In Vivo Fluorescence Imaging of Large-Vessel Thrombosis in Mice

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Objective—Experimental studies of large-vessel thrombosis have been adapted for applications in mice, but they proffer limited quantifiable information in outcome measures. This study presents a novel approach for evaluating large-vessel thrombogenesis with temporally/spatially quantifiable measures and normalization methods for interanimal comparisons.

Methods and Results—Shuttered, beam-expanded lasers provided uniform narrow-wavelength illumination of a \( \times 100 \) microsurgical field with a large depth of focus. Thrombosis was generated in murine carotid arteries and femoral veins by brief vascular surface electrolytic injury. Thrombus-targeting fluorophores were injected systemically and subsequently localized at the site of thrombus induction. A low-light digital video camera with filter wheel provided target-specific image acquisition over a 60-minute interval. Platelets accumulated with a subsequent fibrin border emerging to stabilize the clot in both arteries and veins. Coagulation enzyme complexes colocalized with fibrin deposition. Large arteries underwent cyclic massive thromboembolization, whereas veins showed gradual shedding of microemboli and clot contraction. Systemic administration of fibrin- and platelet-inhibiting compounds reduced their respective targets but also often inhibited their clotting counterparts (platelets and fibrin, respectively) in both arteries and veins.

Conclusion—Intermediate-level magnified image capture represents a novel approach for analysis of fluorescence-based in vivo imaging, with quantitative application to the study of large-vessel thrombosis. (Arterioscler Thromb Vasc Biol. 2011;31:1351-1356.)

Key Words: arterial thrombosis ■ blood coagulation ■ imaging agents ■ thrombosis

Studies seeking to understand the basis of thrombosis or to evaluate antithrombotic therapies have increasingly turned to murine systems, exploiting the capacity for genetic manipulations of the mouse genome to dissect the roles of various proteins and related factors on in vivo clot formation. Experimental thrombosis models in mice, however, have outcome measures that offer little informational content, in part because of the restrictions of working with the very small vascular structures inherent to this species. Large-artery thrombosis studies have most often applied measures of the time to occlusion after a free radical–mediated injury (usually applying topical FeCl3\(^{1,2}\) or using laser irradiation with circulating Rose bengal for photochemical induction of localized free radicals\(^{3-5}\)). Many venous thrombosis models rely on vascular occlusion via total or partial ligation of the vena cava, with subsequent measurement of stasis-induced clot weight, dimensions, or other properties at a fixed time after clot induction. These models may not accurately simulate clotting phenomena of relevance to clinical thrombosis: a site of thrombotic activity with maintained flow for which the therapeutic intent is to minimize thrombotic growth, embolization, or both and to prevent subsequent vascular occlusion.

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In the past several years, elegant in vivo thrombosis imaging systems have been developed\(^{4-6}\) that use thin-tissue structures that are adaptable to microscopic viewing and that incorporate fluorescent probes for specific cellular- and molecular-targeted imaging. These studies have provided insight into how intravascular blood clots form in microvessels; however, they address neither large-vessel thrombosis nor microvessel hemostasis (cessation of bleeding in transected microvessels). Furthermore, the mechanisms for generating blood clot formation in these systems, most often using broad-surface perfusion with free-radical generating solutions or laser-photonic heat injuries, have qualified clinical relevance. Many early studies of large-vessel thrombosis used circulating radiolabeled platelets and fibrinogen to identify and quantitate clot development\(^{7,8}\); however, these experiments suffer from poor spatial resolution and related shortcomings (eg, labeled fibrinogen does not distinguish fibrin-clotted versus platelet-bound fibrinogen in the clot). The study of large-vessel thrombosis would greatly benefit from the high temporal and spatial resolution of the recently developed microvessel imaging systems.

To this end, an imaging system was developed for evaluating in vivo large-vessel thrombosis that uses moderate magnification (\( \times 100 \)) with increased depth of focus (for larger vessels), a beam-expanded and shuttered laser array for uniform field illumination with narrow bandwidths to stimulate fluorophore excitation, and specific sets of fluorophores...
linked to clot-targeting molecules and cells to achieve quantifiable measures of multiple targets in the same clot site. Thrombus induction was generated by electrolytic injury via iron ion shedding to a discrete spot on the vessel surface, controlled by voltage and application time to yield reproducible subsequent thrombogenesis. A normalization procedure was developed to permit direct and highly quantitative interanimal comparisons of thrombus element localization within the site.

Methods

Imaging System

Three lasers—532, 594, and 650 nm—with power levels of 10 to 40 mW were beam-expanded to approximately 1-cm diameters and aimed to converge (after downward mirror reflection) on the surgical field (target) and subsequent emitted light passing through an operating microscope, with filter wheel emission-specific capture by a monochrome camera and image data acquisition.

Fluor 532, 594, or 647 using standard kits (Invitrogen). A monoclonal antibody specific for fibrin<sup>9,10</sup> was isolated from ascites from a hybridoma clone (59D8) kindly provided by Dr Marshall Runge; other antibodies included those for mouse factors VIII/VIIIa, IX/Xa, and X/Xa (Hematologic Technologies, Essex Junction, VT) and P-selectin (Chemicon/Millipore, Billerica, CA). An antibody specific for the activated platelet receptor αIIb/β3 (JON/A) was prelabeled with phycoerythrin (Emrtet, Eibelstadt, Germany).

The following antithrombotic agents were administered independently of the fluorophore/platelet injections at concentrations comparable to clinically relevant doses: heparin (IV, 200 U/kg), hirudin (IV, 1 mg/kg), aspirin (intragastrically 4 hours before thrombus induction, 15 mg/kg), and clopidogrel (intragastrically 24 and again 4 hours before thrombus induction, 10 mg/kg).

Surgery and Thrombosis Models

All animal studies were done under protocols approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee. Wild-type C57Bl/6 mice were purchased from Harlan SD (Indianapolis, IN), and factor IX-null mice were from the Jackson Laboratory (Bar Harbor, ME). Factor VIII-null mice were kindly supplied by Dr Haig Kazazian, and factor V Leiden mice were from Dr David Ginsburg. Mice were anesthetized with intraperitoneal pentobarbital (50 mg/kg) with maintenance dosing as required. Simple skin incisions permitted vessel exposures of the jugular vein (for intravenous injections), carotid arteries (with an external diameter of 0.3 to 0.5 mm), and femoral veins (diameter, 0.5 to 1.0 mm). Simple skin incisions permitted vessel exposures of the jugular vein (for intravenous injections), carotid arteries (with an external diameter of 0.3 to 0.5 mm), and femoral veins (diameter, 0.5 to 1.0 mm).

Labeled platelets (up to 1×10<sup>11</sup> injected per mouse) or fluorophore-labeled antibodies/proteins were injected into the femoral vein in volumes up to 100 μL, 3 to 5 minutes before thrombus induction; labeled antifibrin was used in the quantitative studies at amounts of 10 to 20 μg per mouse. Electrolytic injuries were induced on the surface of the carotid artery with a 30-second, 3-V direct current application, touching the surface of the vessel with the blunt end of a 140-μm-diameter steel needle (Surgical Specialties Inc., Reading, PA) connected to the anode, completing the circuit by contacting local subdermal tissue with the cathode. A similar injury was made on the femoral vein, touching the surface for 30 seconds, 1.5 V, with a 70-μm blunt-end needle. Applications under similar conditions with wires of copper, zinc, silver, or titanium did not yield detectable clots, supporting an iron ion-mediated electrolytic injury of clot induction, operating similarly to ferric chloride models of clot induction.<sup>3,4</sup> A modified FeCl<sub>3</sub> model was also used for comparison: the corner of a piece of filter paper soaked in a 20% FeCl<sub>3</sub> solution was touched to the surface of the carotid artery for 30 seconds, comparable to the contact time and footprint used for the electrolytic injuries. Several other methods of thrombus induction were evaluated that showed less consistency; these are described and presented in the Supplemental Section, available online at http://atvb.ahajournals.org.

Fluorophore Normalization

Primary measures for most experiments used platelets and antifibrin, labeled with distinct fluorophores. Labeled compounds were injected in a 100-μL volume through a branch of the jugular vein. A 1-μL volume of the fluorophore-containing injectate was spotted on a slide and imaged under the same laser illumination and magnification conditions used for the in vivo experiment. The total intensity of each fluorophore was determined thus: [Fluorescence intensity of 1 μL]×[100 (Injectate volume in μL)]/[Total injected fluorescence intensity for a given fluorophore). For each clot, the pixel intensity within the area of clotting activity was measured, with background subtraction, on temporally spaced images of a time-lapse video. A comparative index of measure was developed to normalize experimental runs, controlling for the injected amount of each fluorophore, the labeled intensity of the fluorophore-bound targeting molecule/cell, and the animal weight: [Area of thrombus]×[Average pixel intensity for a given fluorophore]−[Background intensity]×[Animal weight ratio adjustment factor]×[Total injected fluorophore intensity (determined under same imaging conditions)]−[Normalized intensity index (of a given fluorophore within the clot)]. The animal weight
Iron-based electrolytic injury to the vessel surface led to nonocclusive accumulation of substantial amounts of both platelets and polymerizing fibrin localized to the site of injury (Figure 2a and 2b). Progressive clot growth was evident starting immediately after thrombus induction and peaking at more than 10 minutes in both arteries and veins. Intriguingly, both clotting elements showed prominent accumulation in both vessel types (Figure 2c and 2d). This is in contrast to the accepted finding that platelets dominate the “white” clot of arteries and that fibrin is the more operational component of the “red” clot in veins.

The rate of clot dissolution showed dramatically different patterns in the different vessel types. Arterial thrombi often underwent massive and occasionally near-total embolization (Figure 2a; minute 37), followed by regrowth, sometimes with repeated embolization over a 60-minute observation period (Supplemental Video I). Furthermore, the extent of platelet versus fibrin embolization was nonuniform (Figure 2a; minute 37), followed by regrowth, sometimes with substantial amounts of both components showing a gradual downstream “drift” in localization that often preceded large and small embolic events in arteries and veins, respectively. Fibrin-specific labeled antibodies localized to a shell-like perimeter that coincided with the apparent mass attached to the clot induction site, sometimes showing a gradual downstream “drift” in localization that often preceded large and small embolic events in arteries and veins, respectively. 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time and appeared to serve as a stabilizing structure, securing the platelet thrombus to the vessel wall and inhibiting platelet embolization. To what molecular or cellular components this fibrin attaches will require further study. Labeled fibrinogen had an intermediate localization seen in large amounts both in fibrin-low/platelet-rich regions and in fibrin-dense sites, in both arteries and veins, consistent with its dual role in platelet aggregation and fibrin clot formation (Supplemental Figure IIa).

Labeled antibodies specific for factors VIII/VIIIa, IX/Ixa, or X/Xa slightly preceded and spatially colocalized with fibrin-dense regions in both veins and arteries (Supplemental Figures IIIa and IIIb). Labeled activated protein C, a natural inhibitor of both factor Va (of the Va/Xa complex) and factor VIIIa (of the VIIIa/Ixa complex), when used in low concentrations that did not inhibit clot formation, followed a colocalization pattern to fibrin similar to that of the VIII/VIIIa, IX/Ixa, and X/Xa antibodies (data not shown). Intriguingly, a fluorophore prone to photobleaching and attached to an antibody specific to the activated form of aIIb/β3 (JON/A, Emfret), when used with unblocked laser illumination to promote photobleaching, revealed late-developing small platelet masses in large-vein clots that appeared to roll in the direction of flow along the outer border of the established platelet mass, embolizing at the tail of the clot (Supplemental Video IV). This finding is consistent with recent studies12,13 that show surface rolling or loose adherence by small platelet masses and reveals the dynamic nature of platelet activity within ongoing thrombogenic stimuli.

Several standard antithrombotic agents were evaluated under both arterial and venous thrombosis conditions using the described imaging system. A method for quantitatively normalizing the amount of clotting element was applied to enable direct comparisons among experimental groups. High therapeutic levels of heparin and hirudin, well-established inhibitors of fibrin clotting that function primarily by blocking thrombin activity, lead to an approximately 10-fold reduction of fibrin in both veins and arteries and similar platelet reduction in veins and at early time points in arteries (solid black versus light and dark gray lines in Figure 3a to 3d). Knockout mouse lines for factors VIII and IX each showed dramatic reductions in both clotting components (Figure 3e and 3f). The factor V Leiden mutation in humans is a strong risk factor for deep vein thrombosis14,15; the mutated factor Va protein is less effectively inhibited by activated protein C. A mutant factor V transgenic mouse,16 with a single amino acid change analogous to the human factor V Leiden gene mutation, showed larger clots with elevated amounts of both fibrin and platelets (Figure 3e and 3f). Inhibition of a number of platelet activating receptors can reduce platelet aggregation in vitro and in vivo. Aspirin and clopidogrel, 2 commonly used inhibitors of platelet activation and aggregation (through the thromboxane and P2Y12 receptor pathways, respectively), reduced platelet accumulation in both vessel types; fibrin formation was also inhibited but to a lesser extent (Figure 3a to 3d). These findings are consistent with central roles of thrombin (for fibrin formation and platelet activation) and platelets (for aggregation and surface-mediated coagulation complex assembly), and furthermore, they underscore the interactive role of fibrin and platelets in blood clot development and stabilization within large vessels.

Discussion

This quantitative, highly informative imaging system provides a unique platform by which to query thrombotic activities under a variety of circumstances. The use of multiple clot-targeting, fluorescently identifiable moieties in the same experimental preparation expands the repertoire of what can be studied in a single experiment. Using independent labels for platelets and fibrin, the dominant components of clots, provides a means for comparative quantitation of thrombogenesis. The resolution of this imaging system, down to 2-by-2 μm per pixel (with higher resolution possible using higher-end cameras), permits spatial discrimination of many dynamic clotting events, including microembolism and clot contraction. Furthermore, time-lapse video replay gives a unique perspective on otherwise slow-developing thrombo-dynamic activities.

Several novel features of large-vessel thrombosis become evident through this imaging approach. Arterial thromboem-

![Figure 3. Intensity-normalized temporal profiles are shown of clot regions for arterial (a, b) and venous (c, d) electrolytic injury models under the influence of various clot-inhibiting compounds, with quantification of normalized intensities within evident clotting regions (arbitrary units) for platelets (a, c) and anti-fibrin (b, d). The antithrombotic compounds were heparin (200 U/kg), hirudin (1 mg/kg), aspirin (15 mg/kg), and clopidogrel (10 mg/kg); controls were vehicle-treated. All compounds were used at presumed high therapeutic-dose equivalence for mice. Similar normalized profiles for venous electrolytic injuries, without antithrombotic therapies, are shown for factor VIII and IX knockout and factor V Leiden transgenic mouse lines, with graph image capture for platelet (e) and fibrin (f) accumulation. Normalized intensities are displayed on a logarithmic scale on the y axis in all graphs. Each line represents the average of 4 to 8 experimental thrombus inductions, with 1 thrombus per mouse.](http://atvb.ahajournals.org/DownloadedFrom/10.1161/01.ATV.0000394059.62811.6f)
bolic events are common (Figure 2a), often occurring at later time points and occasionally seen to have highly repetitive regrowth and reembolization. Platelets and fibrin do not consistently embolize and regrow to the same extent. Venous thrombi have a more stable growth, usually with only small embolic events seen after peak growth (Figure 2b). The differences in embolic quality are likely due to the higher shear forces under arterial flow conditions. Clot contracture can also be observed, appearing as a consolidation of the clot around its vessel wall attachment site. This bulk change through clot contracture was clearly evident only in venous, not arterial, thrombi, and it was only occasionally seen; the mechanism of this contractile action is currently under investigation. Coagulation factors appear more densely in colocalization with fibrin-intense regions, but they only slightly precede fibrin formation in these localized densities (Supplemental Figures II and III). Despite potential differences in attenuation rates for different fluorophores, creation of a thrombotic nidus on the topmost portion of the vessel surface, directly imaged by the camera/microscope, minimized these attenuation effects (Supplemental Figure IV), thus permitting relatively accurate measurement and quantitation of these clotting events. The normalization approach for data analysis has reasonable linearity with moderate changes in the amount of injected fluorophore, whether this variation was due to altered levels of fluorophore incorporation in the target during its preparation or to the total amount of injected fluorophore-bound targeting cell/molecule. The resulting image intensity, which is proportional to the injected fluorophore intensity, is thus normalized offline through calculation, allowing interanimal quantitative comparisons of clotting components.

Other microscopic methods of murine in vivo thrombus imaging have been developed, primarily designed for and applied to thrombi induced in microvessels. Rosen et al. used laser irradiation alone or in combination with circulating Rose bengal to generate thrombi in mouse ear vessels; thrombi were directly imaged with incident light. Fluorescence microscopy has been used to image mesenteric or cremaster preparations, with thrombus induction within arterioles/venules by ferric chloride superfusion or laser spot injury, respectively. The fluorophore-tagging methodologies of the current report were adapted from those described in these earlier studies, using a similar ex vivo platelet-labeling strategy and thrombus-targeted antibodies labeled with selected fluorophores. Whereas these microvessel imaging models can use the shorter excitation/emission wavelengths of calcein AM (for platelet labeling) and Alexa 350 and 488 for antibody labeling, because of the thin tissues studied, these fluorophores are more strongly attenuated by the thicker tissues and clots of large-vessel thrombosis evaluation, necessitating a shift to longer-wavelength-activated fluorophores described in the current report.

A major difference between the results presented in this report and findings from microvessel thrombus analyses is the time course of thrombogenesis. Site-localized thrombus induction by laser injury generates rapid clot growth, generally seen to reach peak activity in 60 to 90 seconds. Superfused ferric chloride also causes faster thrombus growth, with peak growth more difficult to ascertain (possibly because of the nonlocalization of induction) but appearing to be less than 10 minutes. In contrast, the electrolytic injuries applied herein displayed more prolonged growth kinetics, usually peaking at more than 10 minutes in arteries and more than 20 minutes in veins (Figure 3). This occurred regardless of inhibitor effects, supporting a general time course for this thrombus induction model. Mechanical injury (with apparent intravascular collagen exposure) showed shorter times to peak growth (Supplemental Figure V), but the general time course was still considerably longer than in microvessel thrombus systems. These longer times may be due to 1 or more factors: (1) sustained thrombotic stimulation, (2) a cumulative synergism related to the net (ie, larger) surface area of thrombus induction, (3) flow-mediated effects in larger vessels, or (4) greater intravascular extension of the thrombus into the lumen. A combination of more than 1 of these factors is likely to be operative, but this will require further study.

Pharmacological control of thrombosis has been a continuously active area of research since the emergence of heparin to reduce venous thromboembolism more than 70 years ago. The long-held view that platelets are more responsible for arterial thrombosis whereas fibrin clotting is central to large vein thrombosis has been based on a variety of in vitro and in vivo experiments, post mortem analyses, and many clinical trials. This has guided most approaches to clinical therapies, with some notable exceptions. For example, vascular surgeons still typically use heparin-based compounds to inhibit fibrin clotting when undertaking many arterial-repair procedures (where platelets are expected to be more thrombotically operative), and debate is still open about the effects of platelet-inhibiting agents on venous thromboembolism. Post-mortem studies of both coronary artery and deep vein thrombosis have found variability, too, with regions of high platelet content (white clot), as well as other regions of fibrin-entrapped red blood cells (red clot) in both arteries and veins. The results herein support this overlap of platelets and fibrin within both arterial and venous thrombi. This unique finding may be dependent on the specific model of thrombus induction (ie, electrolytic injury), and thus may be a limitation of the application of this model to the comparison of platelets and fibrin within a thrombus. However, other models of thrombus induction were evaluated and found to yield various degrees of dual platelet/fibrin accumulation (Supplemental Figure V). A better understanding of how thrombogenesis progresses and the effects of various pharmacological agents on these processes is still needed to advance therapeutic care.

Blood clot imaging with current fluorophore-based approaches has limitations in terms of depth of field (red blood cells attenuate all wavelengths, particularly those less than 660 nm), 3-dimensional profile (with the depth dimension approximated by pixel intensity measurement of the 2-dimensional captured image), and precision (red and infrared wavelengths have lower scatter in biological tissues, but their low attenuation leads to significant levels of marginal diffusion that can reduce the precise determination of spatial localization). Despite these drawbacks, the system and ap-
proach presented herein provide a substantial improvement over previous quantitative methodologies. Platelets and fibrin, the principal components of intravascular clots, can be quanti-
tated over 2 orders of magnitude, for comparisons ranging from extremely hypothrombotic phenotypes (eg, hemophilia models) to more prothrombotic conditions. A limitation of the study is
the time lag between thrombus induction and the first useful data point (2 minutes in the data presented here). This lag leads to a “blind spot” in early thrombotic events with this imaging approach, which is evident from the differences in starting values among the pharmacologically inhibited experimental conditions in Figure 3.

The system as described used inexpensive components and relatively low-power lasers with long (up to 1 second) camera exposure times. The components of the system described, minus the microscope, were purchased for less than $20,000, with most of this cost (~$16,000) represented by the low-light camera/filter wheel. The red and green lasers, the mirror, and the filters can be purchased for a relatively low cost with thrifty shopping, with adaptation to an existing dissecting/operating microscope/camera system, to assemble a functional system cost-effectively. Higher-end cameras and more powerful lasers can be used to permit shorter exposure times, which would substantially improve resolution without tissue heating or other modulatory effects. Thus, by customizing the system assembly, specific experimental needs of a laboratory can be met within budgetary constraints. A cautionary word is in order with regard to inherent safety issues with use of class IIIb and IV lasers (above 5 mW for most visible wave-
lengs): until a laser beam enters a beam-expanding lens, its power density presents an eye hazard to experimenters and research animals, so following proper laser safety procedures is essential.

In summary, this approach to large-vessel thrombosis imaging can be used for evaluating clotting components in a flexible, quantitative, data-rich, and cost-effective manner.

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Disclosures
None.
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SUPPLEMENTAL MATERIAL (Methods/Experiments)

Supplemental Experimental Methods

Several other models of thrombus induction were applied in the carotid arteries and femoral veins of mice, using systemically infused labeled platelets and anti-fibrin and subsequent quantitative image analysis as described in the main text. Veins underwent either a mechanical pinch (with a jeweler's forceps) or 30-gauge needle puncture/stab. Arteries received a similar pinch injury. The arteries were also subjected to intraluminal wire injury\(^1\) or vascular transection and repair with 6-8 interrupted 11-0 sutures (microvascular anastomosis);\(^2\) these last two injury models were done while the artery was clamped, with clamp release and imaging done subsequently.

Commercial antibodies used for labeling and in vivo infusion are as follows:

From Haematologic Technologies, Inc. Essex Junction, Vermont:

Rat anti-mouse Factor VIII (Cat # AMVIII-9035; Lot # W0612-0.1MG); 20-25 μg infused from a 1 mg/mL fluorophore-labeled stock solution.

Rat anti-mouse Factor IXA (Cat # AMIXA-9042; Lot # X0116); 30-50 μg infused from a 1 mg/mL fluorophore-labeled stock solution.

Rat anti-mouse Factor X (Cat # AMX-9050; Lot # W0302-0.1MG); 20-50 μg infused from a 1 mg/mL fluorophore-labeled stock solution.

From Chemicon/Millipore, Billerica, Massachusetts:

Rabbit anti-P-Selectin (Cat # CBL40; Lot #Q14242); 20-40 μg infused from a 0.5 mg/mL fluorophore-labeled stock solution.

From Emfret, Eibelstadt, Germany:

Rat anti-activated platelet receptor αIIb/β3 (JON/A; Cat # M023-2; Lot #0232-B); 15-30 μL infused from the supplied phycoerythrin-labeled stock solution.
**Supplemental Results**

Single experimental traces for the various thrombus induction models are presented in Supplemental Figure 5a and 5b for platelet and fibrin accumulation, respectively. The pinch injuries caused a rapid rise and fall of platelets in veins and arteries, seen to peak at 8 and 4 minutes, respectively (Suppl. Fig Va), with a more sustained progression of fibrin in both vessel types (Suppl. Fig. Vb). These injuries, as well as the arterial wire injury, generated an early platelet response at the first data acquisition time point (2 minutes), nearly an order of magnitude higher than the other injury models. In contrast, the electrolytic injury models in both arteries and veins, for both platelets and fibrin, had a delayed initiation of activity. The vein stab injury resulted in apparently less platelet accumulation relative to fibrin. The arterial anastomosis model showed relative peaks for platelets and fibrin at 15-20 minutes. These findings overall show similar high involvement of both platelets and fibrin in thrombus growth in both vessel types, with some variability due to the different induction mechanisms. All of these additional models, exclusive of the electrolytic and ferric chloride models, likely caused direct exposure of either subendothelial or adventitial collagen to the flowing blood, which may account for the more rapid development of thrombus in some of the models.

In general, the electrolytic injury models were more consistent, with the findings amenable to statistical analysis. Data were extracted from the Figure 3 data at the 10-, 30- and 60-minute time points (approximate growth, peak, and stabilization phases of thrombogenesis, respectively), for the control, aspirin and heparin experimental series. These data are presented in Suppl. Fig. VI (four graphs). Statistical analyses were done using ANOVA, with post-hoc Fisher LSD tests for between-group comparisons, using a p<0.05 value for assigning statistical significance. There were significant differences noted for all of the venous electrolytic injury times when comparing the controls to either the aspirin or heparin series, for both platelets and fibrin accumulation (p < 0.025 for these comparisons; Suppl. Fig. VIa, VIb). The arterial electrolytic injury revealed statistically significant differences for controls versus either aspirin or heparin at 10 minutes in the platelet data, and for controls versus heparin at all times for the fibrin data (p < 0.05; Suppl. Fig. VIc, VIId). Other apparent trends seen in the data did not reach statistical significance; power analyses indicated that larger experimental series (n = 8-12) would be needed to verify statistical differences in many cases.
System and Experimental Limitations

Laser Power

After a 30-minute warm-up, lasers showed less than 5% power level variation over the course of 8 hours’ continuous use. The laser power levels yielded relatively low power densities across the imaging field, ranging from 5 to 30 mW/cm², which required long camera exposure times (typically 1 second); this created movement distortions of images with animal movement, particularly for pulsating arteries and for hemostatic wounds (while flushing the bleeding site with saline irrigation). Simultaneous continuous illumination with the defocused green, yellow and red lasers used in the study yielded a total field power density of 50 mW/cm², only ~20% of the power density generated by the operating microscope’s standard white light source. Reduction of movement distortions would be possible using higher-power lasers with reciprocally shorter exposure times, enhancing imaging quality without substantial light- or heat-associated detriment to the in vivo imaged site.

Absorption/Attenuation/Background Effects

Each fluorophore showed a strong linear response for image intensity with increasing fluorophore concentration (data not shown). Creation of clot induction models on the topmost portion of vessels was used to reduce circulating red blood cell attenuation of both excitation and emission wavelengths. In vitro studies showed less than 5% total attenuation through murine vascular tissues, platelets, and clotted plasma for all wavelengths over distances/thicknesses up to 500 μm, whereas red blood cells at hematocrits of 1 to 40 (volume percent) caused significant attenuation, with much greater attenuation for wavelengths less than 650 nm (highly absorbed by hemoglobin). The yellow laser (594 nm) stimulated a moderately apparent autofluorescence in the surrounding skeletal muscle tissue (though not in the vessel wall or circulating blood stream), making total-field images non-ideal for video viewing, but satisfactory for vessel-specific image-captured quantitative evaluations.

Continuous laser illumination of fluorophores for up to 1 hour caused a moderately progressive photobleaching, generally less than a 30% attenuation of the signal; the shuttering of laser irradiation reduced the total illumination to cause less than 10% signal attenuation over 1 hour. Phycoerythrin was an exception among the fluorophores used in this regard, with greater than 60% attenuation over 10 minutes, allowing its use in photobleaching experiments (Supplemental Video 4).

Model Limitations

Absolute amounts of platelets and fibrinogen within the thrombus could be determined based on the intensity of the spot for ex vivo-labeled platelets and fibrinogen, using platelet counts and fibrinogen
concentrations for determining platelet/fibrinogen molecule numbers: control clots in large veins and arteries typically had approximately 1-3% of total circulating labeled platelets and fibrinogen incorporated by 30-60 minutes. Labeled antibodies are not amenable to absolute quantitation because of unknown binding characteristics to their targets under the various in vivo clotting conditions; these antibodies were able to provide comparative quantitation among the various experimental conditions for select targeted antigens.

The quantitation method for estimating total clot content of a given fluorophore uses a 2-dimensional image with an intensity profile that approximates the quantitative levels for the third dimension; in vitro studies with fluorophore-containing clots within cover-slipped "sandwiches" revealed that stacking of these sandwiched clots led to nearly 100% additive fluorescence intensities, indicating that excitation and emitted radiation can penetrate up to 500 μm of clot with little attenuation (data not shown).

The electrolytic injuries occasionally deposited opaque pitted metal on the vessel surface that attenuated fluorescence at these sites; the total attenuated area was generally much less than 5% of the total area.

The electrolytic injury models have less parallel to clinical thrombosis, but provide reproducibility for quantitative studies and have direct analogy to the frequently used ferric chloride-based experimental thrombosis models, as both thrombus inductions are based on localized free radical generation.

The sizes of large arteries and veins are much smaller than those of comparable human vessels. However, the mouse carotid artery and femoral vein have diameters very similar to those repaired by hand surgeons undertaking pediatric finger replantations; such repairs have shown little difference in thrombotic outcomes when compared to adult replant cases, for which vessel diameters are comparable to those encountered in coronary artery bypass surgery, a common clinical procedure with well-studied thrombotic complications. Thus, these murine vessels offer reasonable analogues for human vessels prone to thrombosis.

Control Experiments

Infusion of fluorophore-labeled nonspecific antibodies or unbound fluorophore under clot formation conditions revealed fluorophore accumulation slightly above background circulating fluorescence (generally under 2-fold increase) within the clot; this may be due to nonspecific binding, but may also be caused in part by red blood cell exclusion from the platelet/fibrin-dominated clot that caused an increase in apparent fluorescence due to loss of red blood cell attenuation of background signal within the clot site. These increases were much less than the overall signal for clot-targeted fluorescence markers, for which greater than 100-fold increases above background were typical.
Infusion of the same antibodies labeled with different fluorophores showed similar rates and changes in antibody accumulation. For example, anti-fibrin labeled with Alexa Fluor-532, -594, and -647, injected into the same animal, showed parallel rises and peaks of accumulation (Supplemental Fig. IV). Use of different types of fluorophore labeling that localized with the same clot target showed similar patterns of accumulation. For example, infusion of Vybrant DiD-labeled platelets (near-infrared fluorescence) into a rhodamine 6G-infused mouse (green/yellow fluorescence of platelets) showed similar rates of change and localization of platelets. Alexa-647-labeled human fibrinogen infused separately from a mixture of a biotin-labeled glycine-proline-arginine-proline peptide (which targets to fibrinogen) and phycoerythrin-labeled streptavidin (which binds to the biotin moiety in the peptide) resulted in spatially and temporally colocalized clot accumulation of the fluorophores.

To confirm the specificity of the anti-fibrin antibody (isolated from ascites obtained from a hybridoma clone generously donated by Dr. Marschall Runge), fibrinogen in unclotting solution or with thrombin/calcium added to induce clotting was spotted onto a glass slide. After 3 hours of clotting/drying, the spots were washed with 2% bovine albumen in phosphate-buffered saline (PBS), incubated with neutral mouse IgG antibody, then incubated for 5 minutes with Alexa-647-labeled anti-fibrin (100 ng/mL), and subsequently washed with PBS. Spots were then imaged with 650-nm laser illumination and the average fluorescence intensity was measured. The fibrin spots had more than 20-fold the amount of fluorescence compared with the fibrinogen spots (Supplemental Fig. VII).

To determine a possible effect of systemic administration of anti-fibrin on fibrin clotting, a murine plasma clotting time assay was done, with recalcification of citrated mouse plasma samples and using a Stat4 Instrument (Diagnostica Stago, Parsippany, NJ) for measuring the clotting time; the anti-fibrin antibody concentration was varied (as a potential “inhibitor” of clotting), using neutral antibody for controls. At anti-fibrin antibody concentrations equivalent to final circulating concentrations from the doses typically administered in the in vivo studies (10-20 μg/mL of blood), no effect on plasma clotting time was noted (Supplemental Fig. VIII).

Reference Citations:

Supplemental Figure I. Comparison of normalized fluorophore intensities, for summed platelets and anti-fibrin at the time of clot harvest, with histomorphometrically measured thrombus size. The Pearson correlation coefficient, r, is 0.915, indicating good agreement over a range of clot sizes.
Supplemental Figure II. Femoral vein electrolytic injuries at approximately 40 minutes after clot induction, using various sets of three labeled clot-targeting molecules/cells. a) Platelets (plts; membrane-labeled, in red), fibrin-specific antibody (fbn; in green), and human labeled fibrinogen (fbg; in yellow); merged image also shows a plot profile line used to graphically capture the relative fluorophore intensities over a region of the clot (aligned graph below merged image), displaying variable spatial intensities.
among the fluorophores, which indicates separate regions of fibrin and platelet clotting activity, with overlapping fibrinogen (dual role).  b) Labeled fibrin (red) and Factors (IX/IXa (green) and X/Xa (yellow), using antigen-specific antibodies; the merged image displays a plot profile line, graphed below the image, showing largely coinciding peaks and troughs, suggesting that fibrin clotting occurs at regions of coagulation enzyme complex formation. All color images in (a) and (b) have been artificially colorized and contrast-adjusted for comparative viewing, with graph intensities transformed for comparisons. Bars are 500 μm.

Fibrin/IX/X Comparison

Supplemental Figure III. Comparison of relative intensities for total fibrin, Factor IX, and Factor X/Xa in an electrolytically injured vein, showing that IX/IXa and X/Xa precede fibrin in their relative accumulation. Data were quantitatively analyzed every frame (10-second intervals) over 20 minutes and normalized to the maximum image intensity for each fluorophore (maximum = 100%).
Supplemental Figure IV. Simultaneous injection of anti-fibrin labeled with Alexa-532, -594, and -647, with corresponding laser excitation and three-channel image capture of an electrolytically injured femoral vein. Data were quantitatively analyzed every frame (10-second intervals) over 30 minutes and normalized to the maximum image intensity for each fluorophore (maximum = 1). Pearson correlation coefficients across the data were 0.98 - 0.99, showing a strong correlation for these different wavelengths and antibody labelings.
Supplemental Figure V5: Graphs of imaged thrombus intensities for various induction models, showing relative platelet (a) and fibrin (b) amounts. Thrombus induction was generated in femoral veins and carotid arteries by electrolytic injury (as described in main text), mechanical pinch injury, 30-gauge needle stab (in the vein), intralumenal wire injury (to the artery), and vascular repair of the artery (artery anastomosis). Data were measured at 2-minute intervals, with each line representing one experimental thrombus.
Supplemental Figure VI: Graphs of extracted data from the main text Figure 3 of the main text, for the 10-, 30-, and 60-minute time points, showing averages and standard errors of control (vehicle), aspirin, and heparin treatment groups, for the venous and arterial electrolytic injury models. a) venous injury model showing platelet accumulation levels; b) venous injury model showing fibrin accumulation levels -- statistical significance was found for comparisons between the controls and either the aspirin or heparin series at all time points, for both platelets and fibrin accumulation (p < 0.025); c) arterial injury model showing platelet accumulation levels; d) arterial injury model showing fibrin accumulation levels -- statistical significance was found for comparisons between the controls versus either aspirin or heparin at 10 minutes in the platelet data, and for controls versus heparin at all times for the fibrin data (p < 0.05).
Figure VII: Determination of anti-fibrin specificity. Fluorescence intensity of fibrinogen or fibrin spots after incubation with Alexa-647-labeled anti-fibrin antibody, showing average spot intensity (error bars are standard errors).
Figure VIII: Plasma clotting times of murine citrated plasma upon recalcification, using added concentrations of anti-fibrin antibody; controls (0 ug/mL) were done with neutral antibody added.
Supplemental Material

A series of video files (QuicKTime movies) present clot formation under various conditions (without audio tracks). Videos 1-3 were created with superimposed images that are false-color generations for the monochrome images, taken under each set of laser-filter-fluorophore-emission capture conditions, with brightness and contrast adjusted for optimal viewing.

**Video 1.** Electrolytically injured mouse carotid artery with injected DID-labeled platelets (red) and Alexa-532-labeled anti-fibrin (green), seen in time-lapse for a 60-minute interval. Frames are separated by 10 real-time seconds. Flow is from left to right. Bar = 300 μm. A massive embolic event occurs late (~37 minutes after induction), with regrowth, and a partial embolization is seen earlier (~20 minutes).

**Video 2.** Electrolytically injured mouse femoral vein with injected DID-labeled platelets (red) and Alexa-532-labeled anti-fibrin (green), seen in time-lapse for a 60-minute interval. Injury site is approximated by small blackened region near the center of the clot. Frames are separated by 10 real-time seconds. Flow is from left to right. Bar = 300 μm.

**Video 3.** FeCl₃-injured carotid artery with injected DID-labeled platelets (red) and Alexa-532-labeled anti-fibrin (green), seen in time-lapse for a 60-minute interval. The FeCl₃-soaked filter paper had its corner touched to the vessel surface for 30 seconds. Frames are separated by 10 real-time seconds. Flow is from left to right. Bar = 300 μm.

**Video 4.** Electrolytically injured mouse femoral vein with injected JON/A antibody (specific for activated αIIb/β3 receptor expression) labeled with phycoerythrin, shown in monochrome black-and-white, with the green excitation laser used continuously without shuttering (to induce photobleaching). The thrombus is shown from 5 to 35 minutes after thrombus induction, with one frame every 3 seconds. The word, “Photobleached”, appears when the main thrombus begins to fade from photobleaching (at about 10 minutes), with subsequent brighter appearances of newly activated or attaching αIIb/β3-expressing platelets. Flow is from left to right. Note the apparent rolling of small, newly forming aggregates along the perimeter of the thrombus, with micro-embolization from the tail.