Application Note

Developmental Biology

Agilent

Trusted Answers

Analyzing Wound Healing in Zebrafish Embryos

Using Agilent BioTek Gen5 to analyze wound healing following tail transection in zebrafish embryos

Authors

Sarah Beckman, PhD
Agilent Technologies, Inc.

Abstract

Proper wound healing is vital for the survival of all higher organisms. Responses to tissue injury can lead to either scar formation or complete tissue regeneration. Understanding repair in regenerative animals can help inform these processes in humans and other species where scar formation is the primary response to injury. This study uses transgenic zebrafish to assess wound healing following tail fin injury. Immunofluorescence was performed on fixed embryos at multiple time points over 3 days to determine changes in regeneration length and immune cell number in embryos following caudal fin transection.

Introduction

The skin is a complex structure which protects the body from environmental challenges including penetration of ultraviolet rays from the sun, invasion of harmful pathogens, and evaporation of water. The skin is constantly exposed to potential damage and injury. Because of this, rapid epithelial wound closure is essential for vertebrate life, as it restricts exposure of the inside of an organism to the noxious outside environments when the inevitable insult does occur. While the significance of wound healing is clear, its frequency might not be. Wounds result not only from trauma but also from a variety of pathological conditions ranging from cancer to infection. Elucidation of the cellular and molecular mechanisms underlying wound healing will ultimately allow better treatments to accelerate this critical process and inform treatments to disease.[1]

Wound healing is a conserved evolutionary process among species and encompasses spatially and temporally overlapping processes including inflammation, blood clotting, cellular proliferation and extracellular matrix (ECM) remodeling. Wound repair requires the integration of multiple cellular signaling networks to efficiently restore tissue homeostasis.[2] Injury and infection lead to the production of chemo-attractants that recruit leukocytes to sites of tissue damage. Immune cells play indispensable roles in tissue regeneration by removing dead cells and debris, preventing invasion of microorganisms, and supporting growth of damaged tissues. Neutrophils are the first immune cells to arrive at the injury site and their main job is to fight off foreign invaders. Macrophages enter later and are thought to participate not only in the removal of dead cells and debris, but also in remodeling and tissue regeneration.[3]

Responses to tissue injury can lead to either scar formation or complete tissue regeneration, which occurs in certain salamanders, amphibians, and fish. Tissue repair in embryos is rapid, efficient, and does not leave a scar. This ability is lost to adults as development proceeds. Understanding repair in regenerative animals can help inform these processes in humans and other species where scar formation is the primary response to injury.[4]

An example of a regenerative animal suited for these types of studies is the zebrafish. Zebrafish embryos are transparent, which makes them well suited to imaging studies. Furthermore, zebrafish have a very similar immune system to humans, and zebrafish leukocytes function like their mammalian counterparts and have been demonstrated to phagocytose bacteria, to home to specific tissues within the developing larvae, and to migrate to sites of injury.[5]

The tail fin fold of a 2- to 4-day-old zebrafish larvae is a double-layered epithelium consisting of a basal epithelial layer that is attached to a basal lamina, and a suprabasal layer in which cells are connected by adheres and tight junctions.[1] Upon tail fin amputation, the wound region undergoes a lesion-induced contraction caused by actin-purse string formation. Shortly after, the epithelium cells cover the lesion to seal the stump surface. From 12 hours post amputation on, the wound begins to regrow, and the blastemal-like cells emerge under the well packed epithelium layer, signifying the regeneration of the injured tail fin. The full recovery of the wound normally requires 3 to 5 days and recapitulates the process of tail fin regeneration in adult fish.[6]

This study uses zebrafish, in combination with Agilent BioTek Gen5 microplate reader and imager software to analyze wound healing. Wounds were created at the tip of zebrafish embryo tails and perform immune staining for neutrophils. After imaging the embryos at various stages over 3 days post wounding, the masking function of Gen5 was used to determine regeneration length and immune cell number.

Materials and methods

Zebrafish maintenance

Zebrafish were maintained essentially as described in Westerfield.[7] Adult zebrafish, both male and female, were mixed and maintained at 28 °C with a 14/10 hour light/dark cycle. In order to collect embryos, male and female zebrafish were put into a breeder basket the night before and embryos were collected in the morning. Zebrafish embryos were kept in a 28 °C incubator in E3 media (5 mM NaCl, 0.33 mM $MgSO_4$, 0.33 mM $CaCl_2$, 0.17 mM KCl, and 0.1% methylene blue).

Tail wounding assay

Caudal fin amputation was performed on 3 day post fertilization (dpf) embryos. The caudal fin was amputated with a sterile scalpel, posterior to the notochord and under anesthesia with tricaine in zebrafish water (Figure 1). Embryos treated with nocadazole were incubated for 1 hour prior with 10 µM nocadazole, wounded, and then kept in nocadazole for 1 more hour before fixing the embryos for immunofluorescence.

Whole mount immunofluorescence

Embryos were fixed in 4% paraformaldehyde (PFA) overnight at 4 °C. Next, they were rinsed with PBST (phosphate buffered saline (PBS) with 0.1% Tween 20) and permeabilized overnight in methanol at −20 °C. Following several washes, embryos were blocked in 5% goat serum in PBST for one hour and incubated with rabbit polyclonal anti-mpx antibody (1:100) overnight. After washing with PBST, fluorescently labeled secondary antibody was added (AlexaFlour 588, 1:200) for 4 hours. Following further washes the embryos were mounted in glycerol for imaging.

Imaging

Images were acquired using a 10x objective on an Agilent BioTek Lionheart FX automated microscope configured with a GFP light cube. In each well, a beacon was placed at the end of the yolk sac (Figure 2). Stacks of images were taken in both the bright field and GFP channels around the beacon. For each embryo, 11 stacks were taken with a height of 12 µm per stack.

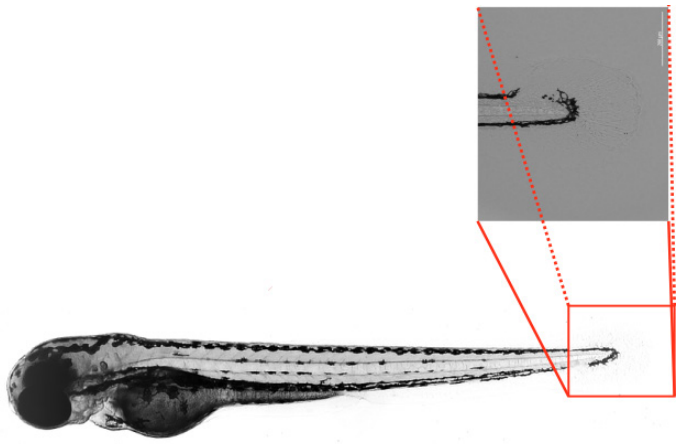

Figure 2. Position of 10x images relative to 3 dpf zebrafish embryo. The beacon is created at the end of the notochord so that the resulting image encompasses the wound area.

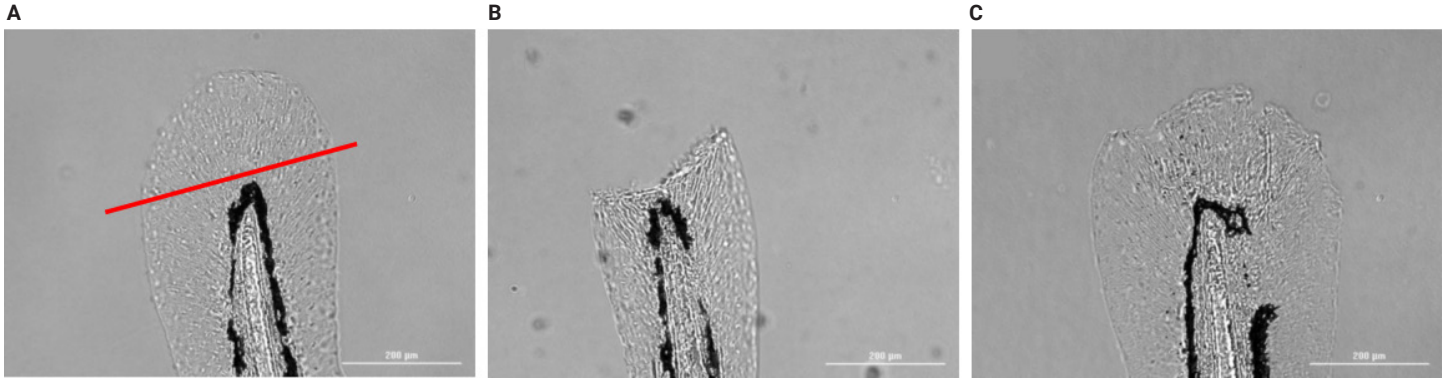

Figure 1. Procedure for tail fin resection. (A) A wound is created at the tip of the notochord. (B) Embryos are fixed at various time points for 3 days post wounding. (C) By 3 days post wounding the tail fin has regenerated.

Image analysis

Focus stacking was used to process the 11 image stacks into one focused image. All of the images were used to create the focus stack. Next, automatic background flattening parameters were used to remove background fluorescence from the GFP channel. No processing was needed in the brightfield channel. Object masking thresholds were then set to identify each cell for counting or to create a mask around the tail and notochord. Image preprocessing and analysis settings are presented in detail in Tables 1 to 4.

Table 1. Agilent BioTek Gen5 image analysis software settings for preprocessing. A z-projection creates a focused stack of the images. Image preprocessing removes background from the stacked imaged to facilitate masking of the neutrophils.

Z-Projection	
Image Set	Brightfield/GFP
Methods	Focus stacking
Size of Max Filter	11 px
Top Slice	11
Bottom Slice	1
Image Processing	
Image Set	ZProj[GFP]
Background	Dark
Rolling Bar Diameter	Auto
Priority	Fast speed
Image Smoothing Strength	0

Table 2. Agilent BioTek Gen5 image analysis software settings for total length. The z-projection image in the GFP channel is used to create a mask around the entire tail in the field of view.

Total Length Cellular Analysis: Primary Mask and Count	
Detection Channel:	Zproj[GFP]
Threshold	2,000
Background	Dark
Split Touching Objects	Unchecked
Fill Holes in Masks	Checked
Min Object Size	100 µm
Max Object Size	11,000 µm
Include Primary Edge Objects	Checked
Advanced Detection Options	
Background Flattening	Auto
Image Smoothing Strength	20

Table 3. Agilent BioTek Gen5 image analysis software settings for notochord length. The z-projection image in the GFP channel is used to create a mask around the notochord.

Notochord Length Cellular Analysis: Primary Mask and Count	
Detection Channel	Zproj[GFP]
Threshold	20,000
Background	Dark
Split Touching Objects	Unchecked
Fill Holes in Masks	Checked
Min Object Size	50 µm
Max Object Size	1,000 µm
Include Primary Edge Objects	Checked
Advanced Detection Options	
Background Flattening	Auto
Image Smoothing Strength	20

Table 4. Agilent BioTek Gen5 image analysis software settings for neutrophils. The transformed z-projection image is used to create a mask around the neutrophils.

Neutrophils Cellular Analysis: Primary Mask and Count	
Detection Channel	Ts[Zproj[GFP]]
Threshold	2,000
Background	Dark
Split Touching Objects	Unchecked
Fill Holes in Masks	Unchecked
Min Object Size	2 µm
Max Object Size	15 µm
Include Primary Edge Objects	Unchecked
Advanced Detection Options	
Background Flattening	20 µm
Image Smoothing Strength	1

Statistics

Statistics were performed with GraphPad Prism. A Student's t-test or one way ANOVA analysis was performed with Dunnett's multiple comparison test.

Results and discussion

Zebrafish cauldal tail fins were transected at 3 days post fertilization (dpf) and then images were taken over 3 days of regeneration. The regeneration length (Figure 3) and number of neutrophils in uncut embryos were analyzed right after the initial wound, 1.5 hours post wounding (hpw), and 1, 2, and 3 days post wounding (dpw) (Figure 4).The length of regeneration (Figure 4G) and the number of neutrophils (Figure 4H) were calculated for each time point. Findings show that embryos regenerate almost completely by the third day post wounding, at which point the regeneration length and neutrophil number return to pre-injury levels.

Immune cells are an important part of the regenerative process. Their size and shape can determine how well they function, or do not function. To demonstrate the effect a microtubule destabilizing drug has on neutrophils, embryos were treated with nocodazole for one hour prior to and following wounding, and then analyzed the effect of this treatment on the shape of neutrophils. Findings show that treatment with nocodazole significantly increased the circularity of neutrophils (Figure 5), as has been documented in the literature.[8]

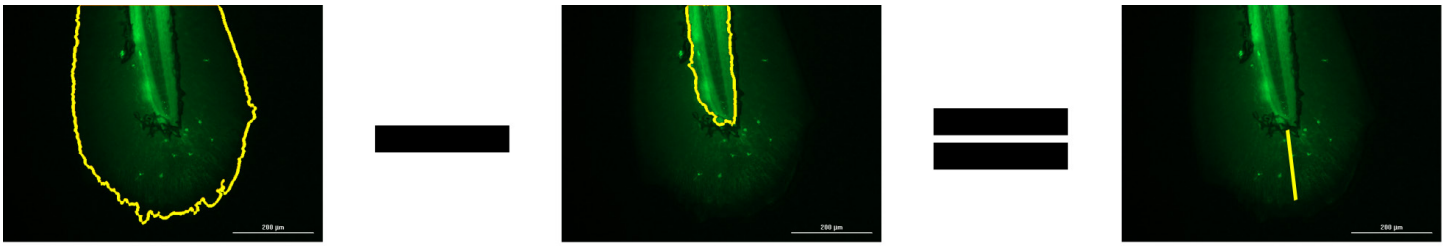

Figure 3. Regeneration length is calculated by subtracting notochord length from total length.

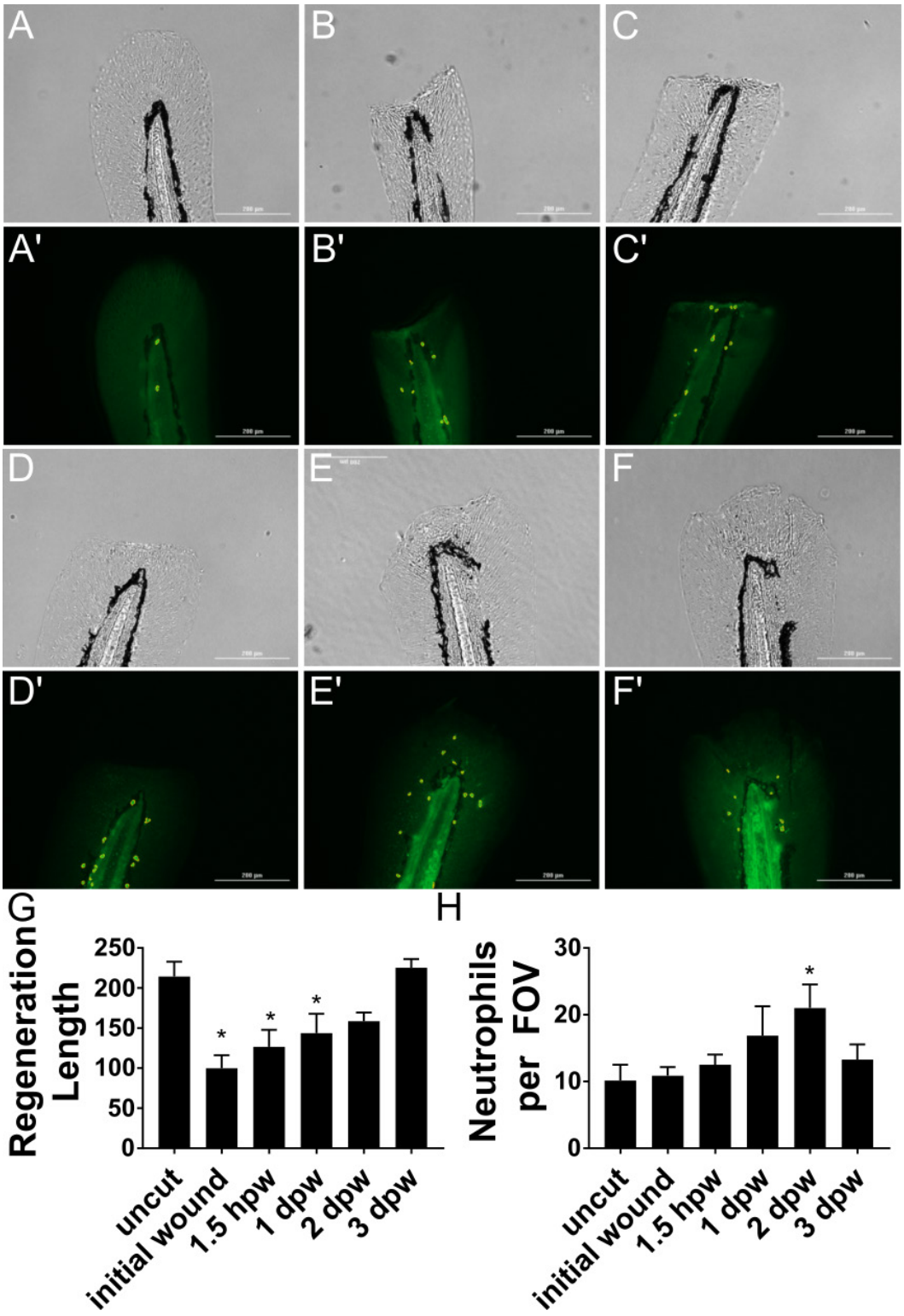

Figure 4. Neutrophil recruitment to caudal tail wound. (A,A') uncut tail (B,B') initial wound at 3 dpf (C,C') 1.5 hours post wounding (hpw) (D,D') 1 day post wounding (dpw) (E,E') 2 dpw (F,F') 3dpw (G) graph of regeneration length (H) graph of neutrophil numbers. (*P <0.05 to uncut); A-F reflect brightfield imaging, A' through F' reflects fluorescence imaging for visualizing immunostained neutrophils.

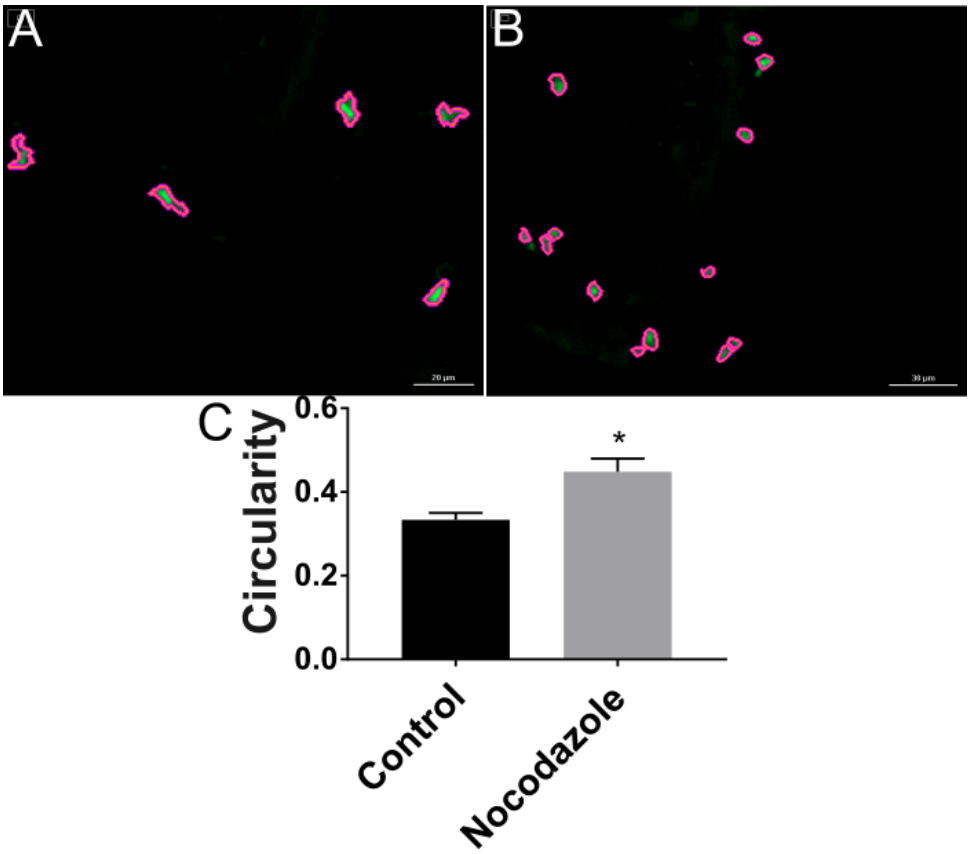

Figure 5. Nocadazole treatment induces rounded neutrophil morphology. (A) Untreated, (B) nocadazole treatment, (C) quantification of neutrophil circularity (*P <0.05).

Conclusion

Wound healing is a conserved evolutionary process critical to the survival of an organism. This study analyzed wound healing after tail fin transection in zebrafish embryos. Specifically, it analyzed regeneration length, neutrophil number, and neutrophil shape after nocodazole treatment. Analysis with Agilent BioTek Gen5 image processing and analysis allows for rapid, consistent, and reliable analysis of these parameters.

References

1. Gault, W. J.; Enyedi, B.; Niethammer, P. Osmotic Surveillance Mediates Rapid Wound Closure Through Nucleotide Release. *J. Cell Biol.* **2014**, *207*, 767–782.
2. Takeo, M.; Lee, W.; Ito, M. Wound Healing and Skin Regeneration. *Cold Spring Harb. Perspect. Med.* **2015**, *5*.
3. Redd, M. J. *et al.* Imaging Macrophage Chemotaxis *In Vivo*: Studies of Microtubule Function in Zebrafish Wound Inflammation. *Cell Motil. Cytoskeleton* **2006**, *63*, 415–422.
4. Redd, M. J. *et al.* Wound Healing and Inflammation: Embryos Reveal the Way to Perfect Repair. *Philosophical Transactions of the Royal Society B: Biological Sciences* **2004**, *359*, 777–784.
5. Hall, C. *et al.* The Zebrafish Lysozyme C Promoter Drives Myeloid-Specific Expression in Transgenic Fish. *BMC Dev. Biol.* **2007**, *7*.
6. Li, L. *et al.* Live Imaging Reveals Differing Roles of Macrophages and Neutrophils During Zebrafish Tail Fin Regeneration. *J. Biol. Chem.* **2012**, *287*, 25353–25360.
7. Westerfield, M. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio), 5th Edition. Univ. Oregon Press. Eugene; 2007.
8. Yoo, S. K. *et al.* The Role of Microtubules in Neutrophil Polarity and Migration in Live Zebrafish. *J. Cell Sci.* **2012**, *125*, 5702–5710.

www.agilent.com/lifesciences/biotek

For Research Use Only. Not for use in diagnostic procedures.

RA44169.1562731481

This information is subject to change without notice.

© Agilent Technologies, Inc. 2006, 2021

Printed in the USA, February 1, 2021
5994-2580EN

Agilent
Trusted Answers