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Quantitative methods for studying hemostasis in zebrafish larvae

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Abstract

Hemostasis is a coordinated system through which blood is prevented from exiting a closed circulatory system. We have taken advantage of the zebrafish, an emerging model for the study of blood coagulation, and describe three techniques for quantitative analysis of primary and secondary hemostasis. Collectively, these three techniques comprise a toolset to aid in our understanding of hemostasis and pathological clotting.

INTRODUCTION

Hemostasis is the process by which blood is prevented from exiting a closed circulatory system, thereby, protecting an organism from exsanguination. Blood coagulation is a critical step in restoring physiologic circulation after vascular injury, which is conserved in vertebrates, from fish to mammals (Jagadeeswaran, Gregory, Day, Cykowski, & Thattaliyath, 2005; Shavit & Ginsburg, 2013). Primary hemostasis initiates with the interaction of platelets and von Willebrand factor at the site of tissue damage along with the binding of collagen to the exposed subendothelial matrix (Shavit & Ginsburg, 2013). This interaction alters platelet morphology and triggers platelet degranulation with release of various agonists, thus, amplifying platelet aggregation. Secondary hemostasis consists of the coagulation cascade, a pathway of serially activating serine proteases and cofactors. This is initiated by tissue factor exposure and release from the subendothelium, leading to the formation of large quantities of active thrombin. Thrombin cleaves fibrinogen to produce the stabilizing fibrin clot.

The zebrafish (*Danio rerio*) is a well-established model for the study of vertebrate physiology. The species exhibits high fecundity, develops rapidly during embryonic and larval periods, and is optically transparent, expediting the study of cardiovascular biology. The zebrafish has also been well established as a model to study human development and disease (Santoriello & Zon, 2012), and shares a high degree of conservation with most human clotting factors (Hanumanthaiah, Day, & Jagadeeswaran, 2002; Howe et al., 2013). These factors make zebrafish an ideal organism for the study of bleeding disorders and pathologic clotting (thrombosis).

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Platelets play an integral role in regulating adhesion and clotting in mammals. Unlike mammals, zebrafish possess nucleated thrombocytes, shown to perform many similar functions as mammalian platelets (Carradice & Lieschke, 2008; Kretz, Weyand, & Shavit, 2015; Weyand & Shavit, 2014). Zebrafish thrombocytes exhibit similar aggregation responses to platelet agonists including ADP, collagen, ristocetin, and arachidonic acid. Additionally, thrombopoietin, a regulator of platelet production, and its receptor Mpl are conserved in zebrafish. The loss of either results in a decreased number of circulating thrombocytes (Kretz et al., 2015; Lin et al., 2005; Weyand & Shavit, 2014). Several studies in zebrafish have led to novel insights into platelet function, demonstrating their relevance as a model organism for studying platelet development and disorders (Khandekar, Kim, & Jagadeeswaran, 2012; Kretz et al., 2015; Lang, Gihir, Gawaz, & Muller, 2010).

During secondary hemostasis in both fish and mammals, the major component of stable clots, insoluble fibrin, is produced by thrombin-mediated cleavage of soluble fibrinogen. Fibrinogen is a homodimer composed of two sets of three polypeptide chains encoded by three syntenic loci, *fga*, *fgb*, and *fgg*, an arrangement conserved from humans to zebrafish (Fish, Vorjohann, Bena, Fort, & Neerman-Arbez, 2012). Deficiency of fibrinogen results in both intracranial and intramuscular hemorrhage, symptoms consistent with human hypo- and afibrinogenemia (Vo, Swaroop, Liu, Norris, & Shavit, 2013). Additionally, targeted disruption of *fga* results in hemorrhage and variable adult lethality (Fish, Di Sanza, & Neerman-Arbez, 2014).

Here, we present three methods for investigation of bleeding disorders and thrombosis in zebrafish larvae. The first assay uses a laser to reliably injure the endothelium of the posterior cardinal vein (PCV) or dorsal aorta (DA) of zebrafish larvae, resulting in thrombus formation. This is facilitated by the optical transparency of zebrafish, enabling quantification of the time to completely occlude blood flow in the injured vessel. This system can be used to study the effect of mutations within the coagulation cascade that result in perturbations to fibrin formation or thrombocyte function. In the second method, we utilize transgenic (*Tg(itga2b:GFP)*, previously *cd41*) zebrafish, in which green fluorescent protein (GFP) is specifically expressed in thrombocytes, to quickly and efficiently quantify the number circulating in larval fish. In the third method, human fibrinogen is chemically labeled with a fluorescent molecule and infused into zebrafish larvae. Under normal flow, the signal is diffused and not visible. However, under certain pathologic conditions that favor fibrin formation, fluorescent deposits can be visualized along the vascular endothelium.

1. METHODS

1.1 LASER-MEDIATED ENDOTHELIAL INJURY

Under normal circumstances, an injury to the vessel wall of zebrafish larvae will result in initiation of the blood clotting cascade, resulting in hemostasis and preventing hemorrhage and/or exsanguination. We simulate traumatic injury to the vessel wall by utilizing laser ablation to expose the subendothelium. This injury results in thrombus formation with complete or partial occlusion of the injured vessel. This technique can be utilized to study the deficiencies of various models of clotting disorders in zebrafish. We provide a modified

description of this technique, which was adapted from the work of Jagadeeswaran, Carrillo, Radhakrishnan, Rajpurohit, and Kim (2011).

1.1.1 Laser and microscope setup—An Olympus IX73 microscope and MicroPoint pulsed laser system (Andor Technology) are used for these experiments. The laser is set to a power of 20 with 99 pulses for venous ablation and a power of 19 with 30 pulses for arterial ablation. This system utilizes a nitrogen laser pumped through coumarin dye, which is attached to an Olympus IX73 inverted microscope fitted with a GFP filter for visualizing fluorescently labeled thrombocyte aggregation. For venous laser injury, embryos are observed using a 20× objective, and a 40× objective is used for arterial laser injury.

1.1.2 Fish preparation

1. Four to seven days prior to laser injury, individual zebrafish breeding pairs are placed in mating tanks and allowed to spawn the following morning. Embryos are collected and kept in system water containing 0.1% methylene blue until the time of experimentation.
2. On the day of experimentation (3 days post fertilization (dpf) for venous laser injury and 6–7 dpf for arterial laser injury) larvae are anesthetized using tricaine (0.16 mg/mL) dissolved in system water.
3. A slide is prepared for larval mounting by drawing an oval on a 24 × 55 mm glass coverslip with a hydrophobic PAP pen and allowed to dry.
4. Five to fifteen larvae are transferred to a 1.5-mL microcentrifuge tube and excess water is removed. A 1.6% solution of low-melt agarose dissolved in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄) is allowed to cool to 37 °C and added to the tube to a volume of 0.5 mL. System water is then added back to the tube to obtain a final volume of 1 mL and final concentration of 0.8% agarose.
5. The larval/agarose mixture is then transferred to the prepared coverslip, and larvae arranged laterally in a vertical line using forceps or a stainless steel probe (Fine Scientific Tools) (Fig. 1).
6. The agarose is allowed to set and the coverslip is transferred to the microscope for analysis.

1.1.3 Venous laser ablation

1. The laser is focused on the dorsal or ventral edge of the PCV endothelial wall within the 5th somite caudal to the anal pore. The laser is set to a power of 20 using the attenuator plates at the laser and MicroPoint bodies, and the endothelium is injured with 99 pulses.
2. Following injury, a developing thrombus can be easily visualized at the site of injury and typically grows to occlude the entire vessel in wild-type embryos (Fig. 2A and B). The time to occlusion (TTO) is determined

beginning at the end of the final laser pulse, and averages 20–30 seconds (s). We find that the most reproducible occlusion occurs at 3 dpf.

1.1.4 Arterial laser ablation

1. *Tg(itga2b:GFP)* larvae are prepared and positioned as described for venous laser ablation, but with the laser focused on the endothelial wall of the DA rather than the PCV.
2. The laser is set to a power of 19 using the attenuator plates at the laser and MicroPoint bodies, and the vessel is ablated for a total of 30 pulses. Fluorescent thrombocytes are first visible at 4 dpf, and the number increases over days 5 and 6 (Huang & Shavit, 2015). Thus, we find that the optimal time for analysis is at 6–7 dpf.
3. After injury thrombocytes will aggregate at the site, eventually occluding the vessel in wild-type embryos (Fig. 2C and D). However, the TTO is typically longer following arterial laser injury, averaging 70–90 s.
4. Additional parameters can be collected as desired. In addition to noting the TTO, we can observe the time to first attachment, as well as the number of thrombocytes present in the thrombus at 120 s. Previous studies suggest that these observations relate to the adhesion and aggregation properties of mammalian platelets (Khandekar et al., 2012; Kretz et al., 2015; Lang et al., 2010).

1.1.5 Analysis

1. TTO is calculated similarly for both arterial and venous laser injury, beginning after the last laser pulse hits the endothelial wall of the vessel. Once the laser is finished firing, the observer continuously visualizes blood flow in the vessel of interest under bright-field settings. The time at which blood flow completely stops is noted and recorded. We have found that if an occlusive clot has not formed within 120 s, it is highly unlikely that one will occur. Therefore, observations are limited to this period. Those that do not fully occlude are noted as “did not occlude” and assigned a value of 120 s.
2. When comparing wild-type larvae to those in which hemostasis has been perturbed, these experiments are always performed by an observer who is blinded to the experimental variables to prevent the introduction of unconscious bias to the results. For the study of genetic mutants, after phenotypic data have been gathered, larvae are removed from the agarose mounting media using forceps and placed in PCR tubes containing 25–50 μ L of lysis buffer (10 mM Tris pH 8.0, 2 mM EDTA pH 8.0, 0.2% Triton x-100, 0.1 mg/mL proteinase K) (Liu et al., 2014). Lysates are then analyzed by PCR for genotyping.

3. Restricting the observation period to 120 s necessitates the use of a nonparametric test for statistical significance. We use the Manne–Whitney *U* test to compare controls to experimental conditions.

1.2 THROMBOCYTE QUANTITATION

This simple and effective method utilizes a *Tg(itga2b:GFP)* zebrafish line with specific expression of GFP in thrombocytes. This enables the relative quantitation of circulating thrombocytes through analysis of short videos obtained with a digital camera fitted to a stereomicroscope. Once captured, a simple algorithm using freely available software is utilized to eliminate background and count thrombocytes. The setup and video capture described is a modification to a method we have previously published (Huang & Shavit, 2015), with the added description of an updated analysis algorithm using nonproprietary software.

1.2.1 Camera and microscope setup

1. A Canon 60D digital single lens reflex camera fitted with an AmScope microscope adapter (United Scopes, LLC, Irvine, CA) is mounted on the viewport of a Leica MZ16FA microscope. Magic Lantern (www.magiclantern.fm), a free firmware add-on, is installed to allow users to adjust sensitivity (ISO) and frame-rate. The camera is also fitted with an external remote, reducing background movement caused by user activation of the shutter.
2. The microscope is adjusted to a zoom of 85× magnification through a GFP2 long-pass filter (Leica).
3. The camera is set to video mode, with an ISO of 6400 and frames per second “(FPS) override” enabled.
4. “Desired FPS” is set to 20 with a “shutter range” of 1/20–1/59 through the Magic Lantern menu.
5. The “Movie REC key” is changed from default to HalfShutter to enable video recording control by the remote.

1.2.2 Embryo preparation

1. Seven to eight days prior to data collection, zebrafish pairs are placed in individual mating tanks and allowed to spawn the following morning. Embryos are collected and stored in system water with 0.1% methylene blue until transfer to 1-phenyl-2-thiourea (PTU) solution.
2. Embryos are placed in 0.003% PTU in system water at 8–24 hours post fertilization (hpf) until time of analysis to inhibit pigmentation.
3. Movies are acquired at 6 or 7 dpf. At that time, larvae are anesthetized using tricaine (0.16 mg/mL) in system water.
4. Mounting media is prepared by heating a solution of 0.7% low-melt agarose in system water and allowing it to cool to 37°C. This solution is

then poured into the lid of a petri dish, and anesthetized larvae are transferred to the media.

5. A 100-mm long, 1.5–1.8 mm outer diameter range glass capillary tube (Pyrex) is fitted to an adapter placed into a pipette pump, and up to four larvae are suctioned into the tube, each separated by an air bubble.
6. Agarose is allowed to set and the capillary is mounted on modeling clay in a plastic tray (Fig. 3). This chamber is filled with system water to submerge the capillary and minimize refraction, followed by transfer to the microscope for imaging.
7. The microscope is set to 85× magnification and larvae positioned by rotation of the capillary to acquire a lateral view. At this age and magnification, the image encompasses the anterior tip of a larva, through the yolk sac extension at the opposite end of the field of vision.

1.2.3 Movie capture and analysis

1. Once positioned, movies are captured through the GFP filter for the duration of 60 s using the remote attachment to control the shutter of the digital camera. After capture, videos are transferred to a desktop computer from the internal SD card, and processed as follows.
2. Video Processing: automated counting of thrombocytes is performed as previously described (Huang & Shavit, 2015) with slight modifications. Videos are processed using the freeware program VirtualDub version 1.10.4 (www.virtualdub.org) and freely available Channel Mixer v1.4 filter from Emiliano Ferrari (emiliano.deepabyss.org). Videos are first filtered using the built-in temporal smoother filter with a setting of five and then the channel mixer (Red: 0,0,0,100; Green: 0,200,0,0; Blue: 0,0,100,0) to remove noise and autofluorescence (Fig. 4A and B). Frame-rate is converted to 20 FPS and exported as a grayscale .avi file. Batch processing is performed using built-in batch functionality.
3. Thrombocyte Counting: a custom macro was generated to facilitate batch processing of .avi files in ImageJ (FIJI package, fiji.sc). The first 1000 frames are imported as an image stack. An average frame is generated using the “Z Project” command with the average intensity option. This is subtracted from the stack using “Image calculator” resulting in images containing only motile (ie, circulating) thrombocytes (Fig. 4C), and a simple binary threshold is applied (7–9255). The lower limit of the threshold is manually determined for each independent day of imaging, usually keeping 0.5–1.5% of the brightest pixels. The resulting stack is then processed using “convert to mask” (Fig. 4D). Circulating thrombocyte quantitation is done using the “Analyze Particles” command (size = 12–6000; circularity = 0.40–1.00). The resulting table is exported as a tab-delimited file and the mean and standard deviation of each larva

are calculated using Microsoft Excel or a custom python script. The results for multiple fish under each condition are averaged.

1.3 FLUORESCCEIN ISOTHIOCYANATE-LABELED FIBRINOGEN INFUSION

This technique utilizes fluorescein isothiocyanate (FITC, Thermo Fisher)-labeled human fibrinogen prepared in our laboratory, as a functional readout of clot deposition in zebrafish larvae. Under normal circumstances, the end point of the coagulation cascade is the cleavage of fibrinogen to form the fibrin clot, resulting in cessation of blood loss from damaged vessels. Over time, fibrin is consumed and recycled through the vasculature and no accumulation will be apparent. However, larvae with pathologic states such as disseminated intravascular coagulation (DIC) will accumulate fibrinogen within the vasculature, and this can be visualized by infused fluorescently tagged fibrinogen molecules (Liu et al., 2014).

1.3.1 Fluorescein isothiocyanate-fibrinogen labeling

1. Purified human fibrinogen (Sigma) is labeled with FITC (Thermo Fisher), followed by PD-10 column purification to remove the free fluorescent molecules.
2. A dye-to-protein molar ratio of 1:1 was used and determined per manufacturer's instructions (Liu et al., 2014).

1.3.2 Fish preparation

1. Four days prior to experimentation, zebrafish pairs are placed in individual breeding tanks and allowed to spawn the following morning. Embryos are collected and maintained in system water containing 0.1% methylene blue until time of experimentation.
2. On the day of experimentation (3–4 dpf), larvae are anesthetized using tricaine (0.16 mg/mL) in system water.
3. A slide is prepared and larvae are mounted as described in Section 1.1.2. Alternatively, larvae can be laid in the wells or trenches of an agarose microinjection tray.
4. Larvae are then transferred to a stereomicroscope for infusion.

1.3.3 Infusion and analysis

1. Once mounted, the coverslip containing larvae is transferred to the stage of a Leica S6E stereomicroscope attached to a Harvard Apparatus microinjector for infusion.
2. A pulled capillary micropipette is backfilled with 1–2 mL (25–50 ng/mL) of FITC-labeled fibrinogen substrate.
3. The needle is inserted into the retroorbital space of the larva and 25–50 ng of substrate is infused.

4. Larvae are kept in agarose in a humidified chamber at 28°C following injection until time of analysis.
5. Larvae are visualized by an observer (blinded to the experimental variables) using a GFP filter on a Leica MZ16FA microscope at 1 hour post injection for accumulation of fibrinogen along the PCV (Fig. 5).

CONCLUSIONS

We have described three methods for studying hemostasis utilizing zebrafish as a model organism. In the first method, we describe a model of thrombosis using laser-induced endothelial injury of the major arterial and venous vessels of the zebrafish larva. The natural response to this injury is formation of a thrombus, resulting in eventual vessel occlusion. Larvae that have defects in proteins affecting the coagulation cascade or thrombocyte activity will fail to create this thrombus, mimicking a bleeding disorder. Measuring the TTO following laser injury provides a simple and high-throughput method for screening larvae for such defects. Clots can be easily visualized using bright-field microscopy, or through analysis of fluorescent thrombocytes such as with *Tg(itga2b:GFP)* fish (Fig. 2). In addition to TTO, time to attachment can be measured by quantifying the period until the first thrombocytes begin to aggregate at the injured vessel wall, as well as the total number of thrombocytes present. The thrombi formed will dissociate without intervention over time, presumably due to the process of fibrinolysis that results in recanalization of occluded vessels (Shavit & Ginsburg, 2013). This time to dissociation is another phenotype that can be quantified for the study of this process.

In the second section of this paper, we describe a method to quickly and efficiently quantify the number of thrombocytes circulating in larval zebrafish. This technique also allows for high-throughput quantitative analysis of thrombocyte number, one of the distinct advantages offered by the zebrafish model system. Rapid quantification of thrombocyte number using this technique can be applied to analyze the effects of small molecule inhibitors and activators on thrombopoiesis. One drawback to utilizing the *Tg(itga2b:GFP)* line is that stationary hematopoietic precursor cells express GFP in addition to circulating thrombocytes. The video processing techniques applied in this method account for and eliminate background autofluorescence as well as these stationary cells, resulting in only quantification of circulating thrombocytes. This was modified from our previous description (Huang & Shavit, 2015) so that video processing now uses freely available nonproprietary software.

We also describe a method for retroorbital infusion of fluorescently tagged human fibrinogen molecules into larvae. This method can be utilized as a functional readout of abnormalities in the blood coagulation cascade. Accumulation of fluorescent fibrinogen within the PCV is a clear indication of a defect in coagulation. Clot dissemination can be easily visualized using a standard GFP filter and stereomicroscope. Because of their small size and high fecundity, hundreds of zebrafish larvae can be injected with this construct in a single setting, making this method easy to use for high-throughput analysis.

To ensure that fluorescent deposits along the PCV represent accumulated fibrinogen, several controls can be utilized. Larvae can be pretreated in a solution containing 4 µg/mL of the anticoagulant warfarin, which completely inhibits the coagulation cascade in this setting. Pretreatment prevents fibrinogen from accumulating along the endothelium in mutant embryos with symptoms of DIC, as was shown previously in serpin peptidase inhibitor, clade C member 1 (*serpinc1*) (also known as antithrombin III) mutant larvae. This results in no accumulation of FITC-fibrinogen along the PCV (Liu et al., 2014). Additionally, retroorbital infusion of tissue plasminogen activator following FITC-fibrinogen reverses fluorescence accumulation (Liu et al., 2014).

These assays have been successfully used to study several different mutant zebrafish lines and models of coagulation factor deficiency, including the *victoria* (Gregory, Hanumanthaiah, & Jagadeeswaran, 2002) and *serpinc1* mutants (Liu et al., 2014), and the morpholino-mediated knockdown of fibrinogen (Vo et al., 2013). With the emergence of CRISPR (Hwang et al., 2013) and the ability to rapidly create targeted genetic mutants in fish, these techniques will continue to be valuable for the characterization of coagulation pathways.

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FIGURE 1. Larvae mounted in agarose for laser injury

Larvae are submerged in 0.8% low-melt agarose on a 24×55 mm glass coverslip, outlined by a hydrophobic PAP pen. Larvae are then arranged in the lateral view across the slide in preparation for laser injury.

FIGURE 2. Thrombus formation through laser-mediated endothelial injury

Following endothelial injury, a thrombus can be seen completely occluding either the posterior cardinal vein (B) or dorsal aorta (D), in contrast to uninjured vessels (A, C). For arterial laser injury, a *Tg(itga2b:GFP)* line is used to visualize thrombocytes through green fluorescence. Aggregated thrombocytes can be seen in the arterial occlusion (D). *Arrows* indicate the site of injury (A, C) and thrombus formation (B, D).

FIGURE 3. Arrangement of larval-loaded capillaries for thrombocyte imaging

Larvae are submerged in 0.7% low-melt agarose and suctioned into glass capillary tubes using a pipette pump. Capillaries are subsequently mounted in modeling clay in a plastic chamber and submerged in system water. The complete chamber setup is shown (A), as well as a magnified view of the larvae (B). The arrow indicates an embedded larva.

FIGURE 4. Representative example of image-processing steps for thrombocyte counts in *Tg(itga2b:GFP)* larvae

Following video capture, raw image data (A) are first processed using VirtualDub software to remove noise and autofluorescence (B). Subsequently, .avi files are further processed using ImageJ software to create files that only identify motile cells (C), and a circularity threshold is applied to further select single thrombocytes (D). All images represent a single frame taken from an individual movie. (See color plate)

FIGURE 5. Fluorescently labeled human fibrinogen deposits along the posterior cardinal vein (PCV) in mutant zebrafish

A zebrafish line with a known mutation in *serpinc1* (Liu et al., 2014) was retroorbitally infused with 50 mg/mL fluorescein isothiocyanate-labeled human fibrinogen. This resulted in fluorescent fibrin deposits along the PCV in homozygous mutant larvae (A) compared to heterozygous siblings which demonstrated no fluorescence (B).