its ubiquitination and degradation. Imbalance of the Nrf2/Keap1 pathway has been implicated in inappropriate redox homeostasis and delayed wound healing in the setting of increased oxidative stress⁹. We have previously shown that suppression of Keap1 stimulates increased Nrf2 activity and promotes rescue of pathologic cutaneous wound healing in diabetic wounds⁹.

Here we describe a protocol that utilizes L-012-assisted bioluminescence imaging to measure ROS levels in an excisional cutaneous wound healing model, which is critical for highlighting the association between ROS and wound healing. This technique demonstrates real-time changes in oxidative burden within wounds and immediate periphery. Furthermore, this method allows for rapid assessment of interventions and mechanisms that affect redox handling. Here we use a model of Keap1 knockdown for the restoration of redox homeostasis to evaluate the applicability of our strategy. Because our technique is non-invasive and wounds are undisturbed, the same animal can be used for further confirmatory analyses on the basis of histology or cell lysates.

Protocol

All methods described here have been approved by the Institutional Animal Care and Use Committee of New York University School of Medicine. All mice are housed behind a barrier and all personnel wear appropriate personal protective equipment.

1. Day 0: Preparation of Murine Model of Excisional Wound Healing

1. Anesthetize diabetic (Lepr^{db/db}) mice, aged 8–12 weeks, with inhalational 2% isoflurane. Confirm that each mouse has been properly anesthetized using the foot pad pinch test. Apply sterile ocular lubricant to each eye to prevent irritation from dryness. Researchers should follow their institutional veterinary staff's guidelines when anesthetizing animals using isoflurane.
2. Weigh the mice and record the body weight of each mouse. Confirm the diabetic status of animals by recording the blood glucose of each animal using a glucometer.
3. Disinfect procedural workspace and anesthesia equipment. Remove dorsal hair of the mice using a hair trimmer, followed by application of hair removal lotion to wipe away excess hair. Use alcohol wipes to clean the exposed skin, twice, and allow to dry.
4. Create two 10 mm full-thickness wounds extending through the panniculus carnosus using sterile 10 mm biopsy punches according to a well-established excisional wound healing technique^{7,8}. Use sterile gloves for all survival surgery. Autoclave all surgical instruments in bags prior to surgery and open only in the surgical field.
5. Splint the wounds open using a 0.5 mm thick silicone sheet with 10 mm circular cutouts and secure the stents in place using interrupted 4-0 silk sutures.
6. Following surgery, remove animals from anesthesia and place on heating pad to facilitate proper recovery. Of note, if animal body temperature decreases during the procedure, the animal may be moved to the heating pad earlier to minimize body heat loss under anesthesia. Monitor the animals until they are awake and mobile.
7. Once fully recovered, return animals to individual cages, containing food and water (ensure some food is on the floor of the cage for post-operative enrichment). Provide shredded paper towels as additional nesting material for 2 weeks. Do not house animals that have undergone surgery with other animals, to prevent adverse interactions and changes to wound healing status.
8. For post-operative pain relief, inject mice subcutaneously with buprenorphine at 0.1 mg/kg of body weight twice a day, starting immediately following the procedure, for 3 days.

2. Day 1: Preparation of *Keap1* siRNA

NOTE: Prepare all treatments inside a biosafety cabinet.

1. Prepare siRNA dilution by combining 37.5 μ L of reduced serum medium with 12.5 μ L of 20 μ M *Keap1* siRNA (si*Keap1*) (250 pmol) in a 1.5 mL microcentrifuge tube on ice.
2. Prepare liposome dilution by combining 25 μ L of reduced serum medium with 25 μ L of liposome mix in a 1.5 mL microcentrifuge tube.
3. Add 50 μ L of siRNA dilution to 50 μ L liposome dilution dropwise (1:1 volume), and gently mix.
4. Incubate for 20 minutes at room temperature.
5. Add 50 μ L of 2% methylcellulose gel in water and mix gently by pipetting up and down.
6. Treat each animal with either a nonsense siNS (control) or si*Keap1* (experimental). Apply the gel to the top of the wound. Wrap the animal's torso with transparent film dressing to keep gel in place, keeping the limbs free to maintain mobility.

3. Day 3: Preparation of L-012 Solution

NOTE: Prepare all reagents in a biosafety cabinet.

1. In a 1.5 mL microcentrifuge tube, prepare L-012 in 1X PBS at a concentration of 0.5 mg/100 mL.
 2. Manually vortex the microcentrifuge tube. The L-012 does not completely dissolve into the PBS, however it should be evenly suspended in the liquid. Of note, do not attempt to dissolve L-012 in water to avoid disturbing physiologic electrolyte balance following injection.
 3. Transfer the solution into a 1 mL syringe using a 27 G needle.
- NOTE: Be sure to protect the L-012 solution from light.

4. Day 3: *In Vivo* Imaging of Diabetic Wounds

1. Anesthetize mice with inhalational 2% isoflurane. Researchers should follow their institutional veterinary staff's guidelines when anesthetizing animals using isoflurane. Confirm that each mouse has been properly anesthetized using the foot pad pinch test. Apply sterile ocular lubricant to each eye to prevent irritation from dryness.
2. Gently remove the transparent film dressing from the mice without disturbing the wounds.
3. Place the mice in the imaging chamber in their respective orders. To maintain proper O₂ levels in the chamber, set the imaging system inflow and the induction chamber O₂ levels to 1.0 L/min.
4. Image the mice for bioluminescence and photograph at baseline before injection of L-012 compound.
5. Wipe the abdomen with alcohol wipes and allow to dry. Perform an intraperitoneal injection of the L-012 solution at 5 mg per 200 g body weight using a 27-gauge needle. For example, a mouse weighing 20 g should receive 0.5 mg of L-012.
6. Immediately following the L-012 injection, place the mice back in their respective locations in the imaging chamber. Image the mice over the course of 60 minutes, for 1 minute at 4 minute intervals. Define the 10-mm wound as the region of interest for determining level of ROS.
7. Following surgery, remove animals from anesthesia and place on heating pad to facilitate proper recovery. Monitor the animals until they are awake and mobile.
8. Once fully recovered, return animals to individual cages, maintaining the same post-operative enrichment environment previously described in 1.6. Do not house animals that have undergone surgery with other animals, to prevent adverse interactions and changes to wound healing status.

Representative Results

Three days after creating bilateral wounds according to an established excisional wound model (**Figure 1A**), diabetic mice are positioned in the imaging chamber. An initial photograph and a measure of bioluminescence are taken before injection of L-012 to account for background signal (**Figure 1B**). Following intraperitoneal injection with the L-012 solution, the mice are repositioned in the chamber and bioluminescence is visualized in areas of the wound where ROS is detected (**Figure 1C**). After selecting the correct imaging and analysis settings as outlined in **Figure 2**, proceed with imaging. Bioluminescence is recorded for 1 minute at 5-minute intervals over the course of 60 minutes (**Figure 3**). This demonstrates the bioluminescence saturation of the region of interest over time. In our animal model, optimal imaging time to reach complete L-012 saturation was determined to be 50 min, after which no notable difference in bioluminescence was appreciated. This time will differ depending on the animal model utilized and should be independently determined and optimized for varying wound models depending on size, animal type, treatment, etc.

A region of interest (ROI) for the purpose of quantifying bioluminescence limited to the diabetic wound area is drawn on the overlay image (**Figure 4**). These ROIs are similarly defined for all wounds including before L-012 injection (**Figure 4A**), and after L-012 injection in nonsense siRNA-treated (**Figure 4B**) and *Keap1* siRNA-treated mice (**Figure 4C**). Bioluminescence was calculated by dividing the total counts of light intensity by the area. **Table 1** shows the calculations graphed in **Figure 4D**. The nonsense siRNA-treated diabetic wounds had 45,775 11,649 bioluminescence/cm², while *Keap1* suppression resulted in 19,405 5,939 bioluminescence/cm² (mean SD). A Student's t-test demonstrated a significantly decreased bioluminescence ($p=0.042$) in the *Keap1* siRNA-treated wound as compared to the nonsense siRNA-treated wound, correlating with lower oxidative burden with higher Nrf2 activity (**Figure 4D**). To confirm that the relative levels of ROS visualized by L-012 on siNS and si*Keap1* correlate with other established means of measuring inflammation, we analyzed 10 day wound tissue sections. H&E stains showed reduced cellular infiltration in si*Keap1*-treated wounds in contrast to siNS-treated ones, indicating reduced inflammatory morphology (**Figure 5A**). Immunoreactivity of F4/80, a protein macrophage marker, (red fluorescence) on wound tissue sections revealed reduced macrophages in si*Keap1* treated wounds as compared to siNS-treated wounds (**Figure 5B**). This further demonstrates that the ROS levels visualized by L-012 are accurate and correlate with other established ROS measurement models. Critically, applying this technique did not necessitate sacrificing the animals for tissue sections as is required for H&E and immunofluorescence staining.

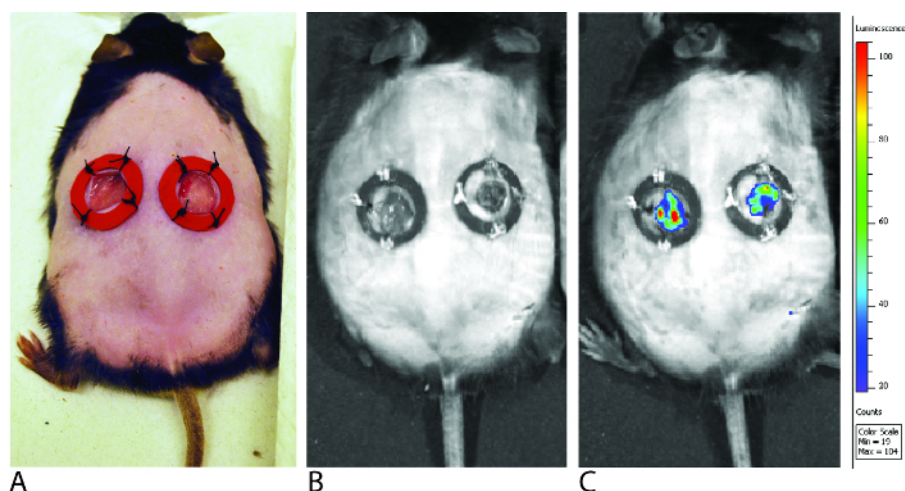
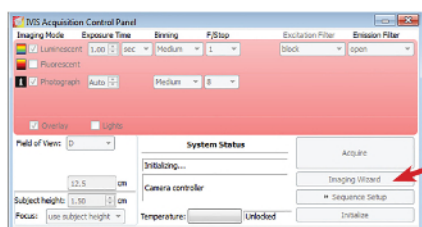
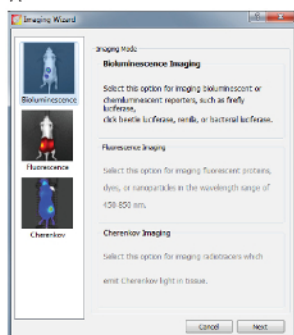


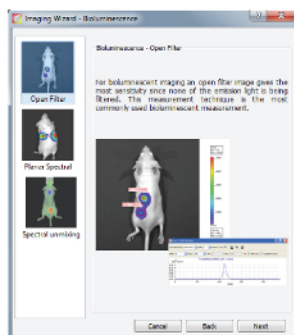
Figure 1: Experimental set-up for imaging. (A) Diabetic mice are wounded using an established excisional wound model. (B) A baseline overlay of photograph with bioluminescence pre-L-012 injection. (C) Following intraperitoneal injection with L-012, ROS is visualized. Luminescence scale for B and C. [Please click here to view a larger version of this figure.](#)



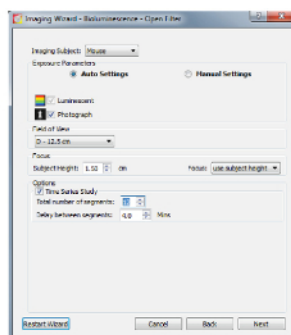
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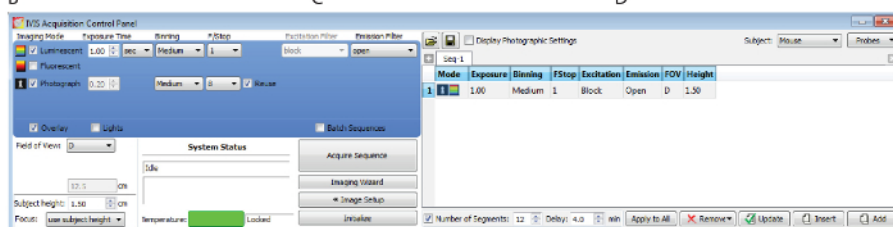
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D



E

Figure 2: Image and analysis settings in *in vitro* imaging system program. (A) Select "Imaging Wizard" on the Acquisition Control Panel (red arrow). **(B)** "Bioluminescence Imaging" is the imaging mode selected. **(C)** "Open filter" is selected as the measurement technique. **(D)** Imaging subject selected is "mouse" and the "time series study" option is selected. The total number of segments and delay time between segments is input. **(E)** Select "Acquire Sequence" to proceed with imaging. [Please click here to view a larger version of this figure.](#)

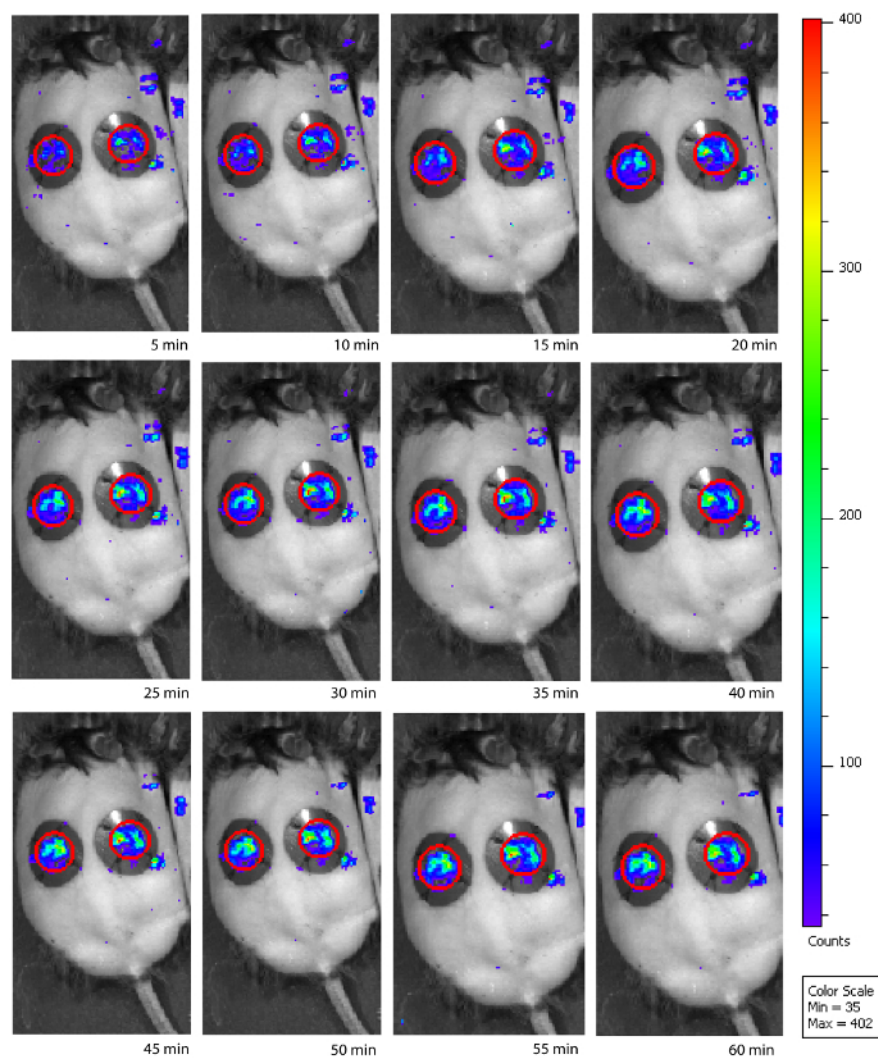
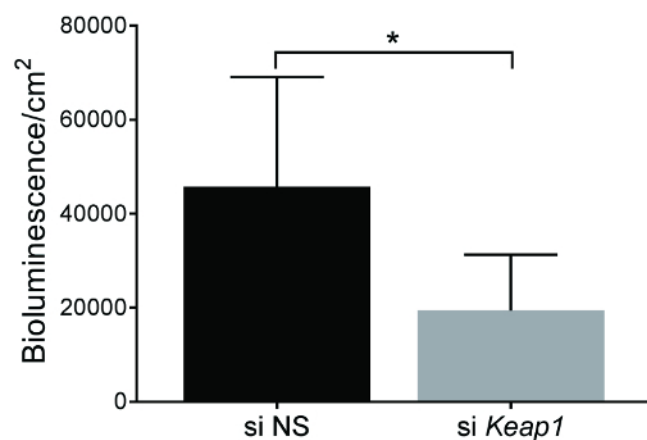
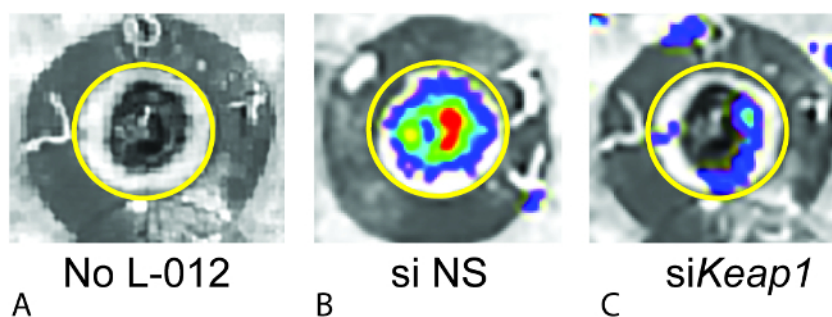


Figure 3: Longitudinal studies of *in vivo* ROS imaging. Diabetic mice are imaged for 1 minute following intraperitoneal injection with L-012 at 5 minute intervals over the course of 60 min. Red circles: ROI. [Please click here to view a larger version of this figure.](#)



D

Figure 4: Quantifying bioluminescence. Photographs with bioluminescence overlay of diabetic wounds (A) prior to L-012 injection, (B) with wounds treated with siNS after L-012 injection, and (C) with wounds treated with siKeap1 after L-012 injection. (D) Quantification of average bioluminescence divided by surface area of the ROIs for siNS and siKeap1 treated wounds. Yellow circles: ROI. Data are represented as mean ± standard deviation; * $p < 0.05$, $n=4$. [Please click here to view a larger version of this figure.](#)

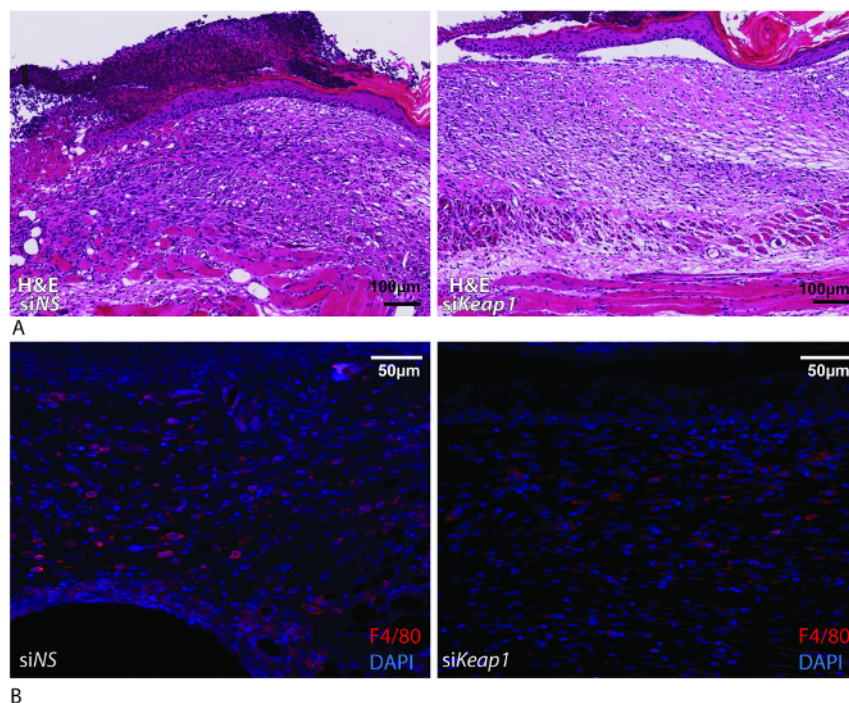


Figure 5: ROS Correlations with immunohistochemical stains. (A) H&E stains of diabetic wound tissue sections at 10 days showed reduced cellular infiltrate as evidence of reduced inflammatory morphology in the siKeap1 treated wounds as compared to the siNS-treated wounds. (B) F4/80 immunofluorescence (red) showed decreased number of macrophages in siKeap1 treated wound sections at 10 days as compared to their siNS-treated counterparts. [Please click here to view a larger version of this figure.](#)

ROI	si	Total Counts	Avg Counts	Stdev Counts	Area [cm ²]	Total counts/area	Average counts/area
ROI 1	NS	9.38E+03	1.14E+02	3.85E+01	2.48E-01	3.78E+04	4.58E+04
ROI 2	NS	4.68E+03	5.37E+01	3.13E+01	2.63E-01	1.78E+04	
ROI 3	NS	1.72E+04	2.18E+02	1.54E+02	2.39E-01	7.20E+04	
ROI 4	NS	1.24E+04	1.68E+02	8.41E+01	2.24E-01	5.55E+04	
ROI 5	Keap1	3.21E+03	3.41E+01	2.76E+01	2.84E-01	1.13E+04	1.94E+04
ROI 6	Keap1	2.56E+03	2.88E+01	1.06E+01	2.69E-01	9.52E+03	
ROI 7	Keap1	4.92E+03	6.48E+01	5.03E+01	2.30E-01	2.14E+04	
ROI 8	Keap1	8.34E+03	1.07E+02	5.25E+01	2.36E-01	3.54E+04	

Table 1: Quantifying bioluminescence for defined ROIs. Bioluminescence is measured for each ROI and standardized relative to surface area. Calculations for mean, standard deviation, and standard error are calculated accordingly. ROI 1, 2, 3, and 4 represent ROI from wounds of biological repeats of siNS treated diabetic wounds and ROI 5, 6, 7, and 8 represent ROIs from biological repeats of siKeap1 treated diabetic wounds.

Discussion

Common techniques for measuring ROS have been limited by complex protocols requiring tissue extraction or similarly invasive techniques. In recent years, measurements of oxidative stress have been reported on the basis of innovative imaging modalities, thereby allowing for spatiotemporal assessments^{9,10,11}. L-012 has several advantages as a chemiluminescent probe relative to luminol, lucigenin, and MCLA^{1,4}. The compound is non-toxic, easily absorbed, and has much stronger bioluminescence as compared to luminol or similar probes¹². Critically, L-012 can be safely administered multiple times in continuum for longitudinal analysis without any adverse effects or harm to animal models⁶. This strategy requires limited special training, and the equipment necessary is readily available in most research laboratories, making it a widely accessible protocol.

For optimal use, we suggest that reagents be properly up to date and protected from light to avoid degradation. Humanizing wounds through stenting allows for murine wounds to heal through migration and re-epithelialization, as opposed to by significant contraction. Sutures should be placed two thirds of the way through the stent perimeter to secure wound edges in place. Wrapping mice with transparent film dressing after gel application ensures that topical treatments remain in place. To prevent self-inflicted wounds in the mice, which may confound results, bitter-tasting spray may be applied to the periphery and suture knots to discourage biting. L-012 has limited solubility in PBS but will form a suspension by repetitive pipetting of the solution. These troubleshooting measures ensure consistency across different wounds, eliminating inter-

user variability while maintaining animal comfort. Since this technique relies on the intraperitoneal injection of a solution into an animal model, there are several interfering factors inherent to the animals which cannot be controlled. For example, local blood flow and subsequent absorption and localization of L-012 to the areas of interest cannot be dictated. However, this variable can be mitigated through the use of an animal as its own internal control such that one wound can be treated with siNS and the other with the siKeap1.

We report on the utility of a strategy for the *in vivo* study of ROS in a cutaneous wound healing model. In our present study, treatment of wounds with Keap1 siRNA resulted in decreased oxidative burden which confirms our understanding of the significance of the Nrf2/Keap1 pathway for antioxidant handling. While this method allows for quantitative analysis, there are a number of important limitations. While L-012 is highly sensitive, it is not specific for ROS and has been shown to also react to reactive nitrogen species⁶. Further analysis with targeted probes and immunoassay-based methods complement this approach through enhanced specificity and subcellular localization of ROS correlates¹³. The proposed mechanism for L-012-based bioluminescence involves the oxidation of L-012 by molecular oxygen through the activity of peroxidase in combination with H₂O₂¹². This limits the use of this technique for studying antioxidant enzyme inhibitors that interact with peroxidase. More detailed analysis intended to specifically quantify ROS other than superoxide anion or reactive nitrogen species metabolites is not possible with the current strategy.

Given the high sensitivity of L-012 for detecting ROS broadly, it is readily adaptable for various cutaneous wound healing and other tissue models. We have demonstrated the expediency of this technique for assessing the redox implications of targeted therapeutics to diabetic wounds such as RTA 408 and the lipoproteoplex delivery of Keap1 siRNA^{14,15}. Given the significant strengths of this protocol, there is immense future potential in applying similar strategies for studying ROS within deeper compartments through *ex vivo* approaches, focused dissections, and organoids.

Disclosures

We have no disclosures to report.

Acknowledgements

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