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

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Cover Letter

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Assistant Professor of Plastic Surgery
Director of Reconstructive Transplantation
Director of Research
Associate Director of Microsurgery

July 6, 2018

Dr. Phillip Steindel
Review Editor
Journal of Visualized Experiments

Dear Dr. Steindel,

Thank you for favorably reviewing our manuscript entitled “In Vivo Imaging of Reactive Oxygen Species in a Murine Wound Model” for publication in JoVE. We have addressed the helpful editorial comments and modified the manuscript accordingly. Please find our responses to the editorial comments enclosed.

Please do not hesitate to contact me about any questions you may have about our manuscript. Again, we are delighted to resubmit our work to the journal, and look forward to hearing your final decision soon.

Best Regards,

Piul Rabbani, PhD.

Daniel Ceradini, M.D.
.

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Title:

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

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Keywords:

In vivo imaging, reactive oxygen species, wound healing, luminol-based chemiluminescent probe, murine wound model, diabetes mellitus

Summary:

We describe a non-invasive *in vivo* imaging protocol that is streamlined and cost-effective, utilizing L-012, a chemiluminescent luminol-analog, to visualize and quantify reactive oxygen species (ROS) generated in a mouse excisional wound model.

Abstract:

The generation of 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 (Keap*1*) 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 ($C_{13}H_8ClN_4NaO_2$) is a luminol derivative that can be used for both *in vivo* and *in vitro* chemiluminescent detection of ROS generated by NAPDH 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.

Introduction:

Oxygen metabolites generated through inflammatory processes contribute to various signaling cascades as well as destructive alteration of cellular components[1]. 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[1-4]. 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 enzymes[8]. 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 stress[9]. We have previously shown that suppression of Keap1 stimulates increased Nrf2 activity and promotes rescue of pathologic cutaneous wound healing in diabetic wounds[9].

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 *Keap*1 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

1.1. 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.

1.2. Weigh the mice and record the body weight of each mouse. Record blood glucose of each animal using a glucometer.

1.3. 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.

1.4. 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.

1.5. 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.

1.6. Following surgery, remove animals from anesthesia and place on heating pad to facilitate proper recovery. Monitor the animals until they are awake and mobile.

1.7. Once fully recovered, return animals to individual cages, containing food and water. 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.

1.8. 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.

2.1. Prepare siRNA dilution by combining 37.5 µL of reduced serum medium with 12.5 µL of 20 µM *Keap1* siRNA (si*Keap*1) (250 pmol) in a 1.5 mL microcentrifuge tube on ice.

2.2. Prepare liposome dilution by combining 25 µL of reduced serum medium with 25 µL of liposome mix in a 1.5 mL microcentrifuge tube.

2.3. Add 50 µL of siRNA dilution to 50 µL liposome dilution dropwise (1:1 volume), and gently mix.

2.4. Incubate for 20 minutes at room temperature.

2.5. Add 50 µL of 2% methylcellulose gel in water and mix gently by pipetting up and down.

2.6. Treat each animal with either a nonsense si*NS* (control) or si*Keap*1 (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.

3.1. In a 1.5 mL microcentrifuge tube, prepare L-012 in 1X PBS at a concentration of 0.5 mg/100 µL.

3.2. 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.

3.3. Transfer the solution into a 1 mL syringe using a 27-gauge needle. **Be sure to protect the L-012 solution from light.**

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

4.1. Anesthetize mice 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.

4.2. Gently remove the transparent film dressing from the mice without disturbing the wounds.

4.3. Place the mice in the imaging chamber in their respective orders. To maintain proper O_2 levels in the chamber, set the imaging system inflow and the induction chamber O_2 levels to 1.0 L/min.

4.4. Image the mice for bioluminescence and photograph at baseline before injection of L-012 compound.

4.5. 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.

4.6. 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.

4.7. Following surgery, remove animals from anesthesia and place on heating pad to facilitate proper recovery. Monitor the animals until they are awake and mobile.

4.8. Once fully recovered, return animals to individual cages, containing food and water. 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.

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 minutes, 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 *Keap*1 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 $\pm$ 11,649 bioluminescence/cm^2, while *Keap*1 suppression resulted in 19,405 $\pm$5,939 bioluminescence/cm^2 (mean $\pm$ 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 si*NS* 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 si*NS*-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*Keap*1 treated wounds as compared to si*NS*-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 and Table Legends:

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.

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.

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 minutes. Red circles = ROI.

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 si*Keap*1 after L-012 injection. (**D**) Quantification of average bioluminescence divided by surface area of the ROIs for siNS and si*Keap*1 treated wounds. Yellow circles = ROI. Data are represented as mean±standard deviation; *$p < 0.05$, n=4.

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 si*Keap1* treated wounds as compared to the si*NS*-treated wounds. (**B**) F4/80 immunofluorescence (red) showed decreased number of macrophages in si*Keap1* treated wound sections at 10 days as compared to their si*NS*-treated counterparts.

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 si*Keap1* 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[9-11]. 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 probes[12]. Critically, L-012 can be safely administered multiple times in continuum for longitudinal analysis without any adverse effects or harm to animal models[6]. 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 si*NS* and the other with the si*Keap1*.

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 species[6]. Further analysis with targeted probes and immunoassay-based methods complement this approach through enhanced specificity and subcellular localization of ROS correlates[13]. 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 H_2O_2 [12]. 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.

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Disclosures:
We have no disclosures to report.

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Figure 1

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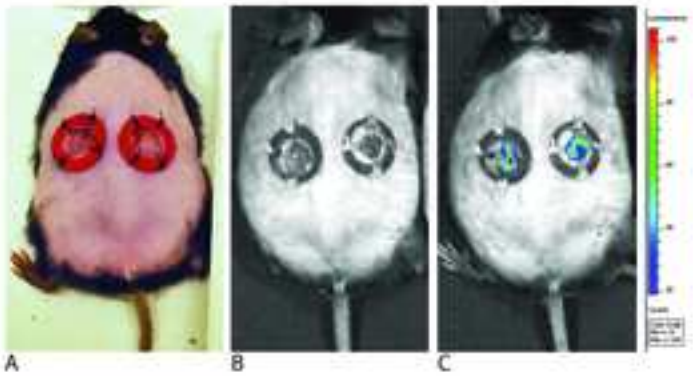

Figure 2

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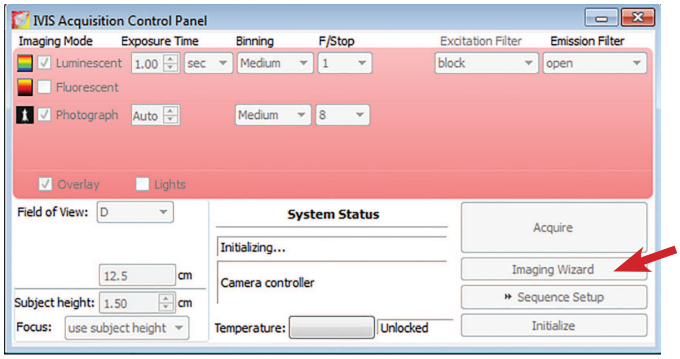

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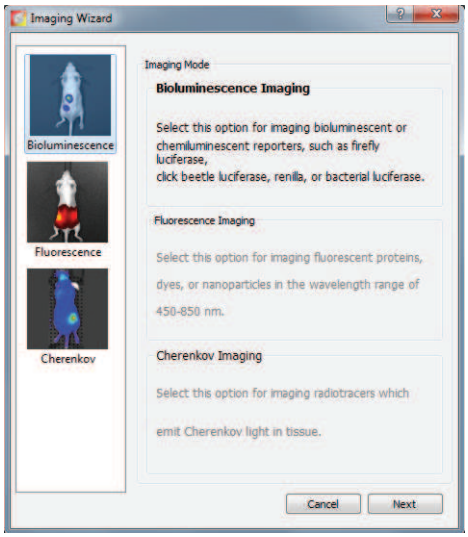

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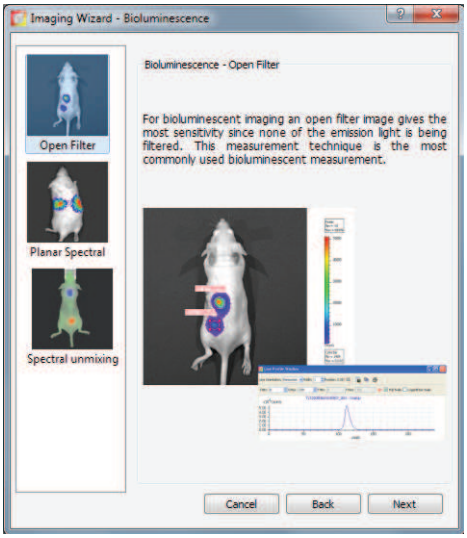

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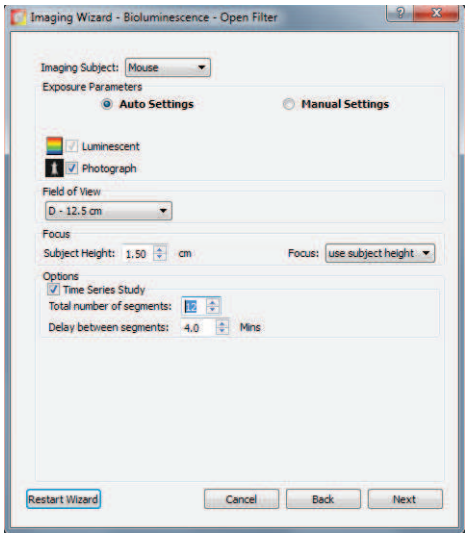

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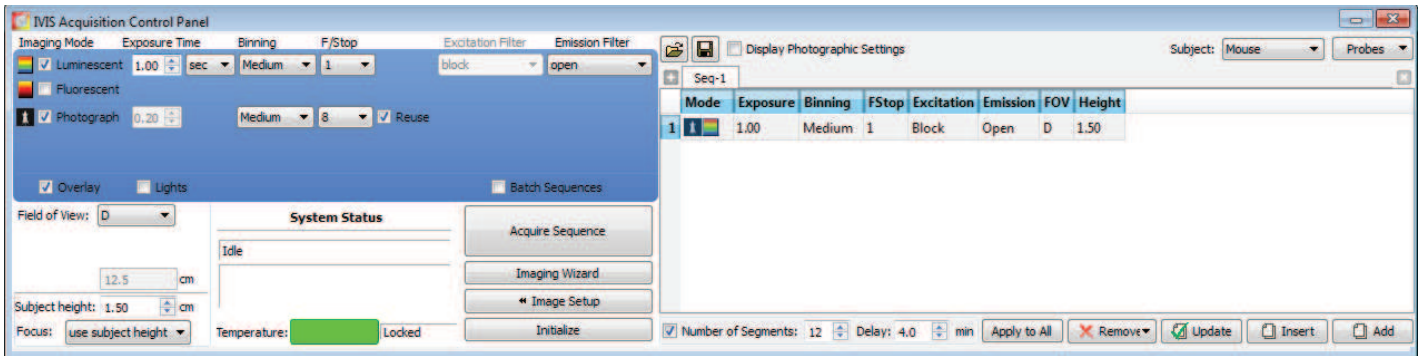

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Figure 3

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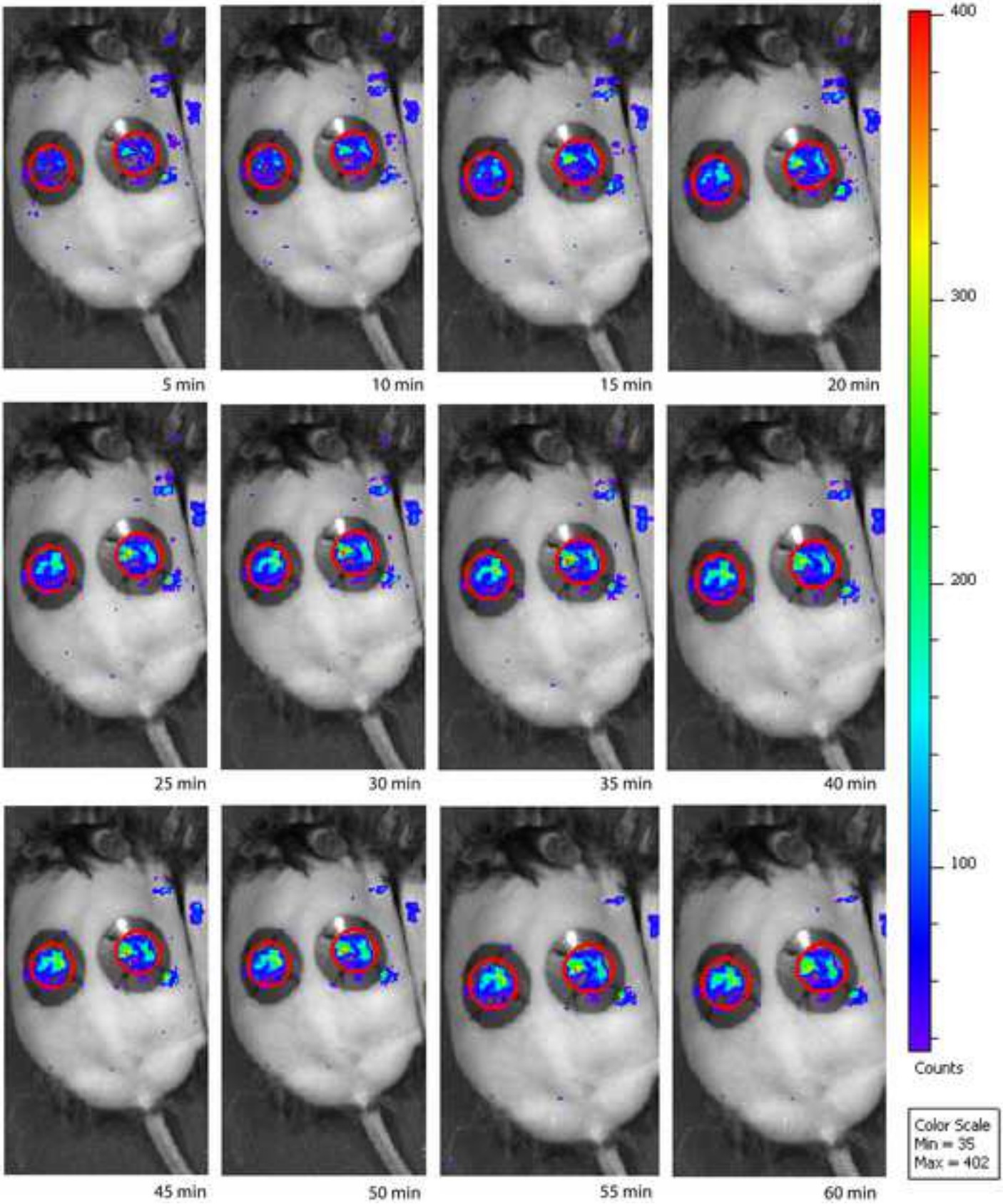

Figure 4

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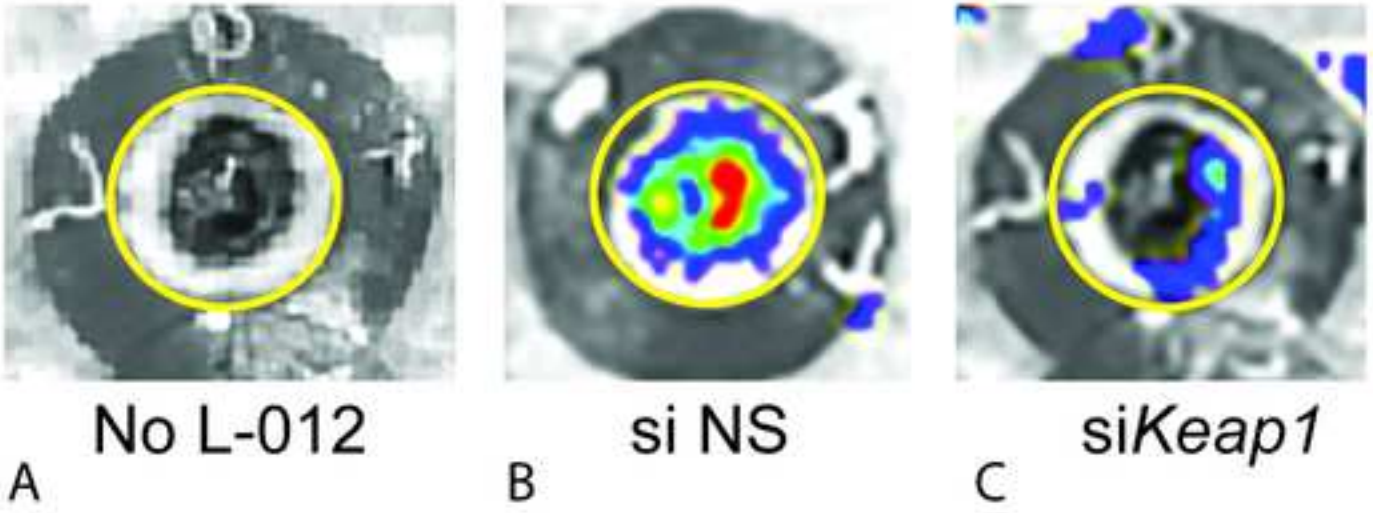

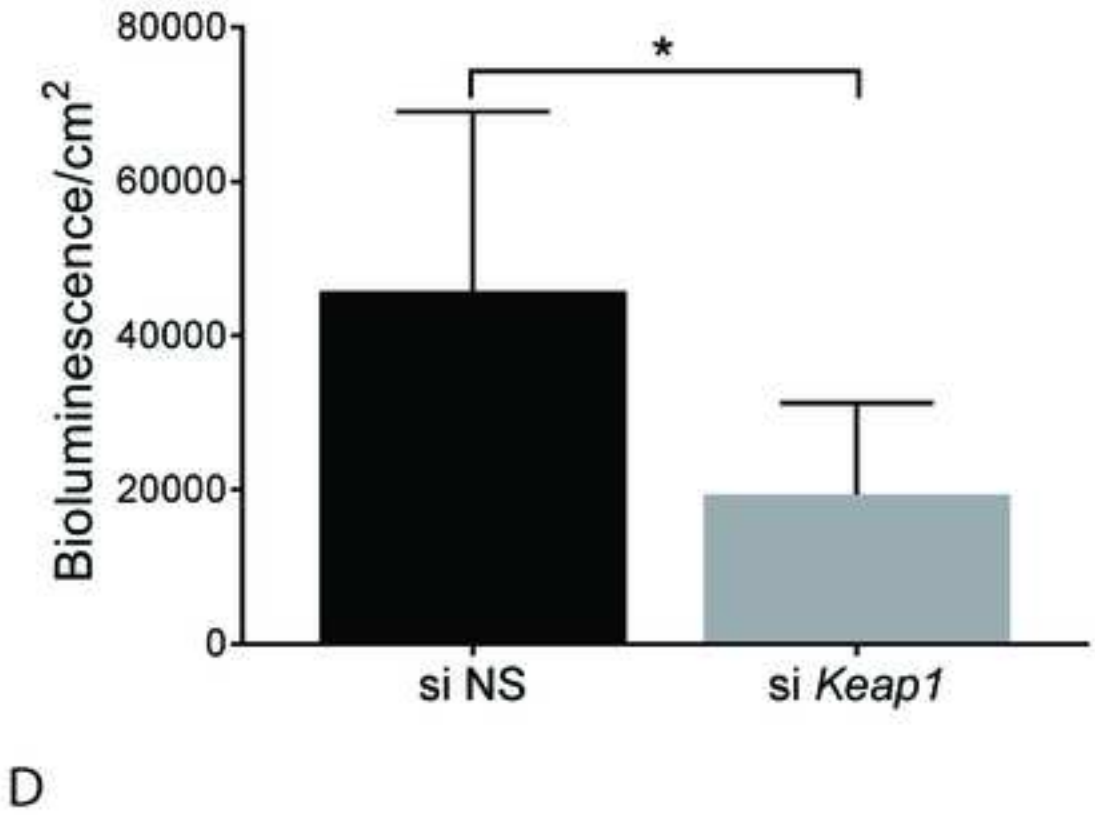

Figure 4

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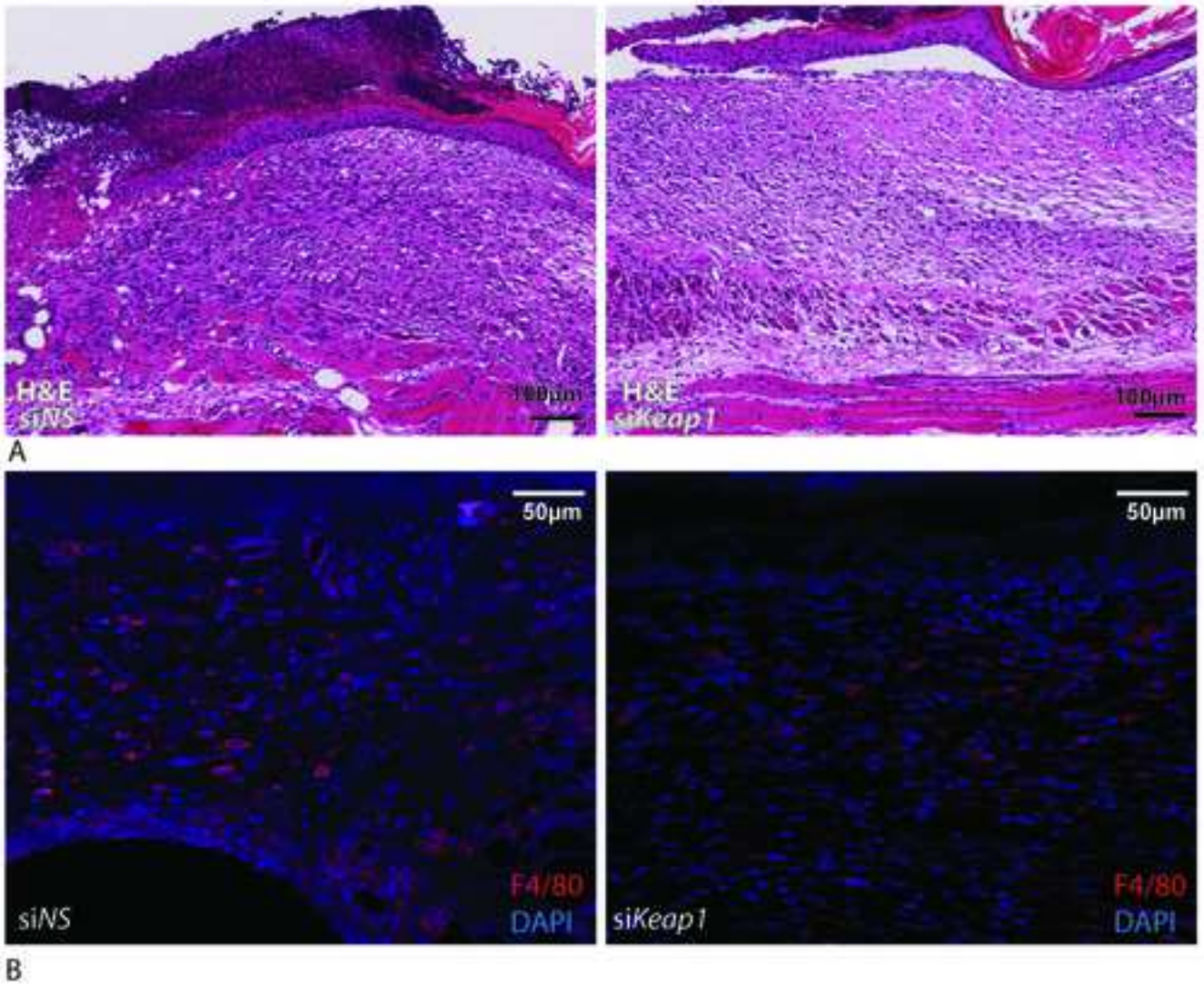

Table

ROI	si	Total Counts	Avg Counts	Stdev Counts	Area [cm²]	Total counts/area
ROI 1	NS	9.38E+03	1.14E+02	3.85E+01	2.48E-01	3.78E+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
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

Average counts/area
4.58E+04
1.94E+04

Materials Table

Click here to download Table of Materials Materials Table.xlsx

Name	Company	Catalog Number	Comments
BKS.Cg-Dock7m+/+ Leprdb/J mice	Jackson Laboratories	000642	
13 cm x 18 cm Silicone sheet (0.6 mm)	Sigma Aldrich	665581	
3M Tegaderm Transparent Film Dressings	3M	88-1626W	
Lipofectamine 2000 Transfection Reagent	Life Technologies	11668027	
Keap1 Stealth siRNA	Thermofisher Scientific	1299001	
Silencer negative control	Thermofisher Scientific	AM4635	
Opti-MEM Reduced Serum	ThermoFisher Scientific	11058021	
DPBS	ThermoFisher Scientific	14040133	
Methyl-cellulose	Sigma Aldrich	9004-67-5	
L-012	Wako Chemicals	120-04891	
IVIS Lumina III XR In Vivo Imaging System	PerkinElmer		

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

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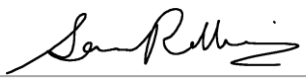
Signature:	 Date: 6/15/2018

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Dr. Phillip Steindel
Review Editor
Journal of Visualized Experiments

July 6, 2018

Dear Dr. Steindel,

Re: JoVE58450 “In Vivo Imaging of Reactive Oxygen Species in a Murine Wound Model”

We would like to thank you for the thorough reading of our manuscript and valuable feedback. Please see below, our itemized point by point response to the editorial comments in bold. We hope that our revisions have satisfactorily addressed your concerns and questions, and we look forward to hearing from you.

Editorial comments:

1. For both steps 1 and 4, we require more specific detail regarding the treatment of animals. Please address the following points, where applicable:

a. Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.

Thank you to the editor for bringing this to our attention. We have added the detail to the manuscript.

b. Please discuss post-anesthesia treatment of animal, including recovery conditions and treatment for post-surgical pain.

Thank you for thorough evaluation of our manuscript. We have now added the pertaining details of post-op and post-anesthesia treatment of the animals to ensure their safety and recovery.

c. Please discuss maintenance of sterile conditions.

Thank you for bringing this detail to our attention. In this revised manuscript, we have added details including the personal protective equipment worn by all personnel and sterile surgical tools.

d. Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.

Thank you for pointing this out. We have revised the manuscript to include steps to monitor anesthetized animals post-op until conscious and mobile, including post-op housing conditions.

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e. Please specify that the animal that has undergone surgery is not returned to the company of other animals until fully recovered.

Thank you for attention to this important point. We do not return animals to the company of other animals after surgery to prevent adverse interactions and changes to wound healing trajectories. The revised manuscript includes these details.

2. Results/Figure 4D: What statistical tests were used?

Thank you to the editor for catching this critical point. We have added that we used Student's t- tests for Figure 4D.

3. Results: It is still somewhat unclear that Table 1 and Figure 4D show different results.

Thank you to the editor for bringing this to our attention. We have now revised Figure 4D to reflect the values in Table 1 to simplify the data presented.

4. Figure 3: You mention in your questionnaire this is reproduced; please indicate as such in the Figure legend, i.e. "This figure has been modified from [citation]." Also, please upload explicit permission (of a letter from the editor or a link to the editorial policy that allows re-prints) to your Editorial Manager account.

Due to the high cost of reproducing our previously published figure, we have created a new Figure 3 with unpublished data.

5. Figure 4D: What is n here?

Thank you to the editor for bringing this to our attention. We have included n=4 in the figure legend.

6. Figure 5: Please explain more how panel A shows 'reduced evidence of inflammatory morphology' and how panel B shows 'decreased macrophages'. Also, please include scale bars in panel B.

Thank you to the reviewer for careful reading of our manuscript. We have re-worded and added more descriptive detail of the figures in Figure 5, both in the text and the figure legend.

Best Regards,

Piul Rabbani, PhD.

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