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Methods for Skin Wounding and Assays for Wound Responses in *C. elegans*

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Abstract

The *C. elegans* epidermis and cuticle form a simple yet sophisticated skin layer that can repair localized damage resulting from wounding. Studies of wound responses and repair in this model have illuminated our understanding of the cytoskeletal and genomic responses to tissue damage. The two most commonly used methods to wound the *C. elegans* adult skin are pricks with microinjection needles, and local laser irradiation. Needle wounding locally disrupts the cuticle, epidermis, and associated extracellular matrix, and may also damage internal tissues. Laser irradiation results in more localized damage. Wounding triggers a succession of readily assayed responses including elevated epidermal Ca^{2+} (seconds-minutes), formation and closure of an actin-containing ring at the wound site (1-2 hr), elevated transcription of antimicrobial peptide genes (2-24 hr), and scar formation. Essentially all wild type adult animals survive wounding, whereas mutants defective in wound repair or other responses show decreased survival. Detailed protocols for needle and laser wounding, and assays for quantitation and visualization of wound responses and repair processes (Ca dynamics, actin dynamics, antimicrobial peptide induction, and survival) are presented.

Video Link

The video component of this article can be found at http://www.jove.com/video/51959/

Introduction

Skin wound healing mechanisms are of basic biological interest and relevant to human health. Wound healing in vertebrates and mammals comprises a complex series of coordinated responses of multiple tissues and signaling[1]. Many simple genetic model organisms are also capable of healing skin wounds[2]. It is therefore of interest to analyze genetically tractable models of skin wound repair. We and others have begun to use *Caenorhabditis elegans* as new model for skin wound healing[3,4]. The goal of this protocol is to enable a broader set of researchers to use *C. elegans* as a tool to investigate molecular and cellular mechanisms of epidermal wound healing.

The *C. elegans* skin comprises the epidermis (also known as hypodermis) and the extracellular cuticle[5]. The adult epidermis is formed from a small number of multinucleate syncytia, of which the largest is the syncytium known as hyp7. The epidermis is a simple epithelium that secretes the cuticle on its apical surface. The skin can actively defend against skin-penetrating pathogens and repair small wounds[4]. Wound repair of the *C. elegans* skin is robust, as almost all wild type animals survive can survive small puncture wounds caused by needles, or local skin damage caused by laser irradiation. *C. elegans* skin wounding triggers a suite of responses, including an epidermal innate immune response, wound closure, and scar formation[4]. The adult epidermis is post-mitotic, and wound healing involves local cellular responses as opposed to epidermal proliferation or cell migration. We have shown that skin wounding triggers a large and sustained increase in epidermal Ca^{2+}, requiring the membrane TRPM channel GTL-2 and internal Ca^{2+} stores[3]. The epidermal Ca^{2+} signal is required for formation and closure of F-actin rings at the wound site. Wounding also induces innate immune responses that activate the transcription of AMPs such as *nlp-29*. The wounding-induced transcription of AMPs is dependent on a TIR-1/PMK-1 p38 MAP kinase cascade acting autonomously in the epidermis[4]. Defects in either the Ca^{2+} signaling pathway or in the innate immune response will lead to lower survival post-wounding. The relatively simple structure of the *C. elegans* epidermis, its genetic tractability and advantages for *in vivo* imaging make it an excellent system to study multiple aspects of wound repair.

Here we present protocols for the two common methods of wounding: needle wounding and laser wounding. Needle wounding requires no specialized equipment (other than the needle puller), and with experience can be performed on hundreds of worms per day. Needle wounding is performed on animals growing on agar plates. In contrast, laser wounding is performed on anesthetized animals mounted on agar pads under a coverslip, and is suited for live imaging of the cellular responses to damage.

Protocol

The following protocols describe the detailed procedure for *C. elegans* skin wounding and for assaying wound responses.

1. Needle Wounding[3,4]

1. Grow healthy unstarved worms on standard NGM (nematode growth medium) plates with *E. coli* OP50 bacteria as food, maintained in a 20 °C incubator.
   NOTE: Methods for NGM agar plates and routine cultivation of *C. elegans* can be found at www.wormbook.org[6].
2. Pick 25 L4 stage worms to a freshly seeded plate 1 day before wounding and culture at 20 °C O/N.
3. Before wounding, pull needles from capillaries using a needle puller. The needles used in wounding are identical to those used for microinjection of *C. elegans*.
4. Ensure that all the worms are at young adult stage. Place the plate of worms on ice for about 30 min. Cooling causes the animals to be sluggish, facilitating needle wounding.
   NOTE: Within 5 min of removal from the ice, the worms will start to move normally. It is possible but challenging to wound moving worms. With experience, 5 min is enough time to wound 25 worms. Immobilize with drugs such as levamisole (as used in laser wounding, see below), although animals will take longer to recover.
5. Remove the agar plate from the ice to a dissecting stereomicroscope. Using a worm pick, move 25 worms into the center of the agar plate.
6. Place the needle into a 100 µl pipette tip to more easily hold the needle. Puncture the anterior or posterior part of the body of the worm with the needle, avoiding the gonad. In general, attempt to wound between the posterior pharynx and anterior gonad, or between the posterior gonad and the rectum. Re-use needles many times, until they break.
   NOTE: Ensure the wounds are brief, gentle pricks that puncture the cuticle and skin, penetrating ~5-10 µm into the animal. The needle should enter the animal at approximately a right angle to the skin. The wounds should not cause obvious damage to internal organs, or immediate rupture of the body. Observe a small amount of cytoplasm oozing out for a few seconds after the wound; however, if cellular contents leak out over a prolonged time the animals will not survive. For optimal imaging, wound the lateral epidermal syncytium (hyp7) or the lateral seam.
   NOTE: This procedure includes the use of a glass needle.
7. Allow worms to recover at RT then culture at 20 °C. Judge the success of needle wounding by a number of assays described below. Greater than 95% of wild type animals survive 24 hr after needle wounding in the young adult stage. The effect of wounding in larvae or older adults has not yet been extensively analyzed.

2. Laser Wounding[3]

Use femtosecond laser irradiation (800 nm) to perform more precise wounding.

1. Prepare agar pads on a glass slide with melted 2% agar[7]. Ensure the pads are similar to the agar pads used for live imaging of *C. elegans*.
2. Transfer 10 young adult worms onto the agarose pad with worm pick, and add a 2 µl drop of 12 mM Levamisole solution. Cover the animals and the liquid with a cover slip. Wait 1-2 min for the worms to paralyze.
3. Place the glass slide onto a spinning disk confocal microscope. Move the stage to find the worms and focus on the lateral anterior or posterior syncytial epidermis with a 100X objective (NA 1.4-1.46).
4. Set the power of the femtosecond laser to 140 mW (measured before the objective). Use the femtosecond laser at a repetition rate of 80 MHz.
   NOTE: Two pulses of 200 msec each (separated by 20 msec) are sufficient for wounding in our hands.
5. Focus on the apical surface of the epidermal cell and wound the epidermis. Observe local disruption of the cytoplasm (bubbling), or local bleaching of any fluorescent marker used.
   NOTE: Follow appropriate laser safety procedures when using the femtosecond laser. Line or point scans using femtosecond lasers such as those in two photon microscopes should provide sufficient power for laser wounding.
   NOTE: While we do not have direct experience of using other lasers, in principle wounding should be possible using conventional UV lasers (as used in *C. elegans* cell ablations). Optionally, use a Fluorescence Recovery After Photobleaching (FRAP) module on spinning disk confocal to wound the epidermis (N. Pujol, personal communication).

3. Visualization of Epidermal Ca^{2+} Responses to Wounding

1. Use transgenic *C. elegans* strains that express Ca^{2+} sensors (such as those of the GCaMP series) in the epidermis, under the control of the adult epidermal specific promoter *col-19* (**Figure 1A**; transgenes are listed in **Table 1**). Use a transgene expressing a red fluorescent protein such as tdTomato as an internal control for transgene expression level.
   NOTE: We have used tdTomato as an internal control for transgene expression as epidermal tdTomato fluorescence is relatively stable and does not interfere with GCaMP imaging.
2. Both needle and laser wounding trigger Ca^{2+} elevation in the epidermis. However, as needle wounding cannot be readily performed on a spinning disk confocal, laser wounding is preferable for quantitative analysis of the Ca^{2+} response (**Figure 1**).
3. Acquire time lapse images in multi-dimensional acquisition mode (interval time 2 sec with 114 msec excitation laser exposure) using a spinning disk confocal with 100X objective (NA 1.4-1.46) and appropriate filter sets (GFP filters will work for GCaMP3).
   NOTE: Acquire time-lapse images every 30 sec for about 2 hr to examine the Ca^{2+} response to wounding over a longer time course.
4. Using image analysis software measure the average GCaMP fluorescence in ten equivalent regions of interest (ROI), five of which are centered on the epidermal cell cytosol and five in the background.
5. Obtain baseline fluorescence (F_0) by averaging fluorescence in 5 ROIs in the epidermis then subtracting the average of 5 ROIs in the background before injury. Express the change in fluorescence ΔF as the ratio of change with respect to the baseline $[(F_t-F_0)/F_0]$.

4. Visualization of F-actin Dynamics After Wounding

1. NOTE: In response to needle wounding, observe an actin ring at the wound site that gradually closes around the wound. This protocol section assays wound closure by measuring the actin ring diameter.

2. Generate transgenic worms that express an F-actin marker such as the Drosophila moesin actin binding domain fused to GFP, expressed under the control of an epidermal specific promoter such as *col-19* (**Figure 2A**) (transgene *juIs352*).
   NOTE: We have obtained similar results using other actin binding domain markers such as Lifeact or F-tractin (unpublished results).
3. Perform needle wounding on P*col-19*-GFP-moesin transgenic worms. After needle wounding, observe a ring of GFP-moesin around the wound site by ~5 min. The actin ring becomes smaller in diameter and eventually closes 2-3 hr post wounding in wild type animals (**Figure 2A**).
4. Prepare a 2% agar pad on a glass slide and transfer 10 worms onto the pad in 2 µl 12 mM Levamisole solution. Image the GFP-moesin rings 1 hr after wounding using conventional confocal microscopy.
   NOTE: Use confocal microscopy to acquire z-stacks (13 x 0.5 µm) of actin rings 1 hr after wounding.
5. Measure the actin ring diameter after wounding. To quantitate epidermal GFP-moesin, first make a maximum intensity projection of the z-stack and then draw 4 line scans (at 45° to each other) over the GFP-moesin ring. Ring diameter is defined as the average peak to peak distance of the four line scans (**Figure 2B**). For rings that have already closed, the diameter is defined as 0 µm.
   NOTE: F-actin rings are conveniently measured 1 hr post wounding as this is approximately half way through the wound closure process in the wild type. F-actin rings can also be measured at multiple time points to derive a time course of wound closure. Time lapse movies of wound closure can be acquired using levamisole-immobilized worms and spinning disk confocal microscopy.

5. Assay Induction of Epidermal Antimicrobial Peptides

NOTE: Wounding also triggers innate immune responses in the *C. elegans* skin. Transcription of genes encoding antimicrobial peptides (AMPs) is induced in the epidermis. The *nlp-29* (neuropeptide-like protein) AMP is strongly induced by wounding[4]. To assay how the epidermis controls AMP expression after wounding, use transgenic reporters or real-time PCR to detect individual AMP transcript levels.

1. Transgenic reporter assay for innate immune response to wounding[4].
2. Perform needle wounding (as in protocol 1) on adult animals expressing the transgenic reporter *frIs7,* which contains P*nlp-29*-GFP*and* *Pcol-12*-DsRed as an internal control. Observe unwounded adult animals as dark red or orange in a GFP long pass filter.
   NOTE: Wounding wild-type worms will induce P*nlp-29*-GFP expression but will not affect P*col-12*-dsRed, resulting in worms that appear orange, yellow, or green under GFP long pass illumination. Loss of function of genes in the p38 MAPK pathway, such as *pmk-1* or *nsy-1* will block *nlp-29*-GFP induction after wounding[4]. P*nlp-29*-GFP is expressed at high levels in larval stages
3. Perform AMP induction assays in young adult animals. Ensure that unwounded control animals have low levels of GFP.
   NOTE:P*nlp-29*-GFP expression is also induced by osmotic stress[8], and may be sensitive to other environmental stresses.
4. 6 hr after wounding, score the numbers of worms that have P*nlp-29*-GFP induction (**Figure 3**)[9] using a fluorescence dissecting scope with GFP long-pass filter.
   NOTE: At 1 hr post-wounding P*nlp-29*-GFP is visibly induced, reaching a peak about 6 hr post-wounding and then remaining elevated up to 24 hr post wounding. Quantify expression by visual scoring[4,9]. Alternatively, quantify expression levels using a fluorescence activated worm sorter[4]. For the worm sorter, at least 50 animals need to be wounded.
5. Real-time PCR assay for quantitating transcript levels of the individual AMP genes. Assay transcript levels for several genes: *nlp-29, nlp-30, cnc-1,* and *cnc-5*.
6. Perform needle wounding on wild-type or mutant worms (see protocol 1)
7. Collect 50 wounded worms and 50 unwounded worms at the desired times (*e.g.* 3, 6, 24 hr) post-wounding.
8. Extract RNA using a commercial kit following the manufacturer's instructions.
9. Perform reverse transcription to get total 20 µl cDNA using a commercial reverse transcriptase kit.
10. For RT-PCR, use 0.2 µl cDNA (1/40) from each sample in combination with a green supermix with fluorescein and 0.2 µM primers. To compare RNA quality, use *ama-1* or *snb-1* RT-PCR as reference; compare the AMP relative expression level by normalizing to internal control (*ama-1* or *snb-1*), or compare fold induction post-wounding (AMP induction in wounded worms/unwounded worms) by normalizing to internal control.
    NOTE: *cnc-1* and *cnc-5* are caenacin family antimicrobial peptides whose transcription is activated by wounding[10]. Typically, needle wounding induces expression (~500 fold for *cnc-1*) over a time course of 4 hr[10]. Primers used in published work are listed in **Table 2**.

6. Assay Survival After Wounding

1. NOTE: Wounding activates at least two independent signaling pathways in the epidermis: the Ca^{2+} dependent wound repair pathway and the innate immune response pathway. Both pathways are required for full survival of sterile wounding. To test whether a mutant or RNAi treatment affects wound healing, measure survival at 24-48 hr post wounding. Optionally, use conventional lifespan assays to determine the effect of wounding on longevity[4,9].
2. Perform needle wounding on young adult (L4 + 24 hr) worms (50 worms, repeat several times) following protocol 1 above. Maintain an equivalent number of unwounded worms as controls.
3. Check survival every 24 hr (transfer wounded worms to a new agar plate every 24 hr). Observe ~50% survival 24 hr after wounding in mutants that are defective in wound repair such as *gtl-2* or *tir-1*, when normalized to unwounded control animals. Death is defined as a lack of locomotor response to touch by worm pick.

Representative Results

Laser or needle wounding will trigger rapid and sustained elevation in epidermal Ca levels, as visualized with Ca sensors such as GCaMPs (**Figure 1**). The Ca elevation occurs within seconds, and remains elevated for tens of minutes. Needle wounding reproducibly results in the formation of F-actin rings at wound sites (**Figure 2**); these appear within minutes, and gradually close over 1-2 hr after wounding. Actin rings are less often formed after more precise laser wounding. Both needle and laser wounding induce expression of epidermal antimicrobial peptides

(AMPs) such as *nlp-29*, detected with transcriptional reporters (**Figure 3**). AMP induction is apparent within 2-4 hr after wounding and lasts approximately 24 hr. Needle wounding of the wild type should not significantly affect viability (measured at 24 hr after wounding) or life span.

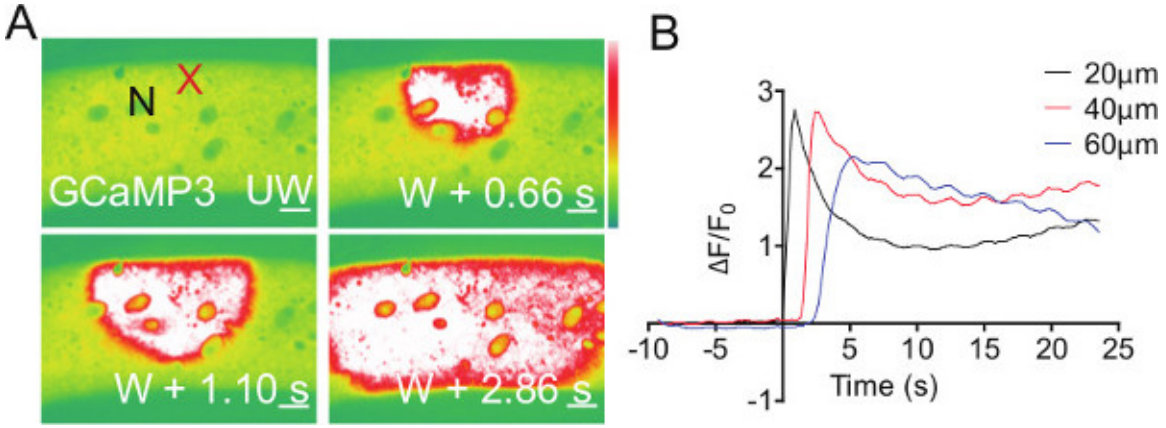

Figure 1. Laser wounding triggers a Ca^{2+} response in the *C. elegans* epidermis. (A) Epidermal GCaMP3 fluorescence (P*col-19*-GCaMP3 *(juIs319)*) levels after femtosecond laser wounding. Lateral view of epidermis in anterior-body; x, laser wound; N, epidermal nucleus. Spinning disk confocal images, intensity code. UW: unwounded, W: wounded. **(B)** Quantitation of GCaMP3 $\Delta F/F_0$ at 20, 40, and 60 µm from wound site; representative trace. Please click here to view a larger version of this figure.

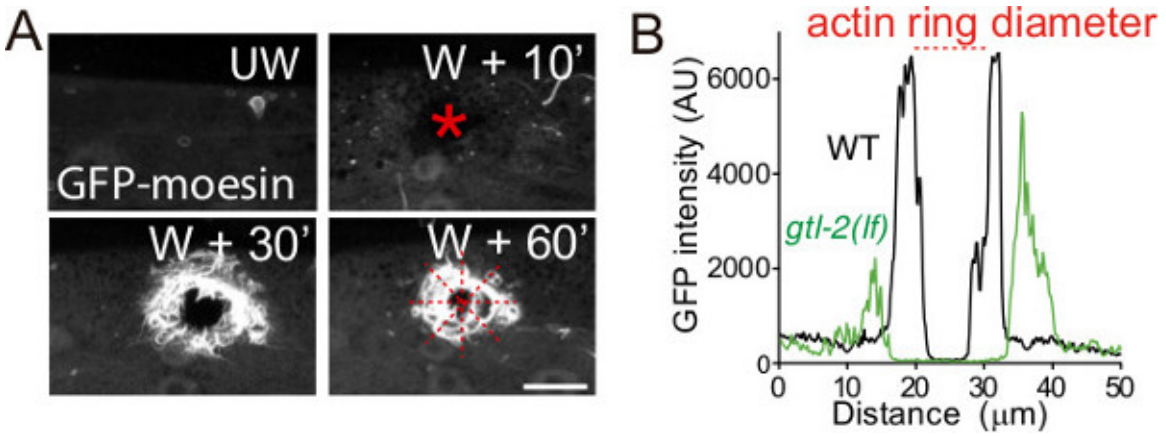

Figure 2. Needle wounding triggers actin polymerization around wound site. (A) Epidermal needle wounds trigger formation of actin rings at the wound margin, visualized with P*col-19*-GFP-moesin *(juIs352)*. Scale: 10 µm. UW: unwounded, W: wounded. **(B)** Quantitation of actin ring diameter from line scans, 4 line scans per animal (dotted red lines in panel **(A)**, WT). Please click here to view a larger version of this figure.

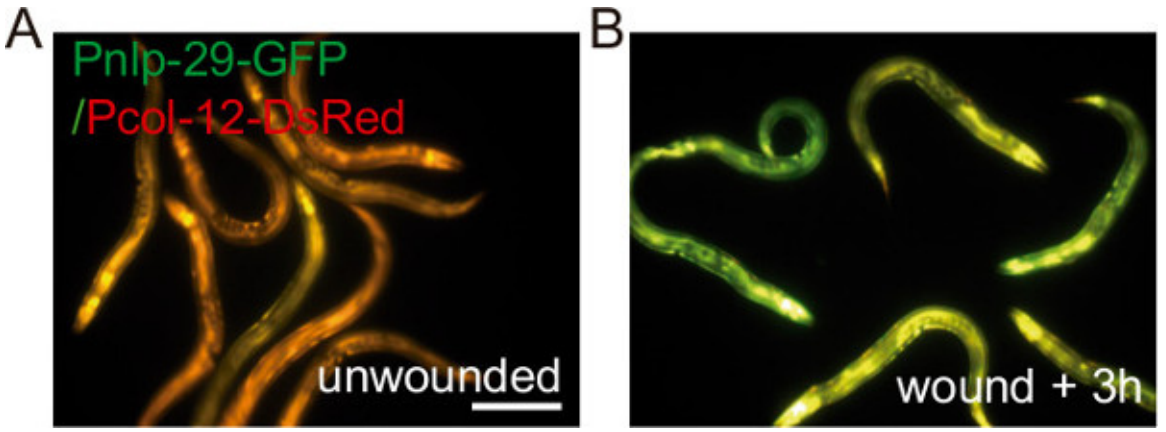

Figure 3. Epidermal wounding activates innate immune response. Representative images (WT unwounded, WT wounded + 3 hr) of P*nlp-29*-GFP*(frls7)* induction after needle wounding. Scale: 200 µm. Please click here to view a larger version of this figure.

Reporter	Transgene	Fluorescence	Allele
Calcium	Pcol-19-GCaMP3;Pcol-19-tdTomato	GFP/tdTomato	juIs319 X
Actin	Pcol-19-GFP::moesin	GFP	juIs352 I
nlp-29	Pnlp-29-GFP/Pcol-12-DsRed	GFP/DsRed	frIs7 IV

Table 1. Transgenes used in wound response assays. This table lists chromosomally integrated transgenes available at the *Caenorhabditis* Genetics Center (http://www.cbs.umn.edu/research/resources/cgc).

Gene name	Sequence
nlp-29	F: cttctcgcctgcttcatggc
R: gtccgtatccaccatatcctcc	
nlp-30	F: TTCTTCTCGCCTGCTTCATGG
R: CATAACCTCTACCATATCCACCG	
cnc-1	F: gccattgtcgccatttcctc
R: cctccatacattggatatcctc	
cnc-5	F: CTTCTTCTAGCAATGCTCGCTC
R: GTATCCTCCACCATACCCTCC	
ama-1	F: ACTCAGATGACACTCAACAC
R: GAATACAGTCAACGACGGAG	
snb-1	F: tccagcagacacaagctcagg
R: gagacaacttctgatcacgctc	

Table 2. Primers used for RT-PCR of AMP transcripts. Working concentration is 0.2 µM. *snb-1* serves as internal control.

Discussion

The methods presented here for needle and laser wounding provide complementary approaches for assessing the ability of the epidermal epithelium to repair damage. Laser wounding is relatively localized and (depending on laser configuration) can be confined to the epidermis, whereas needle wounding disrupts the epidermis, cuticle, and likely internal basement membranes. Needle wounding may more accurately resemble wounds inflicted by pathogens or mechanical damage in the natural environment. As a rule, needle wounding requires more practice to achieve precise wounds that are compatible with animal survival. The cellular responses to needle and laser wounding display many similarities. However it should be noted that formation of actin rings is not reproducibly observed after femtosecond laser wounding.

A limitation of needle puncture is that it is not a precise wound: the needle also damages internal tissues whose contributions to worm survival have not been investigated. A possible modification of this protocol would be to combine microfluidic-based microinjection system[11] and fluorescent reporters to perform more precise physical wounding.

The needle and laser wounding methods detailed here are simple yet fairly labor intensive, and at best medium-throughput, making it challenging to use genomic or biochemical approaches to characterization of the wound response. Cuticle penetrating pathogens such as fungi or protozoa[12,13] can be delivered to larger populations of animals, and may trigger responses that overlap with the response to wounding, yet introduce additional complexities of pathogen specific responses. Further work will be required to explore whether other abiotic methods such as ballistic bombardment[14] or arrays of micromechanical piercing structures[15] can be adapted for large-scale wounding.

Disclosures

The authors are not aware of any competing interests.

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