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Systems Biology of Blood Coagulation and Platelet Activation

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Systems Biology of Blood Coagulation and Platelet Activation

Abstract
Blood clotting is a highly conserved physiological response that prevents excessive blood loss following vessel injury. It involves a sequence of plasma reactions leading to the formation of thrombin (the coagulation cascade) as well as tightly controlled intracellular reactions mediating platelet activation. These two events are inextricably coupled, with the active platelet surface serving as a cofactor for coagulation factor assembly and thrombin serving as a potent platelet agonist. Using the technologies of automated liquid handling, high throughput experimental systems were developed that allowed individual exploration of these two components of the thrombotic response under diverse initial conditions. Based on this high dimensional experimental exploration, a “bottom-up” mechanism based Ordinary Differential Equation (ODE) description of thrombin generation kinetics and a “top-down” data driven Neural Network model of platelet activation were developed. In the first study, “contact activation” (and not “blood-borne TF” alone) despite the best available inhibitor to prevent it, was found build up enough autocatalytic strength to trigger coagulation in the absence of exogenous tissue factor, particularly upon activated platelets. Further, the “Platelet-Plasma model” successfully predicted the stability of blood under multiple perturbations with active enzymes at various physiologically realizable conditions. In the second study, “Pairwise Agonist Scanning” (PAS), a strategy that trains a Neural Network model based on measurements of cellular responses to individual and all pairwise combinations of input signals is described. PAS was used to predict calcium signaling responses of human platelets in EDTA-treated plasma to six different agonists (ADP, Convulxin, U46619, SFLLRN, AYPGKF and PGE_2). The model predicted responses to sequentially added agonists, to ternary combinations of agonists and to 45 different combinations of four to six agonists (R=0.88). Furthermore, PAS was used to distinguish between the phenotypic responses of platelets from healthy human donors. Taken together, these two studies lay the groundwork for integration of coagulation reaction kinetics and donor specific descriptions of platelet function with models of convection and diffusion to simulate thrombosis under flow.

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SYSTENMS BIOLOGY OF BLOOD COAGULATION AND
PLATELET ACTIVATION

Manash Shankar Chatterjee

A DISSERTATION

in

Chemical and Biomolecular Engineering

Presented to the Faculties of the University of Pennsylvania

in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

2011

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Finally I would like to thank my parents and sister. This work would not be possible without their unending support.
ABSTRACT

SYSTEMS BIOLOGY OF BLOOD COAGULATION AND PLATELET ACTIVATION

Manash Shankar Chatterjee

Supervisor: Scott L. Diamond

Blood clotting is a highly conserved physiological response that prevents excessive blood loss following vessel injury. It involves a sequence of plasma reactions leading to the formation of thrombin (the coagulation cascade) as well as tightly controlled intracellular reactions mediating platelet activation. These two events are inextricably coupled, with the active platelet surface serving as a cofactor for coagulation factor assembly and thrombin serving as a potent platelet agonist. Using the technologies of automated liquid handling, high throughput experimental systems were developed that allowed individual exploration of these two components of the thrombotic response under diverse initial conditions. Based on this high dimensional experimental exploration, a “bottom-up” mechanism based Ordinary Differential Equation (ODE) description of thrombin generation kinetics and a “top-down” data driven Neural Network model of platelet activation were developed. In the first study, “contact activation” (and not “blood-borne TF” alone) despite the best available inhibitor to prevent it, was found build up enough autocatalytic strength to trigger coagulation in the absence of exogenous tissue factor, particularly upon activated platelets. Further, the “Platelet-Plasma model” successfully predicted the stability of blood under multiple perturbations with active enzymes at various physiologically realizable conditions. In the second study, “Pairwise
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Chapter 1

Introduction

1.1 Thrombus formation

Thrombus formation is an efficient mechanism to prevent excessive blood loss following vascular damage. Upon vessel wall damage the continuous lining of the endothelium is broken and flowing blood is exposed to the connective tissue matrix of the subendothelium. Tissue Factor (TF) at the site of vascular damage binds to minute quantities of the circulating form of activated factor VII. This triggers the coagulation cascade, involving a series of reactions happening either in solution or upon the catalytic surface provided by nearby activated platelets. This cascade culminates in the formation of the master enzyme thrombin, which once formed can activate platelets, as well as participate in its own production and destruction. Platelets are the primary cells involved in mediating the thrombotic response. In addition to thrombin formed downstream of the coagulation cascade, platelets are efficiently activated by collagen exposed at the site of injury. Moreover, platelets can self activate via released ADP or thromboxane. Mediators like prostacyclin (PGI$_2$) or Nitric Oxide (NO) from the intact endothelium can down- regulate platelet activation and keep them quiescent. But once the platelets encounter a net activating stimulus as a function of the concentration of all of these simultaneous signals they proceed via a sequence of signaling steps (one amongst which is the elevation of cytosolic calcium concentration) to become fully activated platelets. These platelets make up the body of the clot and are held in place by a meshwork of
polymerized fibrinogen formed downstream of thrombin in the coagulation cascade. This process is schematically represented in Figure 1.1-1 and the two individual mechanisms that are critical for the formation of the stable platelet plug (platelet activation and the cascade of reactions leading to thrombin production) are introduced below.

Figure 1.1-1 Coagulation and platelet activation pathways during thrombosis.

Haemostasis system involves a delicate balance of mechanisms that maintain fluidity of blood under physiological conditions, yet allowing the body to mount a robust response to limit blood loss following vascular injury. The coagulation stimulus at the site of injury leads to two interlinked responses. First: the exposure of Tissue Factor (TF) at the sub-endothelium triggers the coagulation cascade leading to the formation of thrombin. Second: platelets are activated according to the prevailing local concentrations of thrombin, collagen, ADP, TXA₂ and adhere to the growing clot. NO and prostacyclin (PGI₂) from the intact endothelium downregulate activation and keep platelets quiescent. Once the platelet encounters a net activating stimulus there it activates via an elevation of cytosolic calcium concentrations. The active platelet surface in turn serves as cofactor for several of the enzymatic reactions of the coagulation cascade.
1.2 Platelet activation

The signaling machinery of circulating platelets makes it possible for them to be rapidly captured at the site of vascular damage and be transformed into a tight layer that stems uncontrolled blood loss. The sequence of events underlying platelet activation can broadly be divided into three stages: initial adhesion to a collagen surface, growth of thrombus via autocatalytic signaling, and stabilization of the platelet aggregate [1].

1.2.1 Initiation of platelet activation on collagen surfaces

Under static conditions, collagen alone at the exposed injury is able to capture and activate platelets. However, under conditions of high shear that exist in the arterial circulation, platelet capture also requires the presence of VWF [2]. Two of the collagen receptors (\(\alpha_2\beta_1\) and GPVI) bind to collagen directly. Of these GPVI is the most potent in terms of generating an activating signal. Collagen binding causes GPVI clustering, leading to phosphorylation of the Fc receptor \(\gamma\) chain (associated with this receptor) by tyrosine kinases of the Src family. Such phospho-tyrosine residues are recognized by Syk [3], and the association and activation of Syk activates PLC\(\gamma_2\). PLC\(\gamma_2\) (like other forms of PLC) converts phosphatidylinositol (PI)-4, 5 P\(_2\) to 1,4,5- IP\(_3\) and diacycglycerol (DAG). IP\(_3\) opens Ca\(^{2+}\) channels in the platelet dense tubular system, and causes rapid store operated calcium entry (SOCE), thus quickly raising cytosolic Ca\(^{2+}\) concentrations [4]. DAG activates Protein Kinase C (PKC) in platelets, which in turn governs several of the serine/ threonine phosphorylation events occurring downstream. PKC activation can cause integrin activation, aggregation and secretion independently of Ca\(^{2+}\) [5]. GP1B, \(\alpha_2\beta_1\) and \(\alpha_{IIb}\beta_3\), present in abundance on the platelet surface, bind to collagen via multimeric VWF (complexed with collagen) under high shear, thus increasing the
probability of collagen mediated signaling via GPVI by bringing the ligand and receptor in close proximity [6].

### 1.2.2 Growth of the platelet aggregate via autocatalytic signaling

Beyond the first monolayer of platelets adherent to collagen/ VWF, the addition of subsequent layers of platelets to the growing thrombus is strongly potentiated by the activity of locally released agonist like thromboxane (TxA2) and ADP, as well as by the activity of locally formed thrombin. All 3 of these agonists act signal through specific G-Protein Coupled Receptors on the platelet surface (GPCRs).

GPCRs are composed of α, β and γ subunits. The binding of an agonist to its receptor causes an exchange of GTP for GDP on the α subunit, and dissociation of the α and βγ subunits. Both these units in turn interact with secondary effectors including PLCβ and Adenyl Cyclase. Human platelets express at least 10 forms of Gα (including members of the Gq, Gi, G12, and Gs families) [7].

Thrombin, ADP, and TXA2 activate PLCβ via Gq. PLCβ generates IP3 from membrane PIP2. IP3 triggers Ca2+ release from the intracellular stores and SOCE, thus increasing intracellular Ca2+ concentrations from ~100 nM to ~ 1 µM. Rising Ca2+ levels activate the Ras family member, Rap1B via Cal-DAG GEF. Rap1B activation is a precursor to αIIbβ3 activation, and allows the platelets to form aggregates with other platelets through fibrinogen bridges [8].

In addition to integrin activation, shape change is another event that occurs downstream of agonist induced platelet activation. There is a Ca2+ dependent activation of myosin light chain kinase (Gq driven), and activation of GTP binding proteins of the Rho family (G12 driven) [9]. Rho activation in turn activates kinases like p160ROCK and
LIM-kinase, which phosphorylate myosin light chain kinase and coflin [10, 11], thus regulating actin dependent cytoskeletal shape. Shape change is mediated via G$_{q\alpha}$ for ADP and G$_{i3\alpha}$ for thrombin and TXA$_2$.

Prostacyclin (PGI$_2$) synthesized by the endothelium causes G$_{s\alpha}$ mediated increase in adenyl cyclase activity. Also NO from the endothelium can inhibit the hydrolysis of cAMP by intracellular phosphodiesterases [12]. Taken together these mechanisms elevate intracellular cAMP levels, which strongly downregulates platelet signaling. Agonists coupled to G$_i$ family members inhibit cAMP production in platelets, thus allowing activation to proceed unhindered [13]. Additionally the $\beta\gamma$ subunits of these receptors can activate PLC$\beta$ and the $\gamma$ isoform of PI3K. The effectors for PI3K include Rap1b and Akt [14]. Signaling downstream of Akt is not completely understood, but deletion of Akt2 in platelets impairs thrombus formation, stability and platelet secretion [15].

ADP is stored in platelet dense granules and is released upon activation. P$_2$Y$_1$ and P$_2$Y$_{12}$ are the primary receptors for this agonist [16]. P$_2$Y$_1$ is G$_{q\alpha}$ coupled and signaling through this receptor causes Ca$^{2+}$ mobilization, shape change and thromboxane generation. P$_2$Y$_{12}$ is the target of the commonly used anti-platelet drug Plavix [17], and is a G$_{i2}$ coupled receptor that inhibits cAMP production in platelets. A third receptor P$_2$X$_1$ is an ATP gated channel that allows Ca$^{2+}$ influx across the plasma membrane [18].

Thrombin is a potent platelet agonist that causes fast mobilization of intracellular Ca$^{2+}$, and activation of phospholipase A2 and subsequent thromboxane generation (all mediated by G$_{q\alpha}$) [19]. Also, thrombin can trigger Rho dependent signaling pathways in platelets (mediated by G$_{i3\alpha}$) [20], that contribute to actin modeling and shape change. Thrombin signals through the protease-activated receptor (PAR) family of GPCRs. PARs
1 and 4 are expressed on human platelets, while PARs 3 and 4 are expressed on mouse platelets. Thrombin cleaves the N-terminus of these receptors, exposing a new N-terminus that serves as a tethered ligand for these receptors. Synthetic peptides are able to selectively activate these receptors and mimic the actions of thrombin (SFLLRN for PAR1, and AYPGKF for PAR4) [21]. Kinetic studies have shown that the human platelet response to thrombin is biphasic and involves first signaling through PAR1 and subsequent signaling through PAR4 [22, 23]. In mouse platelets signaling occurs primarily via PAR4, and is facilitated by PAR3. Conflicting evidence exists Gi signaling in platelets downstream of thrombin can occur independently of thrombin. In addition to the PAR receptors, GP1bα has high affinity for thrombin. Absence of this receptor reduces responses to low doses of thrombin and diminishes PAR1 signaling, suggesting that this receptor facilitates signaling through the PARs [24, 25].

Ca\(^{2+}\) mobilization activates phospholipase A2 (PLA\(_2\)), which in turn converts membrane phospholipids to Arachidonic Acid. TXA\(_2\) is produced from membrane arachidonate by the aspirin sensitive cyclooxygenase (COX-1) enzyme [26]. TXA\(_2\) causes Ca\(^{2+}\) mobilization, aggregation, secretion, phosphoinositide hydrolysis and protein phosphorylation. TXA\(_2\) can diffuse across the membrane and activate nearby platelets, but its activity is limited by the molecule’s short half life (~30sec). Its effects in vitro can be studied using the stable analog U46619. This agonist signals through the TP receptors (α and β forms) [26] that signal through \(G_\alpha\) (Ca\(^{2+}\) mobilization) [19] and \(G_{13}\) (shape change) [20]. No Gi mediated inhibition of cAMP formation is caused via TP signaling.

The signaling events involved in the initial accumulation of platelets and the extension of the platelet aggregate are schematically represented in Figure 1.2-1.
Platelet signaling during development and growth of the thrombus.

Platelet activation during thrombosis is governed by several simultaneous activating signals. Activation by collagen happens predominantly via the receptor tyrosine kinase GPVI. Subsequent activation involves signaling through the GPCRs P2Y1 and P2Y12 (ADP), PAR1 and PAR4 (thrombin), TP (TXA2) and IP (PGI2). Receptors involved are shown in red. Under conditions of high shear, VWF allows the platelet to adhere to collagen via the GP1b receptor.
1.2.3 Stabilization of the platelet aggregate

Because of the close proximity of individual platelets, several close contacts are formed between the surface molecules of individual cells in the growing aggregate. This sets of additional waves of signaling in the platelets as well and supplies an additional source of adhesive force between the cells. Further, interactions between the intracellular domains of $\alpha_{\text{IIb}}\beta_3$ (bound to its ligands fibrinogen and VWF) and the platelet’s actin/myosin machinery causes the aggregate to retract upon itself [27], and thus prevent complete occlusion of the vessel.

Outside-in signaling occurs downstream of the formation of fibrinogen bridges that link activated platelets together. Several proteins interact with the cytoplasmic domains of $\alpha_{\text{IIb}}\beta_3$ to either regulate integrin activation or outside in signaling. Some of these include talin, myosin, C1B1 and the tyrosine kinases Src and Syk. Talin binding is one of the final events preceding activation. C1B1 can interact indirectly with actin via PAK. Fibrinogen binding to the extracellular domains of causes a rapid increase in the activity of Src family members and Syk. These lead to the phosphorylation of the residues Y773 and Y785 on the $\beta_3$ cytoplasmic domain of the integrin. These events are required for full aggregation, spreading and clot retraction. [28]

Platelet Specific Cell Adhesion Molecule (PECAM-1; CD31) is a cell adhesion molecule capable of homotypic interactions. The C-terminal of PECAM-1 is phosphorylated at tyrosine residues upon activation and can bind to the phosphatases Shp-1/2 [29], bringing these enzymes close to their substrates including the Fcγ chain of GPVI. This limits the extent of collagen signaling [30].
JAM (Junctional Adhesion Molecule)-A and C are cell adhesion molecules that localize to platelet-platelet and platelet-leukocyte contacts and mediate adhesive and signaling interactions between these cells. These molecules support heterotypic interactions by binding to integrins. Also JAM-A contributes to adhesion by forming *trans* interactions involving the N-terminal Ig domain [31, 32]. ESAM (Endothelial Cell Specific Adhesion Molecule) [33] and CD226 [34] two other members of the CTX family of immunoglobulin domain containing adhesion molecules detected on active platelet surfaces. SLAM (signaling lymphocyte activation molecule) [35] and CD84 [36] are members of the CD2 family of homophilic adhesion molecules expressed on platelets, and become phosphorylated on activation at platelet-platelet contacts.

Eph kinases are receptor tyrosine kinases that interact *in trans* with their cell surface ligands called ephrins triggering bidirectional signaling. Blockade of this interaction causes reversible platelet aggregation and limits the growth of platelet thrombi on collagen surfaces. Signaling downstream of these molecules promotes further integrin activation (by activating Rap1B) and integrin signaling (by phosphorylating the $\beta_3$ cytoplasmic tail of $\alpha_{IIb}\beta_3$) [37]. Similarly growth arrest specific gene - 6 (Gas-6) secreted from platelet $\alpha$ granules can serve as a ligand for receptor tyrosine kinases Tyro3, Axl and Mer and cause signaling that promotes platelet plug formation and stability [38]. Aggregation in Gas-6$^{-/-}$ platelets terminates prematurely, and produces a phenotype resistant to thrombosis [39].

In addition to the numerous proteins secreted from the platelet granules, platelets shed several surface molecules including GP1B$\alpha$, GPVI and P-selectin [40, 41]. Such shedding has been shown to be mediated by metalloproteases such as ADAM17 and
serves to limit signaling [40, 42]. CD40 ligand (CD40L) is a membrane protein that appears on the surface of activated platelets, and gradually releases an 18-kDa exodomain fragment [43]. The extracellular portion of CD40L can bind to their receptors (CD40) as well as integrin. Growth of platelet plugs in CD40L−/− show defective thrombus growth over collagen, decreased stability, and delayed occlusion. These effects are presumably mediated by integrin since loss of CD40 has no appreciable effect [44].

1.3 The blood coagulation cascade

Under normal conditions in vivo, the intact endothelium inhibits platelet activation and the chain of plasma reactions known as the coagulation cascade. Upon vascular damage, exposed collagen initiates platelet activation, and TF initiates the clotting cascade. The cascade involves a series of sequential enzymatic reactions, wherein at every step an inactive zymogen is converted into an active enzyme typically by the product of the previous step, ultimately leading to the formation of thrombin. With the exclusion of thrombin, such newly formed enzymes have low activity, until they bind to specific protein cofactors or suitable phospholipid surfaces (typically the active platelet).

This cascade is traditionally divided into “extrinsic” (or Tissue Factor) pathway and “intrinsic” (or contact) pathway leading to the formation of factor Xa. The portion of the cascade beyond factor Xa, leading to the formation of thrombin is referred to as the “common” pathway. These pathways happen in the presence of plasma inhibitors that limit the activity of the active proteases that are formed. These events are schematically represented in Figure 1.3-1. Downstream of thrombin, fibrinogen is polymerized during the process of fibrin formation, and the fibers of fibrin hold the loose platelet aggregate together. The formed fibrin strands are degraded during the process of fibrinolysis. [45]
The Coagulation cascade involves multiple sequential plasma reactions that lead to the generation of thrombin. Zymogens (red) are converted to active enzymes (green) by the products of the preceding reactions in the cascade, aided in some cases by active cofactors (light blue). Several plasma inhibitors (red) serve to inhibit the active proteases. The cascade is triggered either by the exposure of TF at the site injury (extrinsic cascade) or by activation of factor XII adsorbed onto negatively charged surfaces (intrinsic cascade). Both pathways ultimately lead to the formation of thrombin. Thrombin formed (in addition to other platelet agonists) can activate platelets. The active platelets provide catalytic surface for several of these reactions. Contact activation can be minimized in vitro by using Corn Trypsin Inhibitor (CTI) to block XIIa.

**1.3.1 The extrinsic pathway**

The predominant initiator of the coagulation cascade in vivo is the extrinsic system, involving exposure of the molecule Tissue Factor (TF) at the site of vascular damage. TF is normally absent from on cells in contact with plasma, but is present on a
variety of cells outside the vasculature [46]. TF exposure, allows it to bind to the activated form of one of the soluble clotting factors: factor VII. Factor VII is one of a group of vitamin K-dependent proteins (including factors IX and X, prothrombin and protein C) that is secreted by the hepatocytes and contains a $\gamma$-glutamyl carboxyl acid (Gla) residue at the N terminal of the molecule. This post-translational modification allows these molecules to bind calcium subsequently assemble on a phospholipid surface.

Once formed, the TF:VIIa complex can activates factor IX via limited proteolysis. Alternatively, TF:VIIa can directly activate X and this is the kinetically favored mechanism in vitro [47]. Activated factor IX can also activate factor X efficiently provided the cofactor activated factor VIII is present. This leads to the formation of the “tenase” complex critical for X activation. Absence of factor VIII or IX underlies the hemorrhagic symptoms of hemophilia A and B respectively [48].

Since all of serine proteases and protein cofactors (excluding TF) in the coagulation cascade predominantly circulate in plasma as inactive zymogens, it is puzzling how this cascade can be triggered via the assembly of inert precursors. It is known that approximately 1 % of total factor VII exists in circulation in the form of VIIa [49]. Proportions of the active forms of other enzymes are miniscule in comparison. Exposure of TF leads to the formation of both inactive TF:VII and active TF:VIIa complexes. Even the minute amounts of TF:VIIa (that can initially exist at circulating levels of VIIa) can set up autocatalytic feedback and activate VII bound to TF. Furthermore, a variety of enzymes including factors IXa, Xa, XIIa and thrombin have been shown to activate VII [50, 51], and this makes the cascade strongly self-amplifying.
1.3.2 The intrinsic pathway

The “intrinsic” ability of plasma to gel when placed in a glass tube, or more accurately “contact” with negative surfaces (glass, clay, kaolin) gives the intrinsic pathway its name.

For many years the contact pathway has been considered physiologically irrelevant because severe deficiency in the initiator of this cascade (Factor XII) causes no bleeding defect [52]. However recent experiments have revealed that mice deficient in Factor XII do indeed have a tendency to form unstable thrombi [53, 54]; suggesting that this often overlooked part of the cascade does indeed have an important physiological role.

This pathway is triggered by the activation of the zymogen factor XII upon contact with a negative surface. The heavy chain of factor XII binds to surfaces, leading to substantial increase in local enzyme concentration, autoactivation and activation of XIIa dependent prekallikrein (and its cofactor high molecular weight kininogen, HK) and factor XI. Subsequently, factor IX is activated by the novel dimeric serine protease factor XIA [55].

Kallikrein in turn cleaves HK to release bradykinin, a potent stimulus which links the coagulation and inflammatory pathways, and the remaining kinin free kininogen (activated HK) binds several fold better than intact HK. This allows more prekallikrein to associate with HK setting up a strong autocatalytic loop. Additionally Kallikrein can reciprocally activate factor XII [55].
This pathway is also characterized by negative feedbacks. Factor XIa can cleave the light chain of HK, which is responsible for the cofactor’s procoagulant activity. This in turn liberates XIa from the activating surface [56].

1.3.3 The common pathway

Factor Xa formed by either the extrinsic or intrinsic pathway converts prothrombin to thrombin. Prothrombin is another vitamin K dependent zymogen that requires calcium to bind to phospholipid surfaces. After cleavage by Xa, the N terminal Gla portion of prothrombin is removed and thrombin is formed. Thrombin occupies a central portion in the formation of the haemostatic plug and limits both its own formation and destruction by acting on multiple substrates including fibrinogen, factors V, VIII, XI, platelet receptors, and proteins S and C. The rate of thrombin formation is increased by more than 300,000 fold (in comparison to Xa alone) by the action of the prothrombinase complex (factors Xa, Va, phospholipid and calcium) on localized prothrombin [57]. Activated factor V is possibly supplied by platelet secretion of α-granule content following activation [58].

1.3.4 Coagulation inhibition

Plasma inhibitors serve to limit the extent of both the coagulation and the fibrinolytic systems. The major plasma inhibitors are C1 inhibitor, ATIII and TFPI.

C1 inhibitor is the major inhibitor of the contact system, and accounts for 95% of the plasma inhibitor capacity of factor XIIa and more than 50% of the inhibitory capacity of kallikrein [45]. α1-Antitrypsin is the major plasma inhibitor of XIa [59].
Antithrombin (ATIII) is one in a class of general serine protease inhibitors (serpins) that limits the action of most active proteases in the coagulation cascade. In particular ATIII strongly inhibits the actions of thrombin, factors IXa and Xa. ATIII deficiency is associated with a striking increase in the risk of thromboembolism. The catalytic site serine of thrombin reacts with an arginine of ATIII to form an inactive covalent complex. The affinity of ATIII for thrombin is strongly enhanced, by the binding of a basic group in ATIII to heparin (a highly negatively charged sulfated polysaccharide that exists on the endothelial surface) [60]. Heparin cofactor II is a serpin that selectively inactivates thrombin in the presence of heparin or dermatan sulfate [61]. Tissue factor pathway inhibitor (TFPI) is a protein that in association with factor Xa, inhibits TF:VIIa [62].

$\alpha_2$-Macroglobulin is a secondary inhibitor of several coagulant and fibrinolytic enzymes including kallikrein, thrombin and plasmin. Enzymes trapped in the cage structure of this inhibitor are protected from other inhibitors [63]. $\alpha_2$-Antiplasmin is the primary inhibitor of plasmin and serves to limit the fibrinolytic response till completion of wound healing [64]. Protein C inhibitor is also a serpin that can function as a procoagulant molecule by inhibiting the action of protein C [65].

### 1.3.5 Fibrin formation

The formation of fibrin strands stabilizes the loose platelet aggregate at the site of injury. Fibrin is formed by the action of thrombin on fibrinogen, a protein present in abundance both in plasma and platelet granules. Thrombin binding to the central domain of fibrinogen liberates fibrinopeptides A and B, resulting in fibrin monomer and polymer formation. Progressive lengthening of the polymer occurs by half overlap, side to side of
fibrin monomers, thus forming long, thin, fibrin strands. The degree of lateral polymerization regulates the tensile strength of the clot. Factor XIIIa serves to cross link these loose fibrin strands and confers resistance to degradation by plasmin [66].

The fibrin mesh holds platelets together and attaches them to the vessel wall, by binding to platelet receptor glycoproteins and by interacting with other adhesive proteins like thrombospondin and fibronectin. After binding to platelet binding sites, these proteins serve as molecular bridges between plasma proteins and the platelet interior, between platelets and the vessel wall, and between plasma fibrin fibers and the subendothelial matrix. For instance, fibronectin is crosslinked by XIIIa to fibrin [67], and its separate binding site for collagen [68] bridges fibrin and the vessel wall. VWF serves as a bridge between platelet membrane and the subendothelium (via platelet membrane GP1B and αIIbβ3) [69]. Additionally, platelet αIIbβ3 joins fibrinogen to intracellular actin, thereby controlling clot retraction and vessel wall constriction [70].

Several mechanisms exist to exist to limit and localize the extent of thrombosis. Firstly, the shear forces of blood flow tear away loosely attached clumps of platelets [71]. Secondly, thrombin in conjunction with thrombomodulin on the endothelial surface, acts as a receptor for protein C. Activated Protein C is a potent inhibitor of factors V and VIII [72]. Soluble pro-coagulant molecules like Xa or thrombin that advect away from the site of the growing clot are efficiently inhibited by plasma inhibitors like ATIII [60].

1.3.6 Fibrinolysis

Fibrinolysis, along with endothelial cell regrowth and vessel regrowth are repair mechanisms set in motion following thrombus formation. Similar to the coagulation cascade, fibrinolysis involves zymogen to enzyme conversions, autoactivation and
inactivation. The inactive precursor plasminogen is present at high concentrations. During the initial process of thrombus formation, activated platelets and endothelial cells release plasminogen activator inhibitors that allow fibrin formation to continue unhindered. However, once the vessel wall is sufficiently repaired, endothelial cells release tissue plasminogen activator, which in turn activates plasminogen to plasmin [73].

Plasmin contributes to its own activation by cleaving an activation peptide from plasminogen, making it more susceptible to surface binding and activation by plasminogen activators. Also plasminogen bound to fibrin, by lysine binding sites on its kringle structures has much higher reactivity. The small proportion of plasma fibrinogen adhered onto fibrin can lead to clot dissolution [74]. There exists a delicate balance dictated by the position and relative abundance of profibrinolytic plasminogen and plasminogen activator molecules and antifibrinolytic α2-antiplasmin molecules (covalently attached via factor XIIIa action) assembled on the fibrin strands. Soluble fibrin degradation products like D-dimers are released into the circulation following fibrinolysis and serve as clinical markers of underlying thrombotic activity [75]. Active plasmin molecules released into circulation are quickly degraded in solution by α2-antiplasmin [76], thus limiting fibrinolysis to the site of the thrombus.

Thus the entire process of clot formation and dissolution is finely regulated by numerous plasma clotting factors, cellular components including endothelial cells and platelets, thrombus specific and distributed inhibitory mechanisms, as well as subendothelial components. This intricate balance of interlinked mechanisms ensures a robust response that prevents blood loss following injury, but is one that does not occlude
blood flow by uncontrolled thrombus growth. Disruption of any portion of these processes can lead to fatal hemorrhagic or thrombotic disorders.

1.4 Outline

Previous studies have often looked at individual parts of the thrombotic response in isolation. In contrast, this dissertation takes a “systems approach” to thrombosis integrating experimental and theoretical studies. This work quantitatively characterizes both the coagulation component of the clotting response leading to the formation of thrombin via sequential plasma reactions, as well as the cellular component involving platelet activation. Fibrinolysis has not been considered. High throughput experimental systems were first developed to efficiently probe the biology of these pathways across several dimensions. Models were then developed based on these experimental observations. In the second chapter, a study of the kinetics of thrombin formation in resting and activated human blood is presented. Thrombin formation is studied in the presence of a fluorogenic thrombin substrate (Boc-VPR-MCA), and a mechanism based ordinary differential equation (ODE) model is developed based on the framework of the Hockin-Mann reaction network. In the third chapter, a study of platelet activation is presented. “Pairwise Agonist Scanning” (PAS) was developed to predict higher order interactions amongst several simultaneous signals from a high throughput experiment sampling of all possible first and second order signals. Data driven Neural Network models were trained on PAS datasets to make donor specific predictions of platelet signaling. In the final chapter extensions of this work are suggested.
Chapter 2

Kinetics of thrombin generation in resting and activated human blood

2.1 Abstract

Blood function defines bleeding and clotting risks and dictates approaches for clinical intervention. Independent of adding exogenous tissue factor (TF), human blood treated in vitro with corn trypsin inhibitor (CTI, to block Factor XIIa) will generate thrombin after an initiation time ($T_i$) of 1 to 2 hr (depending on donor), while activation of platelets with the GPVI-activator convulxin reduces $T_i$ to ~20 min. Since current kinetic models fail to generate thrombin in the absence of added TF, we implemented a Platelet-Plasma ODE model accounting for: the Hockin-Mann protease reaction network, thrombin-dependent display of platelet phosphatidylinerine, VIIa function on activated platelets, XIIa and XIa generation and function, competitive thrombin substrates (fluorogenic detector and fibrinogen), and thrombin consumption during fibrin polymerization. The kinetic model consisting of 76 ordinary differential equations (76 species, 57 reactions, 105 kinetic parameters) predicted the clotting of resting and convulxin-activated human blood as well as predicted $T_i$ of human blood under 50 different initial conditions that titrated increasing levels of TF, Xa, Va, XIa, IXa, and VIIa. Experiments with combined anti-XI and anti-XII antibodies prevented thrombin production, demonstrating that a leak of XIIa past saturating amounts of CTI (and not “blood-borne TF” alone) was responsible for in vitro initiation without added TF. Clotting was not blocked by antibodies used individually
against TF, VII/VIIa, P-selectin, GPIb, protein disulfide isomerase, cathepsin G nor blocked by the ribosome inhibitor puromycin, the Clk1 kinase inhibitor Tg003, or inhibited VIIa (VIIai). This is the first model to predict the observed behavior of CTI-treated human blood, either resting or stimulated with platelet activators. CTI-treated human blood will clot in vitro due to the combined activity of XIIa and XIa, a process enhanced by platelet activators and which proceeds in the absence of any evidence for kinetically significant blood borne tissue factor.

2.2 Introduction

Blood coagulation is a surface catalyzed protease cascade with autocatalytic amplification and multiple modes of inhibition [77]. Clotting in vivo is triggered by exposed tissue factor (TF) during vascular injury or plaque rupture. There is a striking sensitivity of coagulation to picomolar levels of TF [78]. Considerable uncertainty exists concerning the relative roles of circulating concentrations of TF [79, 80] and the mechanism(s) of platelet display of active TF via synthesis [80, 81], de-encryption [82] or changes in membrane composition [83]. Some of these mechanisms are illustrated in Figure 2.2-1. The detection of circulating concentrations of activation peptides, such as F1.2 and fibrinopeptides A and B, inactivation products such as thrombin-antithrombin (TAT) [84-87] and activated factors such as VIIa and activated Protein C (APC) is proof of some low level of activated proteases in normal individuals coexisting with flowing blood in vivo. Exploring this endogenous “engine-idling” active state of blood ex vivo is central to clinical diagnostics.
Figure 2.2-1 Triggers of the extrinsic cascade

Although TF exposure at the site of vascular injury is considered to be the predominant trigger of in vivo coagulation (Also see Figure 1.1-1), several other controversial explanations have been proposed in existing literature that could account for initiation in the absence of exogenous TF. “Bloodborne TF”, “Platelet synthesized TF (either by releasing preformed TF molecules or translating new protein from existing mRNA)”, “de-encryption” of cryptic TF, and TF independent VIIa activity (using platelet GP1b as cofactor) have all been proposed as possible mechanisms.

Once initiated with TF, the coagulation cascade proceeds by sequential enzymatic steps to produce thrombin, which in turn polymerizes fibrinogen to fibrin to form the protein polymer mesh of the thrombus. Thrombin also has complex regulatory action by activating platelets and further enhancing thrombin formation by activating V, VII, VIII, and XI. In vivo, thrombin can limit its own production by binding thrombomodulin and
converting protein C to activated protein C (APC), which subsequently inhibits Va and VIIIa. Upon activation, platelets express anionic phospholipids on their surface, an event that is critical for several surface bound reactions of the cascade [88].

Given the central role of thrombin in clotting, much effort has focused on experimentally detecting the transients of thrombin generation [89]. ‘Calibrated Automatic Thrombography’ measures thrombin formation by following the cleavage of more than 100 μM of the thrombin substrate Z-Gly-Gly-Arg-MCA [90]. However, the use of this fluorogenic substrate has been shown to interfere with the kinetics of the cascade by competitive inhibition of thrombin [91]. Furthermore, inhibitory influences of anticoagulants such as citrate may influence conclusions drawn from experiments with whole blood [92]. Complications due to artificial material activation of the contact pathway in vitro can be minimized by the inhibition of the contact system through the use of CTI to inhibit XIIa [93].

We have built upon the existing Hockin-Mann reaction network of the extrinsic coagulation cascade [94] to predict the function of blood studied in vitro with contact pathway inhibition. The Hockin-Mann model is a homogenous ordinary differential equation (ODE) reaction scheme that assumes a maximally activated platelet at $t = 0$. The Hockin-Mann model predicts accurately the initiation times of blood coagulation at picomolar levels of TF, but breaks down at zero or sub-picomolar levels of exogenously added TF [95]. Other models have attempted to consider the platelet as a separate entity which exists in discrete activation states and contributes binding sites once activated for the catalysis of several coagulation reactions [96, 97]. Such approaches have shed light on the critical importance of platelet activation mediated changes on the reaction surface.
area, the fragility of the cascade to thrombin induced activation of the platelet, stochastic effects at low molecular counts and experimentally observed variability in the initiation of clotting. However, these models still do not explain why blood drawn into CTI will clot without exogenous addition of TF.

Quantitatively, the systems biology analysis of blood clotting must account for strong autocatalytic feedback, nonlinearity of kinetic rates, and extreme sensitivity to initial conditions, specifically to help predict system outcomes that lead to insufficient or overactive responses (i.e. bleeding or thrombosis). The present study explores the stability of normal donor blood (i.e. what tips the balance towards clotting) as indicated by prolongation or reduction of the clotting time. We have developed and validated a high throughput assay to measure thrombin production initiation in static blood under minimal inhibitory influences of fluorogenic substrate and anticoagulant. We used this assay to examine several possibilities in existing literature that could account for initiation without exogenous TF and conclude that a leak of XIIa past saturating CTI is responsible for such initiation. Further, our studies demonstrated blood’s graded sensitivity to exogenously added TF, IXa and XIa; a dramatic switch like response to exogenously added Xa; little discernible effect of exogenously added Va; and statistically significant VIIa activity (independent of TF) when used above nanomolar concentrations. From a systems biology perspective that seeks to accommodate patient-specific variations, modeling can help address central questions regarding: (i) the regulation of response to perturbation, (ii) the stability of blood in vivo or ex vivo in the face of zero or near-zero levels of active TF or active proteases, and the (iii) safe pharmacological alteration of blood function.
2.3 Materials and methods

2.3.1 Materials

The fluorogenic thrombin substrates Boc-Val-Pro-Arg-methylcoumarinamide (Boc-VPR-MCA) and Z-Gly-Gly-Arg methylcoumarinamide (Z-GGR-MCA) were obtained from Bachem (King of Prussia, PA). Lipidated recombinant TF (baculovirus expressed, amino acids 1-263; 43kDa), monoclonal anti-human FVII/VIIa, and TF ELISA kits were obtained from American Diagnostica (Stamford, CT) and used to determine the available TF antigen concentration of 18.2 nM. Monoclonal anti human FXI and FXII were obtained from Enzyme Research Laboratories (South bend, IN). Monoclonal anti human P-selectin was obtained from R&D Systems (Minneapolis, MN). Monoclonal anti human Gp1bα was obtained from Lifespan Biosciences (Seattle, WA). Polyclonal anti human cathepsin-G was obtained from Molecular Innovations (Novi, MI). Monoclonal anti human PDI was obtained from was obtained from Affinity Bioreagents (Rockford, IL). Thrombin-antithrombin ELISA kits were obtained from Dade Behring (Deerfield, Illinois). Active site inhibited VIIa (VIIai) was gifted by Novo-Nordisk, Denmark. Other reagents included: adenosine diphosphate (ADP), benzamidine hydrochloride, EDTA, HEPES, NaCl, NaOH, sodium citrate, apyrase, PGE2, Tg003 and Puromycin (all from Sigma, St. Louis, MO); corn trypsin inhibitor (CTI), Phe-Pro-Arg-chloromethylketone (FPRCK), human α-thrombin, polyclonal anti-human TF, Xa, Va, VIIa, IXa, XIIa, human plasmas deficient in prothrombin and factors V, VII, XI or XII (all from Hematological Technologies, Essex Junction, VT); convulxin (CVX, Centerchem, Norwalk, CT); and black 384-well plates (Corning, Corning, NY). All reagents were stored and prepared according to the manufacturers’ recommendations.
The buffer used for all dilutions was HEPES buffered saline (HBS, sterile filtered 20 mM HEPES and 140 mM NaCl in deionized water adjusted to pH 7.4 with NaOH).

2.3.2 Blood collection and preparation of washed platelets

Unless otherwise noted, blood was collected in a syringe containing 1 part sodium citrate to 9 parts blood and 50 μg CTI/mL. To obtain washed platelets for the experiments in Figure 2.4-10, blood was first centrifuged at 120g for 12 min to obtain platelet rich plasma (PRP). PRP was then treated with 200 μM PGE2 and 1 unit/ml apyrase to prevent activation during subsequent spinning. A platelet pellet was obtained by centrifuging PRP at 800g for 10 min and this pellet was resuspended in HBS to yield a final concentration of ~3×10⁸ platelets/ml.

2.3.3 High throughput experimentation

Prior to phlebotomy, all reagents were prepared in a 384-well plate using a Thermo Multidrop and a Perkin-Elmer JANUS workstation. For all experiments, blood was added to the whole (or segments) of a well plate using a PerkinElmer Evolution P³. To ensure a simultaneous activation time, citrated blood was recalcified in all 384 wells simultaneously, yielding a final added calcium concentration of 10 mM. To detect thrombin activity with time, we added the fluorogenic substrate (SIIa), Boc-VPR-MCA (10 μM) to every well and detected the fluorescence of the released aminomethylcoumarin (AMC) with Thermo Fluoroskan fluorimeters preheated to 37 °C. Each well was read once per minute for 4 hr. After 4 hours, 5 U thrombin/well was added and then the plate was read again once per minute for 20 minutes to determine the maximal signal. This procedure is illustrated in Figure 2.3-1.
Figure 2.3-1 High Throughput Experimental Procedure to study coagulation

(A) Experimental protocol. Calcium and the fluorogenic thrombin substrate (S\textsubscript{IIa}) were added to the 384-well plate on a Thermo Multidrop. The plate was placed on a Perkin-Elmer Janus where various concentrations of each individual species were added to each well. After phlebotomy, the plate was moved to a Perkin-Elmer Evolution P\textsuperscript{3} where the blood was added to all wells simultaneously (\(t = 0\)). The plate was read in a Thermo-Electron Fluoroskan where the fluorescence was measured for 4 hr. The time from vein to first measurement was under 5 min. (B) Initiation Time. The time required to reach 5% conversion of the fluorogenic substrate was set as the initiation time (\(T_i\)). Relative prolongation or reductions in \(T_i\) were used to quantify coagulation initiation. (Thanks to Bill Denney for his contribution in developing this assay).
In experiments to study the effect of fluorogenic substrate concentration, Boc-VPR-MCA or Z-GGR-MCA were used at final concentrations between 1 and 100 μM. All experiments were carried out in 5x diluted whole blood. Fractional conversion of the fluorogenic substrate was determined according to:

\[ C = \frac{F - F_{\text{min}}}{F_{\text{max}} - F_{\text{min}}} \]  

(2.3.1)

where \( C \) is the fractional conversion, \( F \) is the instantaneous fluorescence at any time, \( F_{\text{min}} \) is the minimum fluorescence for that well, and \( F_{\text{max}} \) is the maximum fluorescence in that well during either the experiment or after thrombin addition. The initiation time (\( T_i \)) was set when \( C \) rises past 0.05.

### 2.3.4 Thrombin-Antithrombin (TAT) immunoassays

TAT serves as a measure of the cumulative thrombin production. Citrated blood was dispensed into all wells of a 384-well plate containing calcium using a PerkinElmer Evolution P3 liquid handling station. Reactions were manually stopped in columns of the well plate by adding a ‘stop cocktail’ of 50 mM EDTA, 20 mM benzamidine and 100 μM Phe-Pro-Arg-chloromethyloleth ketone (PPACK) at specific time points [98]. The plate was spun at 120g for 12 min to separate out plasma, which was subsequently assayed for TAT by ELISA (Dade Behring).

### 2.3.5 Simulation

Simulations were performed in Matlab R2008b (version 7.7.0.471) using the Systems Biology Toolbox 2 (SBTOOLBOX2) with the SBPD extension package ODE solver with an absolute tolerance of 10^{-30} and a relative tolerance of 10^{-7} [99].
A function of thrombin concentration was fit with a Hill function to published experimental data that quantified ‘Platelet Activation Status’ by reporting surface Phosphatidylserine exposure (measured by fold increase in Annexin V binding) in response to thrombin (IIa) [100] (Figure 2.3-2):

\[
f([IIa^*(t)]) = \frac{[IIa^*(t)]^{1.6123}}{[IIa^*(t)]^{1.6123} + (2.4279 \times 10^{-5})^{1.6123}}
\]  

(2.3.2)

where \([IIa^*(t)] = \max_{t' \in [0,t]} ([IIa(t')])\). Since thrombin concentration physiologically starts decreasing after monotonically rising to a peak, it was necessary to use the maximum transient thrombin concentration \(\max_{t' \in [0,t]} ([IIa(t')])\), to ensure that \(f([IIa^*(t)])\) never decreases once it has reached its maximum magnitude. \(IIa^*(t)\) is exactly equivalent to \([IIa(t)]\) until \([IIa(t)]\) reaches its peak, whereafter \([IIa^*(t)]\) is constant at that maximum value. This ensures that the platelet stays activated even when thrombin declines.

![Figure 2.3-2 Definition of \(\varepsilon_{\text{max}}\) for a given thrombin concentration](image)

Phosphatidylserine exposure measured by fold increase in annexin V binding was obtained from published values [100] and are shown in blue circles. The maximum platelet activation state attainable at a given thrombin concentration (\(\varepsilon_{\text{max}}\)) was obtained by fitting a hill function to this data (green line).
For a given [IIa], we define the maximum platelet activation state, $\varepsilon_{\text{max}}$:

$$\varepsilon_{\text{max}} = \varepsilon_{\text{max}_0} + (1 - \varepsilon_{\text{max}_0}) \times f([\text{IIa}^*(t)])$$

(2.3.3)

where $\varepsilon_{\text{max}}$ describes the fractional activation state of the platelet. $\varepsilon_{\text{max}_0}$ defines the basal activation state of the platelet at $t=0$, and is set to 0.01 in most simulations (assuming a basal 1% binding strength of coagulation factors to the resting platelet surface). For full activation of platelets at [thrombin] $> ~10$ nM, $\varepsilon_{\text{max}}$ equals 1 and protein dissociation is minimized (Figure 2.3-2).

The instantaneous platelet activation state $\varepsilon$ is governed by the differential equation

$$\frac{d\varepsilon}{dt} = k(\varepsilon_{\text{max}} - \varepsilon)$$

(2.3.4)

with the initial condition at $t = 0, \varepsilon = 0.01$, this solves to,

$$\varepsilon = \varepsilon_{\text{max}} - (\varepsilon_{\text{max}} - 0.01) \times \exp(-kt)$$

(2.3.5)

The constant $k$ is inversely proportional to the time scale of platelet activation, and was set to 0.005. This is consistent with the fact that it takes $\sim 200$ s for platelets to mobilize calcium from intracellular stores upon stimulation with thrombin or other platelet agonists [101]. Such a form of the platelet function ($\varepsilon$) ensures that the platelet achieves its maximum attainable activation state ($\varepsilon_{\text{max}}$) not instantaneously, but on a physiologically relevant timescale. Transients of $\varepsilon$ are shown for values of $\varepsilon_{\text{max}} = 0.25$, 0.5, 0.75 and 1.0 are shown in Figure 2.3-3.

To account for changes in the reaction rates with platelet activation, we modified the Hockin-Mann model rate constants as follows,

$$k_{\text{modified}} = k_{\text{original}} \times (\eta / \varepsilon)$$

(2.3.6)
where $\eta$ was a parameter that was used to alter the magnitude of the rate constant used in the Hockin-Mann model. Many of the Hockin-Mann model parameters were originally fitted empirically to global experimental data. For $\eta = 1$ and full platelet activation ($\varepsilon = 1$), the modified value becomes the original value. For $\eta \neq 1$, the modifications may be regarded as further fits to experimental data, consistent with published values of rate constants (footnotes to Table 2.4-1).

![Figure 2.3-3 Representative transients of $\varepsilon$ for $\varepsilon_{\text{max}} = 0.25, 0.5, 0.75$ and 1.0](image)

The instantaneous platelet activation status ($\varepsilon$) approaches its maximum attainable value ($\varepsilon_{\text{max}}$) on a time scale consistent with the time it takes for the platelet to mobilize intracellular calcium. Shown are $\varepsilon$ transient profiles at various values of $\varepsilon_{\text{max}}$.

To estimate the sensitivity of our model’s output to the choice of kinetic constants, a global sensitivity analysis of the models output thrombin concentration at 0 and 10 pM TF concentration was carried out by the method of weighted averaging of local sensitivities. The results of this analysis are shown in Figure 2.4-15 A and B; Table 2.4-3 and Table 2.4-4.
2.4 Results

We have used a high throughput fluorogenic assay to measure coagulation initiation in 384-well plates where 5x-diluted, citrated human blood (CTI treated) was recalcified within 5 min of venipuncture (Figure 2.3-1 A). A level of 5% cleavage of the fluorogenic substrate Boc-VPR-MCA was used as the coagulation initiation time ($T_i$). A large burst in thrombin production was always detected within minutes after $T_i$. Relative prolongation or reductions in $T_i$ were used to quantify coagulation initiation in response to varying conditions (Figure 2.3-1 B). The observed bursts in fluorescence were completely abolished by the thrombin inhibitor PPACK, and were not observed in prothrombin deficient (platelet supplemented) plasma (not shown), proving that we were indeed detecting thrombin and not some other non specific protease mediated cleavage of the fluorogenic substrate.

The Platelet-Plasma model (Figure 2.4-1, Table 2.4-1 and Table 2.4-1) was built on the existing framework of the Hockin-Mann model [94] and our experimental observations. During the course of thrombin generation in the well plate, platelets become activated and this activation was modeled using a coarse-grained description of platelet function by equating platelet activation ($\varepsilon$) to phosphatidylserine (PS) exposure as a function of thrombin concentration (Figure 2.3-2 and Figure 2.3-3). The complete topology of the model, its constituent reactions, and initial conditions are shown in Figure 2.4-1, Table 2.4-1 and Table 2.4-2. (Also see Supporting Information of [102] for full listing of ODEs and reactions). Justifications for alterations to kinetics are explained in the footnotes to Table 2.4-1.
Figure 2.4-1 Schematic of the Platelet-Plasma model.

A Wiring diagram of the Platelet-Plasma model is shown above. Blue portions represent additions to the Hockin-Mann model [94]. Binding and unbinding equilibriums are shown in black. Reactions highlighted in pink occur on the activated platelet, and the respective unbinding reaction rates decrease by two orders of magnitude during the course of platelet activation. Proteolysis reactions are represented by the green arrows. Inhibition reactions are shown in red.
<table>
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<th>Rxn</th>
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<th>( k_1 )</th>
<th>( k_{-1} )</th>
<th>( k_{\text{cat}} ) (s(^{-1}))</th>
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<th>( \eta )</th>
<th>Platelet-Plasma Model ((\epsilon = 1) K_m ) or ( K_d ) (M)</th>
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<td>$4.9 \times 10^7$</td>
<td>[128, 129]</td>
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<td>[110, 132, 133]</td>
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<td>$9 \times 10^{-9}$</td>
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<td>[134]</td>
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<td>50</td>
<td>Fbg + IIa ↔ Fbg = IIa → Fbn1 + IIa + FPA</td>
<td>$1.0 \times 10^8$ M⁻¹ s⁻¹, 636 s⁻¹, 84</td>
<td>-</td>
<td>$7.2 \times 10^{-6}$</td>
<td>[135]</td>
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<td>51</td>
<td>Fbn1 + IIa ↔ Fbn1 = IIa → Fbn2 + IIa + FPB</td>
<td>$1.0 \times 10^8$ M⁻¹ s⁻¹, 742.6 s⁻¹, 7.4</td>
<td>-</td>
<td>$7.5 \times 10^{-6}$</td>
<td>[135]</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>52</td>
<td>2Fbn1 ↔ (Fbn1)₂</td>
<td>$1.0 \times 10^6$ M⁻¹ s⁻¹, 6.4 x $10^{-2}$</td>
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<td></td>
<td>[135]</td>
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<td>(Fbn1)₂ + IIa ↔ (Fbn1)₂ = IIa → (Fbn2)₂ + IIa + FPB</td>
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<td>-</td>
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<td>[135]</td>
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<td>$k_2$ $s^{-1}$</td>
<td>$k_3$</td>
<td>$k_4$ $M^{-1} s^{-1}$</td>
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<td></td>
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<td>54</td>
<td>$Fbn2 + IIa \leftrightarrow Fbn2 = IIa$</td>
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<td>$1.0 \times 10^7$</td>
<td>-</td>
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<tr>
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<td>$5.0 \times 10^3$</td>
<td>-</td>
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<td>56</td>
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<td>$1.6 \times 10^4$</td>
<td>$1.0 \times 10^5$</td>
<td>-</td>
<td>$1.6 \times 10^4$</td>
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<td>57</td>
<td>$Fbn2 = IIa + ATIII \rightarrow Fbn2 = IIa: ATIII$</td>
<td>$1.0 \times 10^4$</td>
<td>$1.0 \times 10^5$</td>
<td>-</td>
<td>$1.0 \times 10^4$</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Table 2.4-1 Reactions used in the Platelet-Plasma model.

**Rxn 1.** Bach *et al.* [103] report a decrease in $K_H$ from 14.9 to 0.58 nM as % phosphatidylserine (PS) increases from 0 to 40.

O’Brien *et al.* [104] report a $k_{assoc}$ of $3.14 \times 10^5$ M$^{-1}$ s$^{-1}$ and a $k_{diss}$ of $6.29 \times 10^{-4}$ s$^{-1}$, which yields a $K_d$ of ~2 nM. With our choice of $\eta$, $K_d$ is comparable to this value.

**Rxn 2.** Shaw *et al.* [105] report a decrease in $K_d$ from ~60 pM to ~10 pM as % PS increases from 10-40. on TF liposomes and a decrease from ~90 pM to ~10 pM as % PS increases from 10-70 on TF nanodiscs.

**Rxn 6.** Shaw *et al.* [105] also report an ~20x decrease in $K_m$ from ~400 nM to 20 nM for X activation as % PS increases from 10 to 40. Baugh *et al.* [106] report an experimental $K_m$ of 238 nM on 25% PS vescicles.

**Rxn 7.** The product inhibition of TF:VIIa by Xa is dependent on local Xa concentrations. Given that Xa and X binding to a PSPC bilayer increases hyperbolically with $K_d$s of 53.9 and 34.2 nM respectively [107] and that the TF:VIIa:X complex is strengthened with increasing PS content (see 6), it is reasonable to assume that the TF:VIIa:Xa complex is also strengthened with increasing PS levels.

**Rxn 8.** Beals *et al.* [108] report that the $K_d$ for bovine IX binding to lipid surface at optimum [Ca$^{2+}$] decreases from 4.9 to 1.7 $\mu$M as % PS increases from 20 to 50. Given that the formation of TF:VIIa is favoured with increasing PS content (See 2) and analogous to the increased strength of the TF:VIIa:X complex with increased PS (see 6), we expect that the formation of TF:VIIa:IX will also increase with increasing PS content.

**Rxn 11.** Jenkins *et al.* [109] report a decrease in $K_d$ from 351 nM to 4 nM on PCPS vesicles. Neuenschander and Jesty [110] report a $K_d$ of 74 pM on activated platelet surfaces as opposed to 550 pM on equimolar PSPC vesicles. A $K_d$ of 10 pM on the activated platelet was required to fit the shape of the IXa titration in Figure 2.4-11 B.

**Rxn 12.** Rawala Sheikh *et al.* [111] report a decrease in $K_m$ from 45$\mu$M to 160nM from using unactivated to activated platelets respectively.
Rxn 13. Fay et al. [112] report a $K_d$ value of \(-260\) nM for this interaction at pH 7.4 in the absence of phospholipids. Fay et al. [113] report that this interaction is stabilized by the presence of phospholipid. In the Platelet-Plasma model this dissociation constant changes from 270 nM to 2.7 nM as the platelet activates.

Rxn 17. Lindhout et al. [114] report a decrease in $K_d$ from 3.3 nM in solution to 30 pM using 10$\mu$M 40% PS.

Rxn 18. Rosing et al. [115] report a decrease in $K_m$ from 34.5 to 0.21 $\mu$M using 7.5 $\mu$M phospholipids.

Rxn 20. Huang et al. [116] report a decrease in $K_i$ from 85.2 to 65.2 pM on using phospholipids.

Rxn 21. Given that the TF:VIIa:Xa (see 7) and the Xa:TFPI (see 20) complexes are strengthened on phospholipids, we expect the stabilization of the TF:VIIa:Xa:TFPI complex with the exposure of phospholipids as the platelet activates.

Baugh et al. [106] report an off rate $3.6 \times 10^{-4}$ s$^{-1}$ for Xa unbinding Xa:TFPI, and on rates experimentally determined to be $9.0 \times 10^5$ M$^{-1}$s$^{-1}$, or numerically estimated to range between $6.8 \times 10^5$ and $1.35 \times 10^6$ M$^{-1}$s$^{-1}$. Their data imply that these constants are comparable to those for the binding of TF:VIIa:Xa to TFPI (ie a $K_d$ between $2.66 \times 10^{-10}$ and $5.29 \times 10^{-10}$ M). The original constants for this reaction in the Hockin-Mann model were fitted empirically, but their choice of constants results in a far stronger complex than can be reasonably expected from literature. Hence we have increased $K_d$ by two orders ($\eta = 100$) of magnitude from their reported value.

Rxn 28. Experimentally determined by fitting initial velocities of AMC release to standard Michaelis-menton kinetics.

Rxn 29. XII activation was coarse grained by assuming a first order dependence on XII concentration and estimating a rate of production ($5 \times 10^{-4}$s$^{-1}$) that would resolve the disparity between the Hockin-Mann model prediction and the experimentally observed control.

Rxn 30. Kinetics of XIIa autoactivation (in the presence of negatively charged dextran sulfate) was from Tankersley et al. [117]. Griep et al. [118] showed that the
autoactivation (and Kallikrein activation, See 32) of XII is strongly promoted by negatively charged sulfatides.

**Rxn 31.** Kinetics of Pre-Kallikrein activation by β-XIIa (in the presence of dextran sulfate) was from Tankersley *et al.* [117]. Pre-Kallikrein activation by XIIa was shown to be facilitated by negatively charged phosphoinositides [119].

**Rxn 32.** Kinetics of XII activation by Kallikrein (in the presence of dextran sulfate) was from Tankersley *et al.* [117]. Walsh and Griffin [120] showed that this reaction is sped up by the presence of activated platelets.

**Rxn 33.** Kinetics of second order Kallikrein autoactivation was from Tans *et al.* [121].

**Rxn 34.** The pseudo first order rate constant for the inhibition of Kallikrein in plasma (by C1 inhibitor, α2-macroglobulin and ATIII) was obtained from Van-Der-Graaf *et al.* [122].

**Rxn 35.** Hojima *et al.* [93] report a $K_i$ of 24 nM for the inhibition of XIIa by CTI.

**Rxns 36 and 37.** Kinetics of XIIa inhibition by C1 inhibitor and ATIII were from Pixley *et al.* [123]. C1 inhibitor is the primary inhibitor of XIIa. ATIII inhibition (although minor) was considering for consistency with other inhibitory reactions.

**Rxn 38.** Rate constants (in solution) for this reaction are from Gailani *et al.* [124]. Some controversy exists over the physiological surface for this reaction. Oliver *et al.* [125] showed that this reaction happens physiologically on the activated platelet surface. However several seminal papers by Baglia-Walsh *et al.* in the laboratory of Peter N. Walsh which originally proposed that this mechanism happens on the active platelet have subsequently been retracted. We therefore chose not to include a dependence of this reaction on $\varepsilon$.

**Rxn 39.** Rate constants (in solution) for this reaction are from Gailani *et al.* [124]. Walsh and Griffin [120], showed that this reaction is sped up by the presence of activated platelets.

**Rxn 40.** Several authors describe this mechanism of XI auto-activation (See for example [124, 136]). However, following the retraction of (Baglia *et al.* JBC 2000) we are not aware of an experimental report of the kinetics of this reaction. Kramoroff *et al.* [126] estimate the second order rate constant of this reaction to be 3.19 $\mu$M$^{-1}$s$^{-1}$ by optimizing an ODE model of the intrinsic cascade to experimental
measurements of APTT. They consider either XI autoactivation or XI activation by thrombin (but not both possibilities) as plausible mechanisms for XI activation (in addition to activation by XIIa), thus their estimated value is likely an overestimate. We utilized a value 4 fold lower than the value they report for this constant, since we consider thrombin activation of XI in addition to autoactivation. This was in keeping with the experimental titration of XIa (Figure 2.4-11 C) where we have noticed strong sensitivity to even minute amounts of XIa.

**Rxns 41 - 44.** Rate constants are for inhibition of XIa in plasma are from Wuillemein et al. [127]

**Rxn 45.** Rate constants (in solution) for this reaction are from Walsh et al. [128]. Gailani et al. [129] propose a mechanism by which this reaction could happen on the platelet surface facilitated by the dimeric form of factor XI.

**Rxn 46.** Rawala - Sheikh et al. [111] report a reduction in $K_m$ from 45 $\mu$M to 390 nM from unactivated to thrombin activated platelets. In later publications from the same lab, Scandura and Walsh [130] report a $K_m$ of 16nM and a $k_{cat}$ of $5.1 \times 10^{-4}$ for the activation of X by IXa alone on SFLLRN activated platelets in a model where platelet bound IXa interacts with zymogen X, and Wilkinson et al. [131] report a $K_m$ of 6.4 nM and a $k_{cat}$ of $7.0 \times 10^{-4}$.

**Rxn 47.** Rate constants were obtained from Leipold et al. [132] using catalytic efficiencies reported in Lollar et al. [133]. Activation of VIII by Xa, unlike activation by thrombin (reaction 10) was reported to be markedly dependent on the presence of either phospholipid or active platelet surface [110].

**Rxn 48.** Rate constants for this reaction were from Komiyama et al. [134]. Unlike the activation of X by VIIa alone (see 49) the $K_m$ for this reaction was reported to be relatively constant over a wide range of added PCPS concentrations, thus unlike other unbinding reactions in the model there was no dynamic change in reaction rate with platelet activation.

**Rxn 49.** Rate constants for this reaction were from Komiyama et al. [134]. The authors report a decrease in $K_m$ from 1.48 to 0.25 $\mu$M with PCPS levels increasing from 1.4 to 21 $\mu$M.
Rxns 50-57. Kinetics of fibrin polymerization are taken from Naski et al. [135]

Reactions 1-27 comprise the original Hockin-Mann Model.
For reactions 1-27, parameter values were from the Hockin-Mann model when a reference is not cited.
Except for reaction 13, ε wherever applicable operates on the off rate usually defined as k_{-1}. For reaction 13, ε operates on k_1 which is the actual unbinding rate of the VIIIa complex. The notation for this reaction is kept consistent with its description in the Hockin-Mann Model.
On-Rates were assumed to be diffusion limited (with a k_j of 1.0 x 10^8 M^{-1} s^{-1}) [96], and the corresponding off-rate was calculated from $K_m$ using $K_m = \frac{k_{off} + k_{cat}}{k_{on}}$.

Supporting Information of [102] provides a complete description of the ODEs for all 76 species in the assembled reaction network, along with the definition of every reaction rate. Essentially all of the model parameters (rate constants and initial conditions) are known or estimated from literature (Table 2.4-1). The use of η clarifies changes from the original Hockin-Mann rate constants that are justified by more recent literature measurements (See Table 2.4-1 footnotes). Only, the rate of XIIa leakage was estimated (i.e. fitted) based on the difference between simulation and experiment at 0 added TF.

To estimate the sensitivity of our model’s output to the choice of kinetic constants, a global sensitivity analysis of the models output thrombin concentration at 0 and 10 pM TF concentration was studied by the method of weighted averaging of local sensitivities (Figure 2.4-15 A and B; Table 2.4-3 and Table 2.4-4). Local sensitivity of $T_i$ to 10 fold variations in important parameters (determined from the global sensitivity analysis) across the entire range of titrated TF concentrations is shown in Figure 2.4-16.
<table>
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<th>Platelet-Plasma Model Initial Conditions (M)</th>
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Table 2.4-2 Initial conditions of species in the Platelet-Plasma model.

The initial conditions for the first 34 values in Table 2.4-2, were set to the values used in the Hockin Mann model [94]. The level of Boc-VPR-MCA was set to 10 μM (this is the level of fluorogenic substrate we use in our experiment). The level of CTI was set to 4.2 μM corresponding to the concentration of 50 μg/ml used in the phlebotomy syringe. XII, XI Pre-Kallikrein, C1-Inhibitor, α1AT, α2AP and Fibrinogen are set to their plasma concentrations [127, 137, 138].

2.4.1 Validation of experimental methods to detect thrombin dynamics

Calibrated Automatic Thrombography (CAT) [90] measures the initiation, propagation and destruction phases of the thrombogram. In seeking to trace the complete profile, CAT uses a thrombin substrate Z-GGR-MCA that binds weakly (K_m = 305 μM) and that is consumed slowly (k_cat = 1.86 s⁻¹) and requires high concentrations (416 μM) to quantify the complete profile. However, fluorogenic substrates can act as competing substrates for thrombin, thereby slowing down positive feedback. Z-GGR-MCA can also serve as a competitive substrate for Xa. The magnitude and importance of such inhibitory mechanisms of Z-GGR-MCA are still debated [91, 139]. Since thrombin has a strict requirement for proline in the P2 position [140], we compared Z-GGR-MCA with the
highly selective thrombin substrate Boc-VPR-MCA \( (K_m = 61 \, \mu M, \, k_{\text{cat}} = 53.8 \, s^{-1}) \) used at \([\text{SIIa}] < K_m\) to detect coagulation initiation.

Titrations of the fluorogenic substrates Boc-VPR-MCA and Z-GGR-MCA were carried out at 0 and 1 pM added TF (Figure 2.4-2 A and Figure 2.4-2 B) to determine the initiation time \((T_i)\). Since increasing substrate will take increasing time to be consumed, the 5\% conversion criterion (see Methods) cannot be used as the metric to quantify coagulation initiation. We defined instead, the time when the second derivative of fluorescence was maximized as \(T_i\) for this experiment. Without addition of TF (Figure 2.4-2 A), the use of Z-GGR-MCA prolonged \(T_i\) by 10 to 20 min at all concentrations from 1 to 100 \(\mu M\) in comparison to Boc-VPR-MCA. At 1 pM added TF (Figure 2.4-2 B), both fluorogenic substrates were found to be inhibitory to some degree as demonstrated by a prolongation in \(T_i\) with increasing substrate concentration. However, Z-GGR-MCA caused a prolongation of \(T_i\) from 13.1 to 33.8 min as substrate concentration was increased from 1 to 100 \(\mu M\), while Boc-VPR-MCA prolonged \(T_i\) from 4.1 to 14.0 min as fluorogenic substrate concentration was increased from 1 to 100 \(\mu M\). Thus, Boc-VPR-MCA used at up to 100 \(\mu M\) provided smaller \(T_i\) (less inhibition) when compared to Z-GGR-MCA even when used at 1 \(\mu M\).

Thrombin-antithrombin (TAT) formation provided an independent method to validate the fluorogenic assays (Figure 2.4-2 C and Figure 2.4-2 D). Saturation of the detector (at maximized gain to detect low substrate concentrations) prevented the testing of 416 \(\mu M\) Z-GGR-MCA typical of CAT, thus the use of 100 \(\mu M\) Z-GGR-MCA in this comparison. In the absence of added TF (Figure 2.4-2 C) clotting initiation was observed in \(~90\) min as indicated by a change from baseline levels of TAT. The addition
of 10 µM Boc-VPR-MCA or 100 µM Z-GGR-MCA slowed the formation of TAT with 0 or 1 pM TF addition compared to reactions without fluorogenic substrate (Figure 2.4-2 C and Figure 2.4-2 D). In the presence of 1 pM TF (Figure 2.4-2 D), clotting initiated at [TAT] ~ 10 nM at a time between 5 to 10 min in the absence of substrate or with 10 µM Boc-VPR-MCA present. However, clotting initiated at [TAT] ~ 10 nM at a time between 10 to 30 min with 100 µM Z-GGR-MCA present. TAT formation in the propagation phase of thrombin production was inhibited by the presence of 100 µM Z-GGR-MCA substrate. Importantly, we have verified that [TAT] at the time of $T_i$ detected by the fluorogenic assay with 10 µM Boc-VPR-MCA was 10 to 20 nM which was in excellent agreement with the initiation phase of coagulation as defined by the time it takes to generate 10 nM thrombin [141].

The use of a more selective substrate Boc-VPR-MCA at a low concentration of 10 µM (used for the remainder of this study) allows for accurate detection of only the initiation phase and not the entire thrombogram, due to the rapid consumption of the substrate much before the complete conversion of prothrombin. However, the time taken for onset of initiation of thrombin generation is one of the most important characteristic of a thrombogram, and the use of the substrate at low levels allows for accurate detection of this metric without appreciable competitive inhibition of thrombin.

This was the basis for adding the thrombin detection chemistry (Reaction 28, Table 2.4-1) to the Hockin-Mann topology. In keeping with our experimental observation, thrombin formation is slightly inhibited by thrombin occupying the fluorogenic substrate and thus unavailable to generate positive feedback upon the zymogens V, VII, VIII, XI or activating the platelet.
(A) A titration of the fluorogenic substrates Boc-VPR-MCA (blue circle) and Z-GGR-MCA (green square) with 0 added TF showed a mild inhibitory influence of Z-GGR-MCA. *s indicates statistically significant difference (p<0.05) between initiation times detected with the two different substrates. (B) A titration of the fluorogenic substrates Boc-VPR-MCA and Z-GGR-MCA with 1 pM added TF showed inhibitory influence of both substrates where Boc-VPR-MCA was found to be the better substrate to detect initiation and exhibited little inhibition at 10 μM concentration. (C) TAT formation with 0 added TF, in the absence and presence of fluorogenic substrates showed less inhibitory influence of Boc-VPR-MCA on initiation defined by a burst in TAT compared to Z-GGR-MCA. Absolute [TAT] after initiation is decreased in the presence of either substrate. (D) TAT formation with 1 pM added TF, in the absence and presence of fluorogenic substrates showed decreased [TAT] during the propagation phase of coagulation in the presence of either substrate. Initiation detected by TAT correlated well with Ti determined by our fluorogenic assay. In panels C and D, * indicates [TAT] significantly greater than baseline levels (p<0.05) and # indicates statistically significant differences compared to no substrate (blue). Experiments in panels A, B, C and D were carried out with blood from the same phlebotomy. (E) TF titration done in blood anticoagulated with CTI alone (green), Citrate + CTI (red) and Citrate alone (blue). Dashed lines indicate controls with no added TF. No significant difference was detected in titrations done with and without citrate, showing no evidence of inhibition by the anticoagulant. Effects of the contact factor pathway were apparent only below 100 fM added TF.

The nonspecific effects of citrate [92] and CTI [142] were also evaluated by performing a titration of TF into blood drawn into CTI alone, CTI-citrate, or citrate alone (Figure 2.4-2 E). Recalcification was done to adjust the concentrations of Ca^{2+} in each of these citrated blood samples to 10 mM. The time difference between recalcification of all the samples to the same [Ca^{2+}] and the drawing of uncitrated blood (which was free to coagulate in the presence of physiological Ca^{2+} concentrations) was less than 3 min. It has recently been suggested that coagulation studies done with recalcified citrated blood are potentially influenced by inhibitory effects of calcium citrate [92]. Similarly, CTI has been reported to interfere with factor VIII coagulant activity [142]. We detect no significant difference in Ti upon carrying out titrations of TF in blood anticoagulated with
CTI alone or CTI-citrate, indicating little inhibitory influence of citrate on the initiation of coagulation. In the absence of added CTI, the effects of contact pathway-induced production of XIIa were apparent only below 100 fM added TF as suggested by a lowering of $T_i$ in the absence of CTI. It should be noted that citrated blood is maintained for < 5 min prior to recalcification in the current study.

### 2.4.2 Coagulation initiation without added TF

CTI additions beyond 32 μg/ml have been reported to saturate prolongations of clot times due to contact activation in otherwise unstimulated blood [77]. We reproducibly observe (Figure 2.4-3) that blood drawn into 50 μg/ml CTI will still clot between 60 to 120 minutes without addition of TF, for all donors tested. Prior activation of platelets with CVX will lower initiation time to ~20 minutes.

![Figure 2.4-3 Coagulation initiation in the absence of externally added TF.](image)

Blood drawn into 50 μg/ml CTI and without added TF, will still reproducibly clot in ~75 minutes. Prior activation of platelets with CVX will lower initiation time to ~20 minutes. Shown are the multiple replicates tested under the same conditions with the same phlebotomy.
Similar results were obtained with other platelet agonists such as PAR1 peptide agonists or ADP (data not shown). Since contact activation is thought to be completely inhibited by the use of CTI, it was reasonable to hypothesize that TF from some “blood borne” source or other proximal triggers of prothrombinase formation were responsible for such initiation.

2.4.3 Evaluating ‘blood-borne’ TF and other mechanisms that could lead to initiation of clotting in blood drawn into CTI without exogenous TF addition

To evaluate the effect of phlebotomy as a source of vessel wall derived TF, experiments were conducted with the first 10 ml, 10-20 ml, 20-30 ml and 30-40 ml of blood (Figure 2.4-4). If TF was indeed produced from the phlebotomy, an increase in initiation time would be expected with subsequent volumes of blood drawn after the initiation of phlebotomy. Clotting initiated in the first 10 ml of resting blood in ~75 min. For all subsequent volumes clotting initiated at ~90 min and no further increase in initiation times was observed. Initiation occurred in 25 min in CVX pretreated blood for all volumes after the blood draw. The amount of TF due to phlebotomy appears minor and has no role in blood activated with CVX. In subsequent experiments, the first 10 ml of blood drawn was discarded.
To evaluate the effect of phlebotomy, experiments were conducted ±CVX using the first 10 mls, 10-20 mls, 20-30 mls and 30-40 mls of blood. No steady increase in Ti was noted showing that TF from phlebotomy was not leading to eventual initiation.

P-selectin is expressed on the platelet surface upon activation and induces procoagulant PS exposure [143] and TF synthesis in monocytes [144]. TF-containing microparticles (MPs) have been reported to be generated by interactions between P-selectin and PSGL-1. Furthermore the presence of P-selectin on activated platelets helps to recruit these MPs to the developing thrombus [145]. However, the addition of a function-blocking P-selectin antibody caused no change in the time when a thrombin burst was detected, in the absence or presence of CVX (Figure 2.4-5). VIIa can bind weakly to activated platelet surfaces and support thrombin generation independently of TF [146]. Weeterings et al. have reported recently that GP1bα can bind rFVIIa with a $K_d$ of ~20 nM and this interaction enhanced TF-independent thrombin generation [147]. However, neither antibodies against VII/VIIa (Figure 2.4-7 B), nor antibodies against GP1bα (Figure 2.4-5) had any effect on the initiation of thrombin formation with or without CVX stimulation.
Figure 2.4-5 Studying the effect of P-Selectin and GP1bα on initiation

Addition of antibodies against P-selectin or Gp1bα did not prolong initiation either in the absence or presence of high dose CVX.

Protein disulfide isomerase (PDI) has been suggested as a possible trigger for tissue factor dependent fibrin generation. PDI originating from adhering platelets [148] can activate ‘encrypted or cryptic’ [82] functionally inactive TF by changing the disulfide status of the Cys186-Cys209 pair on the TF molecule [149]. Fibrin formation, following infusion of TF microparticles were shown to be strongly inhibited by an anti-PDI antibody in vivo [150]. However, in our hands an anti-PDI antibody had no significant effect in prolonging $T_i$ with or without CVX treatment (Figure 2.4-6), suggesting that TF de-encryption via PDI was not a mechanism at play. This is consistent with earlier reports that: (1) exogenously added PDI or anti-PDI antibodies had no effect on TF-VIIa coagulant activity shown by MDA-MB231 cells stimulated with HgCl$_2$; or (2) PDI silencing with PDI shRNA had no effect on procoagulant activity [151].
Addition of antibodies against PDI or cathepsin G did not prolong initiation either in the absence or presence of high dose CVX. The integrin Mac-1 (CD11b/CD18) on neutrophils or monocytes can bind FX which can be converted to Xa by cathepsin G. Inflammatory stimuli or Mac-1 engagement of its ligands (including fibrinogen and FX) stimulates degranulation and release of cathepsin-G [152]. Such a mechanism of X activation could lead to initiation in the absence of TF. However, the addition of a anti-cathepsin G antibody had no effect in prolonging the time at which a burst in thrombin production occurred with or without CVX treatment (Figure 2.4-6).

We note that in all experiments utilizing antibodies and without prior platelet activation there was slight reduction (relative to the control where no antibody was added) in the time taken for initiation to occur. For instance, sheep IgG by itself reduces $T_i$ (Figure 2.4-5) from 91.82±13.35 (control) to 70.08±10.57 min. Such effects were possibly mediated by non specific Fcγ-R mediated platelet activation [153] and were not apparent when the platelets were already fully activated with CVX.
Figure 2.4-7 Effect of TF translation and activity

(A) The ribosome inhibitor puromycin; the Clk1 kinase inhibitor Tg003; (B) antibodies against TF, VII/VIIa; or (C) VIIai did not prolong initiation either in the absence or presence of high dose CVX. This shows that initiation is unaffected by either ‘bloodborne’ or platelet synthesized TF on the time scales of our experiments.

Platelets may have the capacity to rapidly splice TF pre-mRNA to a mature transcript and rapidly translate TF protein intracellularly [154]. We added Tg003 and puromycin to inhibit Cdc2-like kinase (Clk)1 and ribosomal function, respectively, two
platelet functions reported to be responsible for splicing TF pre-mRNA and rapid TF mRNA translation [154]. A level of 10 μM Tg003 and 5 μM puromycin had no effect on the initiation (Figure 2.4-7 A), either in the absence or presence of CVX. This suggests that the capacity of platelets to splice TF pre-mRNA and rapidly express TF protein does not contribute to clotting initiation at least on the time scales of the experiment. Also, the addition of 20 μg/ml anti-TF, 20 μg/ml anti-VII/VIIa (Figure 2.4-7 B) or 1 μM active site inhibited factor VIIa, VIIa (Figure 2.4-7 C) caused no increase in $T_i$ with or without CVX activation of the platelets. This further suggests that in whole blood endogenous TF mediated mechanisms are not responsible for clotting initiation.

### 2.4.4 Evaluating the role of CTI

In the absence of evidence for kinetically-significant blood borne TF, we evaluated the efficacy of CTI (Figure 2.4-8). Without inclusion of CTI in the syringe or in the well plate, clotting initiated in 57.3 ± 9.9 min. When 10 μg/ml CTI (final concentration) was included in the well plate, initiation occurred in 77.4 ± 7.0 min due to inhibition of XIIa production. When the same final concentration was achieved (after a 5x final dilution of blood in the well plate) by including 50 μg/ml CTI in the phlebotomy syringe, initiation time was markedly prolonged to 110.0 ± 17.0 min. Liquid handling of citrated blood after phlebotomy takes ~3 minutes before recalcification and this result points to the significant production of XIIa during this initial period. When very high concentrations (100 μg/ml) of CTI were achieved by including CTI in the syringe (50 μg/ml, diluted 5x in the well plate) and 90 μg/ml preexisting CTI in the well plate, initiation occurred in 96.6 ± 15.1 min and was not prolonged further. Thus, the inhibitory effect of CTI saturates, pointing to the possibility of a XIIa leak past CTI.
Figure 2.4-8 Saturation of the effects of CTI

To evaluate the possibility of leakage past CTI, experiments were conducted with no CTI, CTI addition in well plate, CTI addition during phlebotomy and large quantities of CTI during phlebotomy as well as in well plate. The inclusion of 50 μg CTI/ml whole blood (before a 5x dilution in the well plate) produced saturating effects.

2.4.5 Anti-XI and XII used simultaneously prevent initiation in resting blood

Combined addition of 50 μg/ml of anti-XI and anti-XII to diluted whole blood (with CTI) completely abolished initiation (Figure 2.4-9) demonstrating that for conditions lacking platelet activation a steady leak of XIIa activity past CTI was the most proximal trigger of platelet activation and subsequent clotting of minimally perturbed blood in vitro. Interestingly, anti-XII when used alone did not prevent initiation (not shown), suggesting that inspite of our best attempts at preventing XII activation in diluted whole blood (CTI and anti-XII used together), some XIIa is still formed on the time scale of our experiments, necessitating the inhibition of XI activity as well. Also, anti-XI by itself did not prevent initiation in whole blood (not shown), suggesting strong autocatalytic amplification by very minute amounts of XIna formed inspite of the use of the inhibitory XI antibody. Despite the efficacy of high dose CTI in conjunction with anti-XI and anti-XII in preventing activation in resting blood, there was no effect of these antibodies on $T_i$ when blood had been treated with CVX. The levels of thrombin produced during the initial moments of the propagation phase of thrombin production (after $T_i$) were however diminished as demonstrated by lowered rates of fluorescence increase. This was possibly a consequence of the inhibition of thrombin-mediated feedback on FXI after initiation [155].
Addition of 50 $\mu$g/ml of anti-XI and anti-XII will completely prevent initiation of clotting in resting blood showing that initiation is a result of leak past saturated effects of CTI. However, on CVX activated platelets initiation is still unaffected by the presence of both CTI and these antibodies. Initial thrombin production during the propagation phase is however diminished due to abolition of thrombin feedback on FXI. Insert shows initial rates of thrombin formation in the presence of these antibodies.

### 2.4.6 Preventing initiation in CVX treated blood

Unlike the case of resting blood, initiation could not be prevented in blood pretreated with CVX by the use of antibodies against XI and XII. To probe further the mechanism responsible for such initiation, we supplemented CTI-treated regular plasma and CTI-treated plasmas deficient in factors VII, XI and XII with washed platelets.
Thrombin generation in these samples as well as samples of plasma that had been treated with antibodies against TF, VII, XI, XII, or XI/XII/VII was studied following CVX activation (Figure 2.4-10). Antibodies against TF had no detectable effect. Thrombin formation was not completely inhibited by blocking any single factor individually, but was inhibited in samples where the activities of XI, XII and VII were simultaneously inhibited (Figure 2.4-10, last row of subplots). In particular clotting was completely inhibited in 6 out of 8 wells in XI-deficient plasma treated with antibodies against XI, XII and VII. Inhibition of VIIa activity alone is insufficient (initiation times are almost unchanged in VII-deficient plasma treated with a VII antibody) because XIa formation beyond the XIIa leak past CTI, is still sufficient to trigger thrombin formation. Inhibition of XI activity (XI-deficient plasma with an XI antibody) has pronounced inhibitory effect, but was still insufficient with VIIa being functionally active on the active platelet. Thus upon activated platelets, in addition to blocking factors XI and XII, VIIa activity too must be inhibited to prevent thrombin generation.

It should be noted that immunodepleted plasmas deficient in coagulation factors have <1% normal activity of the particular factor, but are still not completely devoid of the depleted protein. This explains why initiation is prolonged further by antibodies against a protein that had already been immunodepleted in plasma. For instance thrombin generation in anti-XI treated, XI-deficient plasma is inhibited compared to that observed in XI-deficient plasma alone. The use of deficient plasmas offers a “cleaner inhibition” because the protein being targeted for inhibition is largely absent from the onset, as opposed to reliance on the inhibitory antibody to exert its effect before the protein is functionally active.
Figure 2.4-10 Prevention of initiation on CVX activated platelets.

CTI-treated regular plasma or plasma deficient in factors VII, XI or XII were supplemented with washed (plasma free) platelets. These samples were left untreated or were treated with antibodies against TF; VII; XI; XII or XI, XII and VII simultaneously; and tested for thrombin generation without exogenous TF addition after activating platelets with 25 nM CVX. Simultaneous inhibition of XI, XII and VII activity was required to completely abolish thrombin generation.

Thrombin formation, once clotting is initiated by a single activator for instance by the addition of TF or Xa (See Figure 2.4-11) yields relatively small variations in initiation time when tested in multiple replicates. However, given the strongly autocatalytic nature of the cascade, complete abolition of a triggering mechanism is much more challenging. Even small localized diffusional or binding limitations of the
inhibitory antibody will allow individual molecules that bypass the inhibitors to trigger the cascade. This accounts for the relatively large variations we observe in trying to inhibit initiation upon activated platelets, where thrombin formation occurs stochastically past inhibitory antibodies against targeted coagulation proteins.

2.4.7 Titrations of TF and active proteases into blood

Addition of exogenous lipidated TF was used to initiate coagulation via the extrinsic pathway. Concentrations from 0.5 fM to 5 pM were added to whole blood (Figure 2.4-11 A). Added TF resulted in a continual, statistically significant reduction of initiation time over a wide dynamic range of 3 orders of magnitude of concentration from 20 fM to 20 pM. Distinct from flow experiments where a concentration threshold exists between 2 and 20 molecule-TF/μm² [156], no switch-like regime was detected under static conditions. This suggests that the barrier to be overcome in the flow system to exceed the threshold is not a chemical inhibition barrier since such a barrier would be present under stasis as well. The observation is consistent with Fogelson and Tania [157] who note the barrier responsible for the threshold under flow is a physical one dependent on competition between flow-mediated removal and rate of production of procoagulants.

Next, we investigated the individual components of prothrombinase (XaVa complex) on coagulation initiation (Figure 2.4-11 B). Addition of Va showed negligible effects with minimally decreased initiation time from 0.3 nM to 4 nM added Va (>10% of the plasma concentration of V). This relative insensitivity indicated that Va was not a limiting during coagulation initiation, in agreement with previous studies that show that Va may be present in platelets or be rapidly displayed or produced during activation [158]. In contrast, Xa additions produced a marked switch-like action by moving from a
negligible change in $T_i$ at 10 pM to nearly saturating effect at 100 pM, a concentration that is <0.1% of the plasma concentration of X. This switch-like behavior was consistent with the well understood rate limiting role of Xa [94].

Further, we explored reactions proximal to thrombin production by addition of increasing amounts of Factors VIIa, IXa and XIa to blood (Figure 2.4-11 C). The nominal concentrations of endogenous VIIa (1% of VII) prior to exogenous addition in 5x blood is 20 pM [159]. Using 5x final blood dilution, we extended these concentrations to reach an effective concentration of VIIa above 1 nM. VIIa has no significant effect until above 1 nM, approximately 10% of the circulating amount of VII. For comparison, high dose rVIIa therapies use up to ~17 nM final concentrations [160]. The dynamic range for added IXa extended from below 5 pM to above 500 pM and that for XIa extended from below 2 pM to above 200 pM. The upper end of these ranges are less than 1% of the circulating IX and XI concentration respectively [161]. Similar to results for TF, the addition of IXa or XIa in this assay did not display a switch-like regulatory function, i.e. changing from no effect to full effect over some narrow concentration range, but rather displayed a broad dynamic response over a wide range of concentrations.

Baseline low level protease activity can exist even in healthy individuals (the “engine idling” theory). Knowledge of the upper bounds of these proteases defines the levels of these enzymes that allow blood to remain in a stable state (flowing normally) in spite of their presence. Experimentally measured values of active proteases for a patient that are significantly different from these values might indicate underlying coagulation disorders or heightened sensitivity to weak triggers of clotting.
Figure A: Graph showing the relationship between concentration (in fM and pM) and time (in minutes) for an initial event. The graph includes data points for 'Control' and 'TF Titration'.

Figure B: Graph showing the relationship between concentration (in fM, pM, and nM) and time (in minutes) for a different event. The graph includes data points for 'Control', 'FXa Titration', and 'FVa Titration'.

Figure C: Graph showing the relationship between concentration (in pM, nM) and time (in minutes) for another event. The graph includes data points for 'Control', 'Xia Titration', 'IXa Titration', and 'Vila Titration'.

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Figure 2.4-11 Titration of TF and active proteases into blood.

(A) Effect of exogenous TF on initiation time. TF was titrated from 0.5 fM to 5 pM in 5x diluted blood. The black solid line is the simulated initiation time for the Hockin-Mann model (with S IIa) and the blue dashed line is the prediction of the Platelet-Plasma model. The light green solid line is the experimental control with no added TF. (B) Addition of prothrombinase components. Xa (red) and Va (green) was added to 5x diluted blood. The black solid line is the simulated initiation time for the Hockin-Mann model (with S IIa). The red and green dashed lines are the prediction of the Platelet-Plasma model for Xa and Va, respectively. The light green solid line is the experimental control with no added proteins. (C) Addition of VIIa, IXa and XIa. Various concentrations of VIIa (green), IXa (red) and XIa (blue) were added to blood at 5x dilution. The dashed lines of the corresponding color are simulations done with the Platelet-Plasma model. The light green solid line is the experimental control. (Experiments courtesy Bill Denney)

2.4.8 Platelet-Plasma model of thrombin production in human blood

The Hockin-Mann model was used to simulate these titrations and was found to be quite accurate for TF concentrations above 1 pM, but diverged dramatically at concentrations below 1 pM (Figure 2.4-11 A, black solid line) of added TF. We have shown experimentally, that a leak of XIIa past saturating levels of CTI is responsible for eventual initiation. This prompted us to include the contact factor pathway to Hockin-Mann topology.

Upon contact with anionic surfaces a conformational change occurs in the zymogen FXII, resulting in the formation of active FXIIa. The exact mechanism for XII surface adsorption, conformation change and activation remain ambiguous and cannot be modeled easily. We therefore coarse grained surface mediated XII activation by assuming a first order dependence on XII concentration (Reaction 29, Table 2.4-1) and estimating a rate of production ($5\times10^{-4} \text{s}^{-1}$) that would resolve the disparity between the Hockin-Mann model prediction and the experimentally observed control (green) at 0 TF (without disrupting its predictive ability at high TF). It should be noted that this
estimated rate of XIIa production was based solely on observations in our experimental surfaces (Corning 384-well plates) and may well be different on other surfaces. The small amount of XIIa that is initially formed auto-activates upon negative surfaces \textit{in vitro} (Reaction 30, Table 2.4-1). Formed XIIa further activates Pre-Kallikrein to active Kallikrein (Reaction 31, Table 2.4-1), which in turn reciprocally activates XII (Reaction 32, Table 2.4-1) and Pre-Kallikrein (Reaction 33, Table 2.4-1). Both Kallikrein and XIIa are inhibited by plasma protease inhibitors (Reaction 34, 36-37; Table 2.4-1). Further, XIIa is inactivated by CTI included at high concentrations specifically to inhibit contact activation (Reaction 35, Table 2.4-1). Despite the efficacy of CTI, it is a reversible inhibitor of XIIa; and thus there is always some active XIIa beyond CTI resulting from the unbinding of the XIIa=CTI complex. Such XIIa that has “leaked past” CTI can in turn activate XI (Reaction 39, Table 2.4-1). Thrombin feedback on XI has also been considered (Reaction 38, Table 2.4-1) as a mechanism for XI activation.

We have experimentally observed (Figure 2.4-11 C) strong sensitivity of blood to very minute doses of XIa (even 2 pM of added XI reduced $T_i$ significantly from that of the control). Also complete abolition of clotting without exogenous TF, required inhibition of XI activity in addition to XII (Figure 2.4-9 and Figure 2.4-10) suggesting that the very little XIa that is formed downstream of XIIa is able to strongly self amplify (especially if the pro-coagulant surface of the activated platelet is readily available). With inhibition by ATIII, C1-inhibitor, $\alpha_1$-antitrypsin and $\alpha_2$-antiplasmin (Reaction 41-44; Table 2.4-1), such sensitivity could not be adequately explained by just XIIa mediated activation and thrombin feedback on XI. Therefore we also included the mechanism of XI auto-activation [136] on negatively charged surfaces to the topology of the network
(Reaction 40, Table 2.4-1). Following the retraction of Baglia and Walsh, JBC 2000 we are not aware of experimental reports on the kinetics of this reaction. However, Kramoroff et al. [126] estimate the second order rate constant of this reaction to be 3.19 μM⁻¹s⁻¹ by optimizing an ODE model of the intrinsic cascade to experimental measurements of APTT. They consider either XI autoactivation or XI activation by thrombin (but not both possibilities) as plausible mechanisms for XI activation (in addition to activation by XIIa), thus their estimated value is likely an overestimate. We utilized a value 4-fold lower than the value they report for this constant, since we consider both mechanisms.

The XIa that is formed activates IX (Reaction 45, Table 2.4-1). Unlike the original Hockin-Mann topology, IXa in the absence of its cofactor is now able to activate some factor X (Reaction 46, Table 2.4-1) albeit very inefficiently. This minute amount of Xa contributes to the activation of VIII (Reaction 47, Table 2.4-1), in addition to the VIII activation by thrombin in the Hockin-Mann structure (Reaction 10, Table 2.4-1). VIIIa once formed dramatically raises the efficacy of IXa and hence subsequent thrombin formation. Thus such participation of Xa in VIII activation (in addition to its ability to directly convert prothrombin) helps it to function in simulation as the dramatic switch like controller of the cascade we observed experimentally (Figure 2.4-11 B).

VIIa above 1 nM concentrations is able to produce significant lowering of $T_i$ independently of TF (Figure 1.1-1 C). Furthermore, with platelet activation, inhibition of VII activity was required (in addition to the inhibition of XI and XII activity) to completely block thrombin generation (Figure 2.4-10). This suggests that VIIa activity independent of TF, is effective at high concentrations and on the activated platelet. This
prompted us to include VIIa’s ability to convert IX and X (independent of TF) in the structure of the model (Reactions 48-49; Table 2.4-1).

Fibrinogen can act as an efficient sink for thrombin in experiments conducted in whole blood, and thus limit its ability to generate positive feedback by converting other zymogens or activating platelets. The thrombin catalyzed formation of fibrin I and fibrin II, release of fibrinopeptides A and B and the irreversible entrapment of thrombin in complexes with fibrin and antithrombin are described in (Reactions 50-57; Table 2.4-1) using published kinetics [135].

With these additions to the structure of the Hockin-Mann topology, we could now obtain quantitative agreement between experimental and simulated titrations of the procoagulant proteins TF, Va, VIIa, IXa, Xa and XIa into 5x diluted blood. The original Hockin-Mann topology could predict finite $T_i$ for only high dose TF/ Xa (Solid lines in Figure 2.4-11 A and B).

Although there is evidence to suggest that TF dependent reactions (Table 2.4-1, Reactions 1, 2, 6-8 and 21) are sped up on acidic phospholipids (See Comments in footnotes to these reactions in Table 2.4-1), no $\epsilon$ dependence was used for these reactions in our simulations of the TF titration in Figure 2.4-11 A. This is because TF was already added in lipidated form in these experiments, and thus the TF-VIIa assembly or activity will not be affected by changing lipid composition upon the platelet surface.

The Kuharsky-Fogelson model [96] for thrombin formation under flow in the presence of collagen activated platelets shows [Xa] remaining at levels of about 10 pM throughout the duration of simulation. In the Hockin-Mann model with a TF stimulus of 5 pM, Xa concentrations are 10-100 pM during the times that substantial thrombin
formation first occurs and subsequently this rises by about 10-fold. Our results, along with the modeling predictions in these papers suggest why it is necessary that clotting be sensitive to low levels of Xa \textit{in vivo}. Namely, if levels produced under flow (where Xa can be rapidly convected downstream) are to be efficacious at all, they must produce substantial effects at very low levels (~10 pM), despite the presence of much higher concentrations of X, or the system’s ability to produce much larger concentrations of Xa under conditions of stasis.

![Simulated transient concentrations for 5 pM TF additions to whole blood](image)

**Figure 2.4-12 Simulated transients of selected species during coagulation with TF.**

Shown above are the time courses of thrombin, Xa, Xa:Va, Va and the platelet’s activation state (ε) for coagulation triggered with 5pM TF in whole blood. Before initiation sufficient catalyst quantities are built up by the minute (subnanomolar levels) of thrombin being formed. Concentrations of all species increase steeply beyond initiation (and full platelet activation) during the propagation phase of the thrombogram. Note, the shift in equilibrium of Va towards Xa:Va following platelet activation. Thrombin concentrations eventually start decreasing because of destruction of the free enzyme by ATIII and consumption of limited reserves of prothrombin inspite of the presence of sufficient prothrombinase catalyst.
Similar to Kuharsky-Fogelson (Figure 7 of reference [96]), we show in Figure 2.4-12 simulations for the concentrations of thrombin, Xa, Xa:Va, and Va for a stimulus of 5 pM TF (Also, compare to Hockin-Mann, Figure 8B of reference [94]). We found free Xa concentrations growing to about 20 pM during the initial instants followed by a much more substantial burst (that increases Xa levels by ~3 orders) following platelet activation. The Va concentrations grow steadily, but beyond full platelet activation (~10 nM thrombin), there is a dramatic shift in the equilibrium towards Xa:Va and thus the amount of free Va drops rapidly, as prothrombinase levels rise steeply.

2.4.9 Simulations of clotting times in whole blood

Our high throughput experimental system allows us to efficiently study coagulation reactions under various perturbations with multiple replicates. However, reaction volumes in our 384-well plate systems are only 50 μl, preventing us from studying undiluted whole blood. Automated liquid handling can dispense with high precision volumes of 5 μl, but for lower volumes experimental variability is high. Since each individual well must contain several reagents (calcium, fluorogenic substrate, platelet agonists, antibodies and coagulation proteases) other than human blood, there is a limitation to the volume of blood that can be used in each well.

De Smedt et al. [162] have recently reported that anticoagulant pathways (particularly the TFPI and APC mediated) are more affected by dilution than the procoagulant pathways. However, they did not observe any significant effect of dilution on the duration of the initiation phase, both for TF and Kaolin stimulated coagulation reactions in PPP upto dilutions of 12x (detectable effects on endogenous thrombin potential and peak height of the thrombograms were however reported). Blood at 5x
final dilution is below this limit of 12x dilution beyond which linear scaling of initial conditions with the dilution factor might not be sufficient to account for the kinetics of thrombin generation initiation, and provides a tractable system for extensive high throughput experimentation. In simulating such experiments, the initial conditions of all species were divided by 5 to account for the dilution in each well.

![Figure 2.4-13 Simulating clotting times in whole blood.](image)

The very small reaction volumes in a 384 well plate prevent us from studying coagulation reactions in whole blood (See text). To simulate the kinetics of initiation in whole blood we simulated clotting times for additions of TF, thrombin (IIa), IXa, Xa or combinations of all 3 proteases at low and high doses reported by Butenas et al. [163] in the Mann laboratory. We found good qualitative agreement between experimental clotting times (blue) and initiation times predicted by the Platelet-Plasma model (green). The Hockin-Mann (with fluorogenic thrombin substrate, S_{IIa}) model (red) predicts finite initiation times only in the presence of high dose TF or Xa.
To test the ability of the model to predict perturbations in whole blood, we simulated clotting times (a good measure of the time to generate a burst in thrombin) for additions of either TF; IXa, Xa, thrombin; or all 3 proteases together reported by Butenas et al. [163]. We found good agreement between experimentally reported values of clotting time and predicted initiation times across all conditions (Figure 2.4-13). The original Hockin-Mann model could predict finite $T_i$ in the presence of high dose TF/ Xa, or when low dose thrombin, IXa and Xa were present simultaneously.

2.4.10 Simulating platelet activation

The Hockin–Mann model assumes a fully activated platelet at $t=0$. In the Kuharsky-Fogelson model [96] or the Cellular Potts model [164], platelets can exist either in discrete “unactivated” or “activated” states. We do not consider platelets explicitly, but all reaction rates reported in literature to be dependent on platelet activation are altered by a single function ($\varepsilon$) of thrombin concentration (See Methods). As the platelets are activated by thrombin, they expose anionic phospholipids like PS on their surface, and the “platelet activation status” $\varepsilon$ increases from a basal state of 0.01 to 1.00. The unbinding rates of all enzyme-substrate complexes are set to their literature values at saturating PS levels, but are all divided by $\varepsilon$. Thus, as the platelet activates the unbinding rates dynamically decrease by two orders of magnitude, consistent with the notion that the platelet surface becomes more procoagulant. In the Kuharsky-Fogelson model of intravascular thrombosis under flow, reaction rates depend on the availability of fully activated platelets (contributing maximal platelet binding sites for the coagulation factors). Since binding sites change with the concentration of activated platelets, the local effective reaction rates change as well. For modeling isotropic coagulation in a well
plate, we account for a continuum of platelet activation states by thrombin and assume an excess of PS once platelets fully activate (as opposed to discrete saturable binding sites in the Kuharsky-Fogelson model).

Reactions that have been reported in literature (See Table 2.4-1 footnotes) to be accelerated by acidic phospholipids are upregulated in the model when platelets are activated by thrombin as $\varepsilon$ increases (and dissociation rates decrease). Platelets provide the negative surfaces for coagulation factor assembly in vivo, yet most current enzymology studies of coagulation enzyme kinetics in vitro use artificial phospholipid surfaces since standardizing platelet surfaces for kinetic assays is difficult.

In reality, surface adsorption of procoagulant molecules might also alter the catalytic efficiency ($k_{cat}$), but for the sake of simplicity all $k_{cat}$ values are set to their reported values at saturating PS levels (with the assumption that loose enzyme substrate complexes will hardly be formed in the first place and thus rarely get converted). The assumption that all unbinding rates change by two orders of magnitude is also made for simplicity. (In reality some enzyme substrate complexes are dramatically strengthened on exposed PS surfaces, while others show only weak dependence. See Table 2.4-1 footnotes.)

We make the assumption that the amount of active surface available upon activation of platelets at normal platelet counts ($3 \times 10^6$ platelets per 50 $\mu$l reaction well for 5×diluted blood with a platelet count $3 \times 10^8$ platelets/ml) is sufficient for coagulation reactions to proceed at their most optimal rates. Thus, like the Hockin-Mann model we assume an excess of phospholipid surfaces once the platelets are activated. Yet, our
approach accounts for the fact that surface reactions occur at a rate dependent on the extent of platelet activation rather than being fixed at the highest level at $t = 0$.

As a pseudohomogeneous and single phase model of coagulation, we do not treat platelets as separate entities (or account for free and plate bound species). Thus, the fact that each platelet may have a limited number of specific binding sites is not considered. Our approach assumes that sufficient binding sites are available in 5×diluted blood, an assumption that may not be valid at greater dilutions. Also, we assume that coagulation in a well plate is well mixed, lacks spatial gradients, and is not transport-controlled. In contrast, intravascular thrombosis occurs under conditions with (1) substantial gradients of reactive species and platelet binding sites, (2) significant effects of convective transport on soluble species, and (3) intrathrombic diffusion limitations.

Setting the initial activation state $\varepsilon_{\text{max}_0}$, allows us to control in simulation the basal level of activation of the platelet (Figure 2.4-14 B). For $\varepsilon_{\text{max}_0} = 0.01$ (akin to assuming 1% PS exposure on the surface of unactivated platelets), we obtain a burst in thrombin production (and hence fluorogenic substrate cleavage) at ~75 min in the Platelet-Plasma model. To simulate experiments where coagulation reactions occur upon pre-activated platelets, $\varepsilon_{\text{max}_a}$ is set to a value of 1.0 (with the assumption that the fully activated platelets have exposed all of their PS) giving $T_i \sim 15$ min. These values are in good agreement (Figure 2.4-14 A) with the mean initiation times obtained upon unactivated platelets (solid lines) and activated platelets (dashed lines) for the 4 donors shown in Figure 2.4-3.
Figure 2.4-14 Simulating platelet activation.

(A) Mean substrate conversion across all replicates for the donors shown in Figure 2.4-3. Substrate conversion traces without platelet activation are shown in solid lines and conversion traces upon activated platelets are shown in dashed lines. (B) Setting the initial activation state \( \epsilon_{\max_0} \) allows us to simulate platelet activation and its dynamic effect on all platelet dependent unbinding rates (see methods). The red line indicates simulations of substrate conversion without prior platelet activation \( \epsilon_{\max_0} = 0.01 \). The blue dashed line indicate simulations of substrate conversion upon instantaneously fully activated platelets at \( t = 0 \). \( \epsilon_{\max_0} = 1.0 \)
We note that the rate of thrombin production (substrate conversion) in simulation beyond $T_i$ increases more sharply than actually observed in experiment. Thrombin concentrations at $T_i$ are ~10 nM, corresponding to a fully activated platelet (Figure 2.3-2) beyond $T_i$. This suggests that a method of prothrombinase inhibition (that has not been considered in our reaction topology) may exist once platelets are activated. Inhibition of XaVa \textit{in vitro} in the absence of endothelial thrombomodulin is poorly understood. Further experiments will be required to pinpoint this inhibitory mechanism, but for the present the Platelet-Plasma model is a reliable indicator of the time taken to generate a burst in thrombin \textit{in vitro}.

### 2.4.11 Sensitivity analysis of the Platelet-Plasma model

We estimated global parameter sensitivities for the Platelet-Plasma Model’s output for 10 pM added TF (Figure 2.4-15 A and Table 2.4-3) and 0 added TF (Figure 2.4-15 B and Table 2.4-4) input stimuli by the method by the method of weighted averaging of local sensitivities [165]. In this method local parameter sensitivities in output ‘$y$’, for a parameter ‘$j$’ ($j = 1, \ldots, 105$) at a point ‘$i$’ in parameter space is first calculated by introducing a 2% increment in the parameter $p_j$:

\[
s_{ij} = \frac{\partial y/y}{\partial p_j/p_j}
\]  

(2.4.1)

We define ‘$y$’ to be the thrombin concentration at the initiation time ($T_i$) of the nominal parameter set. Note $T_i$ cannot be used as the output during sensitivity analysis because 5% conversion of the fluorogenic substrate is not achieved in some perturbed parameter sets. Local sensitivities were calculated at multiple random points ‘$i$’ in a very large parameter space where each of the 105 parameters has a spread of half an order of
magnitude around their nominal value. In total 10,000 points were explored \((10,000 \times 10^5\) simulations) and global sensitivity indices were determined by calculating a weighted average of local sensitivities. Similar to Bentele et al. [165] we use the Boltzmann-Distribution \(\exp(-E/k_B T)\) for weighting, in which \(E\) is the difference in thrombin concentration between the nominal and perturbed states and \(k_B T\) is a scaling factor (we use minimum \(E\) for scaling). Based on the assumption that parameter sets resulting in output values close to the experimental observation are the most probable, this approach statistically amplifies the sensitivities of parameter sets that result in outputs close to the nominal solution. For ease of visualization sensitivity indices were normalized between 0 and 1 and the values for ranked according to decreasing sensitivity.

Other methods of global sensitivity analysis like ‘Sobol’s method’ [166] or the ‘Extended FAST method’ [167] provide non-biased sampling over the entire parameter space. Such methods were attempted (not shown) and provide similar rank orderings of sensitivities. However convergence was not achieved in a feasible amount of time.

For 10 pM added TF (Figure 2.4-15 A and Table 2.4-3), the rate of binding of TF to VIIa was found to be the most sensitive, followed by the rate of binding of X to TF:VIIa. Interestingly, thrombin production was also found to be strongly sensitive to TF independent VIIa binding to IX, as well as VIIa’s ability to convert X by itself. Reactions regulating prothrombinase formation (Ranks 7 and 8) were also found to be sensitive. Contact activation (XIIa) mediated reaction rates were generally too slow too have any appreciable effect, although thrombin feedback on XI was found to be moderately sensitive (Ranks 17 and 18).
Figure 2.4-15 Global sensitivity analysis of the Platelet-Plasma model
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Table 2.4-3 Rank order of sensitivities at 10 pM added TF

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Table 2.4-4 Rank order of sensitivities at 0 added TF
Figure 2.4-16 Effect of variation of important parameters across a TF titration.

The experimental values of $T_i$ for a titration of TF are shown in blue. The experimental value of the control is shown in green. Simulated values of $T_i$ are shown by the blue dashed line. Important individual parameters were locally perturbed by either $10\times$ (red dashed line) or $0.10\times$ (black dashed line) and the resulting simulated titrations are shown above. This local sensitivity analysis was performed for the 3 most globally sensitive parameters (subplots 1-3) in the absence of added TF (Figure 2.4-15 B and Table 2.4-4), the estimated rate of XIIa produced from the wall (subplot 4), as well as for the 4 most globally sensitive parameters (subplots 5-8) in the presence of 10pM added TF (Figure 2.4-15 A and Table 2.4-3).
In the absence of external TF (Figure 2.4-15 B and Table 2.4-4), regulation of XIa activity resulting from its production by autoactivation or inhibition by C1-inhibitor, alpha1-antitrypsin or ATIII was found to be the most sensitive. The estimated XIIa production rate was moderately sensitive (Rank 19) but not the most crucial determinant of contact activation in the presence of CTI. This is in accordance with our observation that very minute amounts of XIIa, leakage past CTI can strongly self amplify via XIa. On the slow timescales of activation in these simulations, inhibition of thrombin activity by ATIII or by its binding to fibrinogen was also found to be important. On expected lines, no sensitivity to TF mediated reactions was observed.

2.5 Discussion

Human blood is the only living tissue that is routinely and easily obtained for in vitro research and clinical diagnostics. Blood is fully amenable to high throughput functional phenotyping and strong genotype-phenotype linkages are the basis of significant human disease. The platelet and the plasma form the basis for hemostasis and have been modeled from a bottom-up systems biology approach [94, 96, 97, 168] with the distinct advantage of relatively well defined reaction topology and freedom from genome-wide transcriptional complexity.

We have developed a high throughput assay that allowed us to study thrombin generation in diluted blood, across several conditions simultaneously with multiple replicates at each condition. The use of diluted blood in this assay is a limitation of the small reaction volumes in a 384-well plate and scales the initial concentrations of all species by the final dilution factor. The presence of an externally added fluorogenic substrate for the purpose of detection represents a subtle alteration of thrombin-mediated
feedback of its own generation by competitively occupying thrombin’s active site. We evaluated the commonly used thrombin substrates Z-GGR-MCA and Boc-VPR-MCA (Figure 2.4-2 A, B, C and D). The latter substrate was found to have acceptably small inhibitory effects on $T_i$ at a concentration of 10 μM. In contrast, Z-GGR-MCA at a concentration of 100 μM was found to markedly hinder $T_i$ for 1 pM TF triggered coagulation. The use of 416 μM of this substrate in the ‘Calibrated Automatic Thrombography’ assay may be necessitated by the objective of tracing the ‘complete’ thrombogram. The use of 10 μM Boc-VPR-MCA suffices for the purpose of detecting coagulation ‘initiation’ and reduces assay interference. Also, the use of citrate as an anticoagulant was not found to have any significant influence on clot initiation times and permitted us to have a well defined activation time ($t=0$) for the coagulation reaction space defined as the time of recalcification of all the wells. Effects of the contact factor pathway were significant at concentrations less than 100 fM TF and were subsequently prevented by the use of CTI (Figure 2.4-2 E).

We and others have consistently observed that CTI-treated human blood clots in the absence of added TF in vitro. In keeping with the notion of “blood borne TF” it is tempting to attribute such clotting initiation to platelet-dependent sources [81, 154]. We explored several possible mechanisms with neutralizing antibodies against TF, VII/VIIa, PDI, P-selectin, GPIb, and cathepsin G but none had affect on thrombin generation. Similar to the experiments of Butenas et al. [169], we have not found any evidence for functional TF in static whole blood (a closed system).

CTI inhibits β XIIa [170] and has relative specificity for this form of XIIa [171]. The β form of XIIa does not bind to surfaces and has little ability to activate XI, although
it does activate Pre-Kallikrein (which in turn amplifies XIIa production). The effectiveness of CTI in prolonging APTT is probably a result of prevention of XIIa amplification by Kallikrein produced by β XIIa. The surface bound α form of XIIa is the main activator of FXI and the intrinsic coagulation system [172]. Such XIa produced by α XIIa on negative surfaces could get past CTI and lead to contact activation. We have demonstrated that in resting whole blood XIIa can leak past even very high concentrations of CTI, eventually leading to a significant burst in thrombin production (Figure 2.4-8). Without CVX activation, the combined use of CTI, anti-XII and anti-XI block all proximal triggers of clotting in the 4 hr assay (Figure 2.4-9). With CVX activation, VIIa activity on the active platelet alone (independent of TF) is kinetically significant, but the local activity of single molecules of XIa (formed downstream of a XIIa leak past CTI) in the proximity of the active platelet surface, can lead to efficient thrombin production (Figure 2.4-10).

We highlight a recent report by Back-Nilsson et al. [173] where the authors showed that the levels of XIIa-AT, XIa-AT and Kallikrein-AT complexes were not affected by CTI treatment if contact activation of XII is allowed to occur on activated platelet surfaces. In contrast, if contact activation is allowed to occur on artificial negative surfaces like glass or kaolin, the formation of XIIa inactivation complexes is completely abolished by CTI (Back et al. Figures 1-3). Thus, there exists strong evidence that CTI is ineffective against XIIa formed on active platelet surfaces. Several reports attribute in vitro thrombin generation following platelet activation to endogenous TF. Caution must be exercised in interpreting such results because contact activation still
occurs upon active platelet surfaces even in the presence of CTI (and antibodies against XII and XI).

Initiation times obtained by titrations of active proteases provide estimates of upper limits for endogenous active proteases. Activation peptides of several proteases have indeed been detected in blood indicating that these proteases do exist in active form albeit in concentrations low enough to have little effect on physiologic time scales [84-87]. Our experimental titrations (Figure 2.4-11) bounded the initial conditions of active proteases (or TF) in blood to: <~500pM Va, <1 nM VIIa, <1 pM IXa, <10 pM Xa, <1 pM XIa and <~40 fM of pre-synthesized TF. Titrations of added thrombin are difficult to deconvolute using fluorogenic thrombin substrates. Circulating thrombin levels must be <100 pM to maintain platelets in an unactivated state (Figure 2.3-2). With respect to initial conditions of active proteases at t=0, we attempted to search the initial condition space of blood using Simulated-Annealing and Particle-Swarm optimization algorithms to find the best fit of initial conditions, subject to the above upper bounds for non-zero initial levels of Va, VIIa, IXa, Xa, XIa and bloodborne TF. Despite more than 100,000 simulations, no acceptable fit was obtained by optimization of initial conditions alone. Importantly, adjustment of XIa (t=0) was insufficient to maintain an “engine idling” model of blood consistent with the results in Figure 2.4-11, Figure 2.4-13 and Figure 2.4-14. It was necessary to include a source term that generated XIIa at a rate slow enough to have little effect on short time scales in the presence of high concentrations of active proteases, but fast enough to limit $T_i$ to that of the control. TF independent VIIa activity was found to be appreciable at high concentrations and upon active platelet surfaces and was therefore also considered as a proximal trigger of coagulation initiation.
The estimation of a rate of XIIa production (Reaction 29, Table 2.4-1) allows us to extend the predictive capacity of the Hockin-Mann model to low concentrations of TF. Further we were also able to simulate titrations of Va, VIIa, IXa, Xa and XIa in 5x diluted blood (Figure 2.4-11); clotting times observed with additions of TF, IXa, Xa and thrombin in whole blood (Figure 2.4-13); and the observed consequences of platelet activation (Figure 2.4-14). However, the Platelet-Plasma model remains a coarse grained platelet and pseudo-homogeneous model. No distinction is made explicitly between bulk and surface phases and binding of reactants onto the platelet surface is not considered separately. Net conversion rates were sped up as the platelet activates by decreasing the unbinding of the enzyme-substrate complex (representing the role of anionic lipid exposure), but no explicit attempt was made to model the change in exposed surface area and chemistry, and its effect on reaction rates. For the sake of simplicity, a single functional form ($\epsilon$) was assumed for the changes in rates as the platelet activates, resulting in a two order change in rate of all desorption reactions on the platelet surface.

We have shown recently that it is possible to construct donor specific models of platelet calcium mobilization as a function of combinatorial agonist concentrations at the site of the thrombus [101]. To incorporate donor specific descriptions of platelet function in models of coagulation, the correlation between intracellular calcium and PS exposure must be defined.

The present study indicates the future feasibility of a full description of blood accounting for intracellular platelet metabolism and heterogeneous reactions on the dynamic platelet surface. In keeping with the static environment in a well plate and the absence of endothelium, no consideration was made of fluid flow, mass transfer from the
bulk onto the platelet surface or thrombomodulin/APC mediated pathways. Our model extends the topology of the Hockin-Mann structure to better simulate *in vitro* clotting initiation of whole blood under diverse initial conditions, however the assumption of a well mixed static system is not appropriate for simulations of thrombosis under flow *in vivo*.

Our high throughput measurement system allows us to rapidly and reproducibly quantify the duration of the initiation phase across numerous conditions from a single blood draw. This has the potential to efficiently identify coagulation pathologies. For instance, an extended duration of this metric for a donor would indicate propensity to bleeding diseases like hemophilia while a shortened metric would indicate predisposition to thrombosis. Titration experiments with added proteases provide a higher dimensional examination of blood function than simply measuring the clotting time after addition of high doses of TF.
Chapter 3

Pairwise agonist scanning of human platelets reveals the high dimensional response to combinatorial mediators of thrombosis

3.1 Abstract

Prediction of cellular response to multiple stimuli is central to evaluating patient-specific clinical status and to basic understanding of cell biology. Cross-talk between signaling pathways cannot be predicted by studying them in isolation and the combinatorial complexity of multiple agonists acting together prohibits an exhaustive exploration of the complete experimental space. Here we describe pairwise agonist scanning (PAS), a strategy that trains a neural network model based on measurements of cellular responses to individual and all pairwise combinations of input signals. We apply PAS to predict calcium signaling responses of human platelets in EDTA-treated plasma to six different agonists (ADP, convulxin, U46619, SFLLRN, AYPGKF and PGE2) at three concentrations (0.1, 1 and 10 × EC50). The model predicted responses to sequentially added agonists, to ternary combinations of agonists and to 45 different combinations of four to six agonists (R = 0.88). PAS could distinguish between the phenotypic responses of platelets from ten healthy male donors and even showed promise in stratifying donors based on ethnicity and gender. Further, PAS was performed in the presence of external calcium for the 3 agonists ADP, CVX and U46619, both without pharmacological intervention and with inhibitors against targeted pathways. This was
used to train patient specific neural network models of regular response, as well as response to therapy, and forms the basis of the platelet activation module in a multiscale Lattice Kinetic Monte Carlo model of thrombosis. Training neural networks with pairs of stimuli across the dose-response regime represents an efficient approach for predicting complex signal integration in a patient-specific disease milieu. Finally this approach can be extended to study not just human platelets, but also murine platelet phenotypes (both regular and genetically modified) across a broad range of conditions using very small volumes of blood.

3.2 Introduction

Since cells can respond to numerous signals and integrate their response to dose-dependent combinations of these inputs, efficient methods are needed to survey such high dimensional systems. The evaluation of response to pairs of stimuli offers a direct and rapid sampling of a response space that can be built-up into a higher level predictive tool through the use of neural networks. Central to cardiovascular disease, platelets are cells that respond in a donor specific manner to multiple signals in vivo and their activation in response to thrombotic signals is central to the thrombotic risks and events surrounding 1.74 million heart attacks and strokes, 1.115 million angiograms and 0.652 million stent placements in the United States each year [174].

During a clotting event, platelets experience diverse signaling cues simultaneously. After capture of the flowing platelet via glycoprotein GPIb-IX-V binding to von-willebrand factor, exposed collagen activates glycoprotein VI (GPVI)-dependent tyrosine kinase signaling leading to phospholipase C\(\gamma_2\) activation, which in turn triggers calcium mobilization and \(\alpha_2\beta_1\) and \(\alpha_{2b}\beta_3\) integrin activation. ADP is released from dense
granules to activate the G-protein coupled receptors (GPCR) P₂Y₁ and P₂Y₁₂, while thromboxane A₂ (TxA₂) is synthesized by platelet cyclooxygenase 1 (COX1) and binds platelet TP receptors. Tissue factor at the site of the damaged vascular wall leads to the production of thrombin which cleaves the platelet GPCR protease activated receptors PAR₁ and PAR₄ on the platelet surface. These activating signals occur in the context of inhibitory signals from endothelial nitric oxide and prostacyclin.

While these diverse signaling events occur simultaneously in vivo during thrombosis, most in vitro studies examine single agonists in isolation. ADP[175], thrombin[176], collagen[177] have been investigated in detail, yet these studies do not capture the highly heterogeneous and dynamic thrombotic environment. Platelet signaling varies spatially and temporally in growing thrombi[178]. Few experimental or computational tools are available for building a global understanding of how a cell integrates multiple stimuli present at varying levels.

We have developed a high-throughput experimental platform that measures the human platelet response to all pairwise combinations of six major agonists. Agonists tested in this study were: convulxin (CVX; GPVI activator), ADP, the thromboxane analog U46619, PAR₁ agonist peptide (SFLLRN), PAR₄ agonist peptide (AYPGKF), and PGE₂ (activator of IP receptor). The method yields high-resolution time-series measurements of intracellular calcium which is the convergent node of these platelet signaling pathways (Figure 3.2-1) and is critical to granule release, exposure of phosphatidylserine, actin polymerization, shape change, and integrin activation[179]. We first tested the platelet response to all pair-wise combinations of 6 agonists at 3 separate doses (0.1, 1, 10×EC₅₀). These 135 calcium traces trained a 2-layer neural network (NN)
to successfully represent an individual’s platelet phenotype. The Pairwise Agonist Scanning (PAS) method demonstrates that sampling all dual orthogonal "axes" (every agonist pair) can successfully predict the dynamic responses and cross-talks of a higher dimensional system (6 agonists in this case).

![Figure 3.2-1 Signaling pathways in human platelets converge on calcium](image)

Platelets integrate signals from numerous simultaneous thrombotic inputs. The signals considered in this study include Collagen (CVX), thromboxane (U46619), thrombin (AYPGKF and SFLLRN), ADP and PGE2. All of these signals regulate the levels of cytosolic calcium and an elevation of calcium concentration precedes several responses including integrin activation, granule release and shape change. (Also see Section 1.2 and Figure 1.2-1).

### 3.3 Materials and methods

#### 3.3.1 Materials

The protease activated receptor-1 (PAR-1) agonist peptide SFLLRN (thrombin receptor agonist peptide, TRAP) and the PAR-4 agonist peptide AYPGKF were obtained from Bachem (King of Prussia, PA). Convulxin (CVX), was obtained from Centerchem,
Thrombin and GGACK were obtained from Haematologic Technologies (Essex Junction, VT). Clear, flat bottom black 384-well plates were obtained from Corning (Corning, NY). ADP, U46619, PGE$_2$, EDTA, HEPES, the fibrin polymerization inhibitor Gly-Pro-Arg-Pro (GPRP), NaCl, NaOH, apyrase, indomethacin and sodium citrate were all from Sigma (St. Louis, MO). Fluo-4 NW Calcium assay kits were obtained from Invitrogen (Carlsbad, CA). The buffer used for all dilutions was HEPES buffered saline (HBS, sterile filtered 20 mM HEPES and 140 mM NaCl in deionized water adjusted to pH 7.4 with NaOH).

3.3.2 Platelet preparation

Whole blood was drawn from healthy male volunteers according to the University of Pennsylvania Institutional Review Board guidelines, into citrate anticoagulant (1 part sodium citrate to 9 parts blood). All donors affirmed to not taking any medications for the past 10 days and not consuming alcohol for the past 3 days before phlebotomy. After centrifugation at 120g for 12 min to obtain platelet-rich plasma, 2 ml of platelet-rich plasma was incubated with each vial of Fluo4-NW dye mixture reconstituted into 8 ml of buffer for 30 min.

3.3.3 High throughput experimentation

An ‘agonist plate’ containing varying combinatorial concentrations of platelet agonists was prepared on a PerkinElmer Janus (PerkinElmer Life and Analytical Sciences) using 10× stock solutions of ADP, CVX, SFLLRN, AYPGKF and U46619. A separate ‘platelet plate’ containing dye-loaded platelets was prepared on a PerkinElmer Evolution.
Figure 3.3-1 High throughput experimental procedure

An agonist plate containing combinatorial agonist combinations and a platelet plate containing dye-loaded platelets were separately assembled. Agonists were dispensed onto platelet suspensions and fluorescence changes were measured to quantify platelet calcium concentrations $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ transients can be represented as overlapping plots (lower right) or parallel heat maps (lower left). RFU, relative fluorescence units.
Final platelet rich plasma (PRP) concentrations were 12% by volume (6 μl/well) after agonist addition, and 5 mM EDTA was included in every well. Agonists (10 μl/well) were dispensed after a 20-s baseline read from columns of the ‘agonist plate’ onto the corresponding columns of the ‘platelet plate’ on a Molecular Devices FlexStation III. Fluo4 fluorescence was measured at excitation 485 nm and emission 535 nm for 4 min in every column of the plate. The fluorescence $F(t)$ was scaled to the mean baseline value for each well $F_0(t)$ and relative calcium concentrations were quantified as $F(t)/F_0(t)$. An entire 384-well plate was read in ~90 min.

### 3.3.4 Agonist selection

The number of agonists tested in a PAS experiment is limited to 6 by the need of testing all the 154 conditions in duplicate in a single 384-well plate. Agonists were chosen to be representative of physiological signaling cascades. Convulxin is a selective GPVI activator [180] and under static conditions this receptor is the predominant determinant of collagen induced signal strength [181]. In contrast, the soluble monomeric form of collagen interacts only with $\\alpha_2\\beta_1$, which regulates platelet adhesion but has little direct effect in mediating signaling [182, 183]. “Horm” collagen preparations are insoluble making them poorly suited for automated liquid handling.

Although ADP stimulates both $P_2Y_1$ and $P_2Y_{12}$, the latter receptor has minor effect on calcium mobilization [184], allowing us to use the physiological agonist ADP instead of specific $P_2Y_1$ ligands. Thrombin signals through two separate $G_q$ coupled receptors PAR1 and PAR4 both of which produce temporally separate calcium signals [185, 186]. This prompted us to use selective PAR agonist peptides (SFLLRN and AYPGKF) to distinguish the separate signal contribution of both these receptor pathways. Moreover
thrombin stimulation of unwashed PRP requires inhibition of fibrin and FXa formation (See Figure 3.4-21). Washing or gel filtering platelets is a processing step that decreases throughput in a large scale experiment and often causes residual platelet activation in the absence of PGE₂ or other PGI₂ analogs. The use of a short lived prostaglandins like PGI₂ [187] is unsuitable for assembly of agonist plates (requiring ~120 min) and plate reading (requiring ~90 min). In contrast, prostaglandins of the E series are chemically stable prompting us to use PGE₂ as an agonist causing elevation in intracellular cAMP. Similarly, for reasons of stability during the course of the experiment the thromboxane analogue U46619 was used instead of its physiological equivalent TxA₂ [188].

3.3.5 Choice of synergy score

To quantify cross talks between agonist combinations, the ‘Synergy score’ was defined as the difference between the observed and the predicted additive response. For ease of visualization this difference was scaled to the maximum synergy score observed in an experiment (or simulation) giving a metric that ranges from -1 (antagonism) to +1 (positive synergy). A similar synergy metric was previously defined as the ratio of the observed and the predicted additive response to demonstrate synergistic calcium signaling between C5a and UDP in RAW264.7 cells and bone marrow derived macrophages[189]. The use of a ratio rather than a difference is prone to numerical errors for small values of the predicted additive response.

3.3.6 Neural network model construction, training and simulation

Neural network modeling and analysis was performed using the Neural Network Toolbox for MATLAB (The MathWorks). Training data consisted of (i) the dynamic
inputs, which represent the combination of agonist concentrations present at each time point for a particular experiment. Because the concentration of agonists remains essentially constant throughout each experiment, these values were generally a constant vector of concentration values repeated at 1-s intervals. (ii) The dynamic outputs, which represent the experimentally measured calcium concentrations, also interpolated at 1-s intervals. To normalize the input data, agonist concentrations of 0, 0.1, 1 and $10 \times EC_{50}$ were mapped to the values ($-1$, $-0.333$, $+0.333$, $+1$) before introducing them to the network, so as to fall within the working range of the hyperbolic tangent sigmoid transfer function, which was used for all processing nodes. Output values (fluorescence measurements) were normalized between $-1$ and $+1$, so that the basal concentration of calcium at $t = 0$ was defined to be 0. After training all 420 possible one- and two-layer neural networks with between 1 and 20 nodes in each processing, or ‘hidden’, layer and testing each network for accuracy, a final neural network topology with a six-node input layer (representing the six agonists), two processing layers (eight nodes/four nodes) and a single-node output layer (representing the intracellular calcium concentration) [190] was most optimal (best predicted the ‘net’ output response $[Ca^{2+}]_i$ for a given multivariate input using the fewest neurons) and thus selected to predict successive time points from all 154 $Ca^{2+}$ release curves gathered experimentally (Figure 3.4-3). For the sake of simplicity and because we already obtain reasonably accurate time series predictions of $[Ca^{2+}]_i$, more processing layers or $>20$ neurons in each layer were not tested.

The work training, testing and utilizing the predictions of the Neural Network model in Section 3.4 was done in collaboration with Jeremy Purvis.
Figure 3.3-2 Dynamic neural network used to train platelet response

A constant sequence of input signals (agonist concentrations) is introduced to the two-layer, 12-node network at each time point. Processing layers integrate input values with feedback signals to predict $[\text{Ca}^{2+}]_i$ at the next time point.

From a purely biological perspective, the model architecture is arbitrary and no particular meaning should be inferred from the narrowing of eight nodes in the first layer to four nodes in the second processing layer. Moreover, this neural network model (Figure 3.3-2) does not correspond to an actual signaling network (Figure 3.2-1) but does provide a highly efficient framework for use as an independent signaling module in multiscale models of thrombosis under flow. From a mathematical perspective this architecture represents a multivariate regression to obtain optimal good fits of high-dimensional data and allow extrapolation onto experimentally unexplored spaces.
NARX (nonlinear autoregressive network with exogenous inputs) models are recurrent dynamic networks with feedback connections enclosing multiple layers of the network, and are well-suited for predicting time series data [191] because they process inputs sequentially, that is, at successive time points. Calcium outputs before the current instant were fed back to hidden layers using a delay line spanning 128 s. Initial states of the delay line were set to 0, corresponding to the steady state of the platelet before agonist stimulation. Such a structure allows the network output to progress over time, using the ‘memory’ of the previous 128 s in calculating the current output. Training was performed using Levenberg-Marquardt back-propagation until the performance of the model (mean squared error between the simulated and experimentally measured PAS responses.) did not improve >1 x 10^{-5}. During training, the pairwise agonist data (154 time-course traces) was divided into training, validation and testing vectors. Validation and testing vectors were each generated by randomly selecting 23 (15%) of the 154 pairwise time-course traces. The training vectors were used to directly optimize network edge weights and bias values to match the target output. The validation set was used to ensure that there is no overfitting in the final result. The test vectors provide an independent measure of how well the network can be expected to perform on data not used to train it.

Mathematically, the output $y$ at an instant $t$, for an input vector $\overline{I}$ of the concentrations of the six inputs species can be compactly described by

$$y(t) = f \left( \overline{L}_3^{(1;4)} \times f \left( \overline{H}_2^{(4;8)} \times \overline{y}_{k(8;1)} + \overline{L}_2^{(4;8)} \times f \left( \overline{H}_1^{(8;8)} \times \overline{y}_{k(8;1)} + \overline{W}_{(8;6)} \times \overline{I}_{(6;1)} + \overline{b}_1^{(8;1)} + \overline{b}_2^{(4;4)} + \overline{b}_3^{(1;4)} \right) \right) \right)$$
where $\text{IW}$ is the matrix of input weights, $L_2$ and $L_3$ are the weight matrices that operate on the ‘inputs’ coming from the first and second processing layers respectively. $H_1$ and $H_2$ are matrices that contain history coefficients that weigh the history vector $y_h$ (containing the output of the system 1, 2, 4, 8, 16, 32, 64 and 128 s prior to the current instant). $b_1$, $b_2$ and $b_3$ are bias vectors that add constant biases to each weighted input and weighted histories to produce the ‘net input’ to each transfer function. $f$ is the hyperbolic tangent function that operates on a vector of ‘net inputs’ to yield the corresponding transformed output. Numbers in parentheses show the sizes of relevant matrices or vectors. The NARX model presented here, represents a nonlinear regression model with input stimuli and system history. The use of simple 1st and 2nd order polynomial terms (with lower number of optimizable parameters) did not produce acceptable fits (not shown), necessitating the use of the NARX architecture. A 3rd order polynomial was not attempted since it requires 316 fitting parameters, far exceeding the number of parameters in the neural network model.

It should be noted that each trained neural network model produces a deterministic prediction of platelet activation. Experimental variations are inherent in replicates of donor-specific training data (Figure 3.4-17), and the tightness of the measured mean will determine the predictive quality of such a donor-specific neural network model.

Busch et al.[192] used a continuous time recurrent neural network (CTRNN), to describe the fold expression kinetics of 9 “top ranked” genes involved in the sustained migration of keratinocytes after hepatocyte growth factor (HGF) treatment. Utilizing the NN weights for a signaling network, they define the modulation and control elements of
the response [192]. Also, previous studies have used Partial Least Squares Regression Analysis (PLSR) to understand the interplay of molecular mechanisms during signaling [193, 194]. PLSR measures multiple intermediate signaling molecules at various time points for a relatively small number of inputs, and identifies principal components that capture the phenotype of the system. In comparison, the PAS approach offers less mechanistic dissection, but provides rapid (a 2-hr experiment) and efficient prediction of dynamic input-output relationships at numerous (~10²) physiologically relevant conditions. Geva Zatorsky et al. [195] have recently reported similar utility of sampling the effects of drug pairs in predicting the dynamics of response to three or four drug combinations in lung cancer cells.

3.4 Results

3.4.1 Defining the dynamic range of individual agonists

To determine appropriate dynamic ranges for the 6 agonists (Figure 3.2-1), each compound was first tested individually. Dose response curves were constructed in terms of the peak fluorescence signal at each agonist dose (Similar dose responses were obtained for the integrated signal). This allows us to define the complete dynamic range for each agonist and to determine their half maximum response (EC₅₀) values (Figure 3.4-1). The inhibitory response of PGE₂ was studied by concomitantly stimulating the platelet with 60 μM SFLLRN. These dose responses were determined both in the absence and presence of external calcium. The removal of external calcium does not affect the ability of the studied receptors to signal, as no appreciable difference in EC₅₀'s were noted with or without external calcium (Figure 3.4-1 a and b).
Figure 3.4-1 Single agonist dose response curves

Single agonist dose response curves were constructed for platelet responses to the agonists ADP, CVX, U46619, SFLLRN, AYPGKF and the antagonist PGE2. All conditions are for a single donor with 8 replicates per agonist dose. EC50 levels were determined by fitting a 4 parameter curve (dashed lines) to the peak calcium signal. EC50 levels were almost unchanged in (a) 1.5 mM extracellular Calcium (with 15 μM Indomethacin to prevent autocrinic amplification via TXA2) or (b) 5 mM EDTA. Values shown are mean ± standard deviation.
3.4.2 Supression of autocatalytic signal amplification

To eliminate the sensitivity of cells to confounding autocrine effects of soluble mediators that are dependent on platelet concentrations and transport processes, we conducted all experiments in 5 mM EDTA, which chelates extracellular calcium. In spite of the absence of external calcium the ability of the receptors studied to signal is not affected (Figure 3.4-1 a and b). Although such an experimental design does not capture the contribution of store operated calcium entry, it offers several operational advantages by (i) lowering background fluorescence without extensive platelet washing, (ii) preventing thrombin production, (iii) inhibiting granule release [196, 197] as well as TxA2 formation [198], and (iv) inhibiting integrin mediated signaling downstream of Ca2+ release [199]. Thus, the resulting traces of Ca2+ are directly dependent only on receptor mediated release from intracellular stores. The operational advantages of using EDTA, however, prevent prediction of important physiologic phenomena like granule release, integrin activation and outside-in signaling.

To test whether the intracellular Ca2+ signal detected was being influenced by endogenously released agonists, we studied the effects of 2 units/ml apyrase (which hydrolyzes released ADP) or 15 μM indomethacin (which inhibits production of TxA2). Both these inhibitors had no effect on individual responses (Figure 3.4-2, and Table 3.4-1 and Table 3.4-2), suggesting that endogenous agonists have no effect on the Ca2+ signal. This confirms that the resulting traces of Ca2+ are directly dependent only on receptor-mediated release from intracellular stores.
Figure 3.4-2 Addition of Apyrase and Indomethacin.

Possible secondary autocrine amplifications by secreted ADP or synthesized thromboxane were evaluated by adding the ADP hydrolyzing enzyme apyrase or the COX inhibitor indomethacin. No statistically significant reduction in (a) the peak calcium signal or (b) the integrated calcium signal was noted upon stimulation with either 0.1, 1, or 10x EC$_{50}$ levels of CVX, U46619, SFLLRN, AYPGKF or PGE$_2$. *s represent conditions where the use of the inhibitor produced a significant reduction ($P$ Value <0.05). The $P$ Values for all the comparisons carried out are listed in Table 3.4-1 and Table 3.4-2. Values shown are mean ± standard deviations.
<table>
<thead>
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<th>Agonist</th>
<th>Dose (×EC50)</th>
<th>15μM Indomethacin</th>
<th>2 Units/ml Apyrase</th>
<th>Both Inhibitors added</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.6504</td>
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<td>0.4686</td>
<td>0.4091</td>
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<td>10</td>
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<td>0.5314</td>
<td>0.3496</td>
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<tr>
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<td>0.7857</td>
<td>0.0325*</td>
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<tr>
<td></td>
<td>10</td>
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<td>0.9535</td>
<td>0.9535</td>
</tr>
<tr>
<td>SFLLRN</td>
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<td>0.5314</td>
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<tr>
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<td>10</td>
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<tr>
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</tr>
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<td>PGE2+60μM SFLRN</td>
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<td>0.9102</td>
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<td>10</td>
<td>0.3312</td>
<td>0.7056</td>
<td>0.5909</td>
</tr>
</tbody>
</table>

Table 3.4-1 Comparison of peak signals in the presence and absence of inhibitors against autocatalytic signal amplification pathways

*P* Values comparing the peak calcium signal in the presence of various inhibitors (columns 3, 4 and 5) with no added inhibitor at various agonist doses (column 2). *s indicate significant reductions in peak signal and were noted in 2 out of 45 conditions tested.
### Table 3.4-2 Comparison of integrated signals in the presence and absence of inhibitors against autocatalytic signal amplification pathways

*P* Values comparing the integrated calcium signal in the presence of various inhibitors (columns 3, 4 and 5) with no added inhibitor at various agonist doses (column 2). *s indicate significant reductions in peak signal and were noted in 3 out of 45 conditions tested.

Each experimental condition was tested in replicates of 6. The non-parametric Wilcoxon-Mann-Whitney test was used to test whether the use of either Indomethacin/ Apyrase or both inhibitors added together resulted in a significant reduction in signal compared to the control experiment where no inhibitors were added. The tests were conducted at 5% significance level. Statistically significant reductions in signal were obtained with these added inhibitors in only ~5% of the conditions tested.
Lack of detectable secondary autocatalytic amplification is expected since diffusion would reduce platelet surface ADP levels by 1000-fold within 2 sec following release of all dense granules [200]. Also assuming release of 75% of platelet dense granule ADP content of 1.74 μmoles/10¹¹ platelets [201] into an instantaneously isotropic reaction volume of 50 μl containing 12% PRP results in a final ADP concentration of 494 nM, a concentration less than the EC50 levels of ADP. In vivo, platelets are concentrated 5-10 fold near the vessel wall due to drift caused by red blood cell accumulation at the vessel center [202]. Furthermore, during thrombosis, platelet concentrations in deposited aggregates can increase 10 to 50-fold over platelet-rich plasma levels. At such close confinement during thrombosis, autocatalytic amplifications caused by secreted ADP and TxA₂ will become significant. However in the controlled, dilute (12% PRP), and unstirred environment inside the well plate containing EDTA which attenuates secretion, we observed only primary effects of receptor mediated calcium release from cell stores.

### 3.4.3 Pairwise Agonist Scanning

We tested all 135 pairwise combinations of low (0.1×EC₅₀), moderate (1×EC₅₀), and high (10×EC₅₀) agonists concentrations (Figure 3.4-3 a). The pairwise agonist synergy score (S_ij) is the scaled difference between the integrated calcium transient (area under the curve) for the combined response and the integrated area for the individual responses (Figure 3.4-3 b) (S_ij>0, synergism; S_ij=0, additivity; S_ij<0, antagonism).

Neural network (NN) models are remarkable in learning patterns of inputs and predicting outputs by optimizing the weights of intermediate connections, akin to a platelet’s ability to respond to multiple thrombotic signals through coupled biochemical reactions. Motivated by the notion that a living cell is essentially a neural network whose connection weights have been selectively adjusted during evolution [203], we took a “top-down” approach [204] to model platelet signaling. A NN model was trained on these 154 time-course traces (135 pairwise responses, 18 single agonist responses, 1 control) and captured both the time-course behavior (R=0.968 for correlation between time points) and the pairwise agonist synergy (R=0.884 for correlation between S_ij scores (Figure 3.4-4) with excellent accuracy (Figure 3.4-3 a and b).
**a** Input

<table>
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<th>PGE2</th>
<th>AYPGKF</th>
<th>SFLLRN</th>
<th>U46619</th>
<th>ADP</th>
<th>CVX</th>
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</thead>
<tbody>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Platelet Response

- Interquartile Range: min
- Maximum: max

Intracellular [Ca²⁺]

---

**b** Pairwise Synergy Score ($S_{ij}$)

$$S_{ij} = \frac{\int AB - (\int A + \int B)}{\max[\int AB - (\int A + \int B)]}$$

---

**Experiment**

<table>
<thead>
<tr>
<th></th>
<th>PGE2</th>
<th>AYPGKF</th>
<th>SFLLRN</th>
<th>U46619</th>
<th>ADP</th>
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**NN Prediction**

<table>
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<th>U46619</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Dose × EC₅₀

Normalized Synergy
Figure 3.4-3 Pairwise Agonist Scanning (PAS)

(a) Pairwise agonist scanning (PAS) All 154 binary combinations of the agonists CVX, ADP, U46619, SFLLRN, AYPGKF and PGE₂ at concentrations of 0, 0.1, 1 and 10x EC50 were combined on the same plate (in replicates of 2) and the dynamic response of the platelet to each combination was recorded. The NN model was trained on this dataset. 

(b) Pairwise agonist synergy scores, which reflect the gain or loss in calcium response due to agonist cross talk, were calculated for both experimental and predicted time course traces. EC₅₀: PGE₂, 24.6 µM; AYPGKF, 112 µM; SFLLRN, 15.2 µM; U46619, 1.19 µM; ADP, 1.17 µM; CVX, 0.00534 µM.

Figure 3.4-4 Predicted versus measured synergy scores for a PAS experiment

Predicted versus measured synergy scores for the 135 conditions in a PAS experiment that contained binary agonist pairs (Figure 3.4-3). This fit is a measure of the “adequacy” of training of the NN on the binary interaction dataset. The strong correlation shows that the NN was suitably trained by the data, but does not yet demonstrate the predictive capability of the model.
3.4.4 Platelet responses to ternary agonist stimulation

![Diagram showing platelet response to ternary agonist stimulation]

Figure 3.4-5 Ternary agonist stimulation

Measurement and prediction of the platelet response to all 64 ternary combinations of ADP, SFLLRN and CVX at 0, 0.1, 1 and $10 \times EC_{50}$. The neural network model was trained only on pairwise interactions but successfully predicted ternary interactions.
Figure 3.4-6 Predicted versus measured synergy scores for ternary stimulation

Predicted versus measured synergy scores for the 27 conditions in the ternary experiment (Figure 3.4-5) that contained 3 agonist combinations. This fit is a measure of the *de-novo* predictive capacity of the NN model (trained exclusively on the binary interaction dataset) on ternary agonist space.

Next, we measured all 64 ternary combinations of the agonists ADP, SFLLRN, and CVX at 0.1, 1, and 10×EC<sub>50</sub> concentrations (Figure 3.4-5). A CVX response requires GPVI multimerization [180] and is characterized by a slow rise to a large peak signal followed by a slow decline. In contrast, G<sub>q</sub>–coupled responses (ADP or SFLLRN) produce rapid bursts that are quickly brought down to baseline. Increasing CVX for a fixed ADP level resulted in a steady increase in calcium concentrations on longer
timescales. In contrast, increasing ADP for a fixed CVX level bolstered early calcium release. A moderate dose of both ADP and CVX (for 0 and low SFLLRN) produced a response that almost instantaneously plateaued at a steady level above baseline.

The NN model, trained exclusively on the pairwise interaction data set (Figure 3.4-3 a), successfully predicted all experimentally observed trends in the ternary interactions among the three agonists studied. Both the time-course behavior ($R = 0.844$) and ternary agonist synergy scores ($R = 0.881$) (Figure 3.4-6) were accurately reproduced for the 27 unique ternary conditions in this experiment that were not present in the training set. Thus, the pairwise interaction data presented in Figure 3.4-3 captured additional information about the interactions amongst 3 agonists.

### 3.4.5 Sequential addition of the platelet agonists ADP, SFLLRN, CVX

We also explored the effect of adding the agonists ADP, SFLLRN and CVX in various sequential combinations (Figure 3.4-7). Several interesting behaviors were accurately predicted by the NN model. Preceding a large dose of ADP with prior treatment with low dose of ADP (Figure 3.4-7, panel 12) desensitized the calcium signal expected from a large dose of ADP (as in Figure 3.4-7, panel 1). This behavior has been observed previously [205] and is attributed to the internalization of $P_2Y_1$. The temporal sequence ADP-SFLLRN-CVX (Figure 3.4-7, panel 1) produced three distinct calcium bursts, whereas the ADP response was completely abolished in the sequence SFLLRN-ADP-CVX (Figure 3.4-7, panel 3). This behavior points to mechanisms of cross-down regulation of the ADP signaling cascade by component(s) of the PAR1 cascade.
Figure 3.4-7 Measured and predicted platelet responses to sequential additions of ADP, SFLLRN and CVX.

The effect of ADP, SFLLRN and CVX added in various temporal sequences was tested. The NN model trained exclusively on binary interactions could predict several of the experimentally observed trends of cross down regulation and desensitization between the ADP, SFLLRN and CVX cascades.
Prior addition of CVX abolished detectable responsiveness to both ADP (Figure 3.4-7, panel 5) and SFLLRN (Figure 3.4-7, panel 6) again pointing to a mechanism where components of the GPVI signal are able to down regulate the G$_t$-coupled ADP or SFLLRN signal. Additions of any two of these agonists in combination followed by the third agonist confirm the observation that any mixture containing CVX down-regulates responsiveness to both ADP (Figure 3.4-7, panel 9) and SFLLRN (Figure 3.4-7, panel 8). CVX-mediated calcium mobilization events were unaffected by pretreatment with either ADP (Figure 3.4-7, panel 2) or SFLLRN (Figure 3.4-7, panels 4 and 3), or a binary combination of these agonists (Figure 3.4-7, panel 7). Activation of GPVI or thrombin receptors phosphorylates the ITIM domain of platelet PECAM [206]. ITIM phosphorylation inhibits response via phosphatases like SHP-2 [207]. Such inhibitory mechanisms, or even the existence of agonist selective stores [208], may explain the lack of ADP/SFLLRN response after prior CVX stimulation and the lack of ADP response after prior SFLLRN stimulus. Simulation traces containing CVX did not decay as observed experimentally after ~260s (Figure 3.4-7, panels 2, 4-10). Such a limitation was expected because the NN was trained on measurements spanning only 260s (Figure 3.4-3 a) and not the entire duration (upto 900s). Importantly, the NN captured such cross-talks of sequential additions despite being trained on purely synchronous interactions.

3.4.6 Sequential additions mimicking the in vivo thrombotic environment

Platelets at the site of injury are likely to encounter specific sequences of thrombotic signals. These are illustrated in Figure 3.4-8 A. The effects of thrombin can be approximated by considering SFLLRN+AYPGKF to be its equivalent in simulation.
Figure 3.4-8 Sequential additions mimicking the \textit{in vivo} thrombotic environment

\textbf{(A)} Various sequences of thrombotic signals likely to be encountered by platelets during \textit{in vivo} thrombosis. \textbf{(B)} Comparisons between experiment and simulations studying sequential additions of CVX, ADP and thrombin (or SFLLRN + AYPGKF)
In a realistic thrombotic setting, the platelet is likely to first get activated upon an exposed collagen surface. Subsequently, platelets adhered to collagen will encounter either thrombin (formed on the active platelet surface downstream of the coagulation cascade) or ADP (released from activated platelets). Platelets that do not directly adhere to the exposed collagen surface might get activated by thrombin or ADP (or sequences of these agonists) and subsequently stick on to collagen adhered platelets or form larger aggregates in solution. These conditions are illustrated in Figure 3.4-8 A. We mimic these 4 conditions by making selective sequential additions (similar to Figure 3.4-7) of the collagen analogue CVX, the physiological agonist thrombin as well as preassembled mixtures of SFLLRN and AYPGKF peptides all at 10x EC50 in Figure 3.4-8 B. We show that the signals generated by thrombin for these selective additions are similar to those generated by a mixture of high dose PAR1+PAR4 peptides. A PAS trained NN for donor A, qualitatively reproduces these sequential additions of PAR1+PAR4 peptides (to approximate the function of thrombin). The results we obtained are similar to those already reported (Figure 3.4-7). Prior GPVI activation abolishes detectable responsiveness to ADP or thrombin. Thrombin (or PAR1+PAR4) abolishes subsequent response to ADP (presumably by cross down regulation of the ADP cascade by PAR1 as was already reported in Figure 3.4-7). In contrast, prior ADP stimulation does not abolish subsequent detectable responsiveness to thrombin (or PAR1+PAR4). We note that the NN was not trained using thrombin as an agonist and peptides have weak μM affinity to PARs compared to the intramolecular signaling that is generated by thrombin cleavage of the PARs. Differences between thrombin and preassembled mixtures of
SFLLRN and AYPGKF were noted when a CVX stimulus followed these inputs. These effects are discussed in Section 4.2.

3.4.7 Predicting the global 6 dimensional human platelet response

To fully test and utilize the predictive power of the NN model, we made in silico time-course and synergy predictions for the complete 6-dimensional agonist space consisting of 4077 unique agonist combinations of 2 to 6 agonists at 0.1, 1, or 10×EC$_{50}$ concentrations. For comparison, the full distribution of synergy predictions for all 4077 agonist combinations is shown as a vertical heat map in Figure 3.4-12. Based on these predictions (Figure 3.4-9), we selected 45 combinations of 4, 5 or 6 agonists that displayed a range of predicted synergy scores from strong synergy to strong antagonism and tested them experimentally (Figure 3.4-10). To prevent any bias in the selection we selected conditions that had maximal dissimilarity in the types and concentrations of agonists.

![Figure 3.4-9 Predicted distribution of synergies scores.](image)

The complete distribution of predicted synergy scores in all 4077 conditions that comprise the 6 dimensional agonist space of the agonists PGE2, AYPGKF, SFLLRN, U46619, ADP and CVX. This space was probed at the indicated positions to sample both ends of the synergy spectrum.
Figure 3.4-10 Measurement and prediction of response in the full agonist space.

45 conditions (containing 4-6 agonists) spanning the entire synergy spectrum were chosen from amongst the NN model’s predictions of the complete experimental space. These were tested experimentally along with 19 controls.

We found strong agreement between both predicted and measured calcium transient shapes \((R=0.845)\) in **Figure 3.4-10** and **Figure 3.4-11**, as well as between predicted and measured \(S_{ij}\) scores \((R=0.883, \text{ slope}=1.08)\) (**Figure 3.4-12**).
Figure 3.4-11 Probing the complete agonist space.

Experimentally observed mean (blue) and NN predicted (red) $[\text{Ca}^{2+}]_i$ time courses for 45 out of the 4077 possibilities (shown in Figure 3.4-9) in the complete 6 dimensional agonist space. Each experiment was done in replicates of 6. Experimental error bars corresponding to standard deviations are shown at only 17 points along a time course for the sake of visual clarity. The 45 conditions tested were chosen to span antagonism, additive interaction and positive synergy.

Conditions containing high levels of all agonists showed especially low measured synergy due to saturation of calcium release. The highest synergy was observed for agonist combinations that contained high levels of the thromboxane analog U46619 with no PGE$_2$ present (Figure 3.4-12, orange bar).
Figure 3.4-12 Predicted versus measured distribution of synergies for conditions containing 4-6 agonists and spanning the complete agonist space.

Predicted versus measured synergy scores for the 45 conditions in Figure 3.4-10 (upper left). Distribution of synergy scores for all 4,077 possible experimental conditions (upper right). Experimental conditions for the 45 sampled combinations of agonists, arranged in order of increasing synergy (bottom). The orange bar denotes the three most highly synergistic conditions, which all contained high U46619, no PGE₂ and low levels of other agonists.
Given that only 8 of 45 conditions had maximal U46619/PGE2 ratio, this ordering of the top 3 conditions was highly significant ($p < 0.004$), considering there are 14,190 possible ways to order the first 3 conditions of which only 56 combinations would contain high U46619 and low PGE2. Thus, the NN model trained on pairwise data facilitated discovery of a high dimensional synergy that occurs at high U46619/PGE2 ratio (at low levels of ADP, SFLLRN and submaximal levels of AYPGKF) consistent with the known cardiovascular risks of COX2 inhibitors that prevent endothelial production of prostacyclin without affecting platelet production of thromboxane [209]. This points to a ‘high-dimensional’ COX2 inhibition risk of high concentrations of thromboxane, in the absence of PGI2, potentiating the effects of other agonists.

Figure 3.4-13 Effect of input silencing on predictive ability

The NN model’s predictive ability of response to multiple agonists does not amount to prediction of the response to a dominant pair in the input set of agonists. To illustrate the ability of the model to integrate all of the inputs in its prediction and not just rely on certain input species, we systematically silenced each input to the model. Single inputs were silenced first, followed by pairs, triples, etc. The performance of such models that considered only smaller input sets (comprised of a lower number of input species) was evaluated across all high dimensional input sets tested.
The results of this analysis are presented above. Presence of a particular agonist is indicated by a black fill in a respective input set. In all but two cases, the overall predictive power decreased as inputs were silenced. This shows that the NN model utilizes information from all inputs to gain accuracy. Any suppression of 1, 2, 3, 4, or more inputs drives down the accuracy. In fact, deletion of data down to only 2 inputs (if a pair is often able to predict as well as the full input set) shown in red reduces the predictive power severely to zero correlation between experiment and the NN. This data demonstrates that no single pair embedded in the experiment that predicts all the tested outputs better than the full input test.

We note that there were two smaller subsets that yielded better prediction than the complete input set. Also there were cases where reducing the number of inputs markedly (for instance even up to two inputs for the two left most red columns) does not make much difference to the overall prediction. It is generally impossible however, to have a priori knowledge of which input to exclude to get better predictive ability than the full input set. Given that there is a strong trend for predictions to do better when given multiple inputs (despite these few exceptions) we conclude that the NN integrates information from all input species.

To investigate whether smaller subsets of inputs, such as dominant pairs, could account for the network’s predictive accuracy, we retrained the neural network on different subsets of inputs. This typically, but not always, reduced predictive accuracy (Figure 3.4-13), suggesting that the neural network does not exclusively rely on smaller subsets of inputs.

### 3.4.8 Donor specific platelet responses

To investigate the reproducibility of the PAS procedure and to investigate the potential for using it to stratify individuals’ platelet responses, PAS was performed twice within two weeks of each other (with 2 replicates of every condition in an individual experiment) for 10 healthy male donors to construct donor specific synergy maps (Figure 3.4-14). Donors (Ages: 22-30 years) spanned several ethnic groups (3 Western Europeans, 2 Asians, 2 Indians, 1 Caribbean, 1 African American and 1 African).
Figure 3.4-14 Donor specific platelet agonist synergisms.

PAS was performed twice for 10 healthy male donors to construct 135 parameter donor specific synergy maps.
Figure 3.4-15 Donor specific PAS clustering patterns

Ten healthy donors were phenotyped for platelet calcium response to all pairwise agonist combinations. Repeat experiments were conducted within 2 weeks. Donors (ages, 22–30 years) spanned several ethnic groups (three Western Europeans, two Asians, two Indians, one Caribbean, one African American and one African). The magnitudes of synergy in each of the 20 donor-specific synergy maps were arranged as columns of the synergy matrix. These vectors were clustered according to similarity using a distance-based clustering algorithm.

The 135 conditions containing pairs of agonists in a single PAS experiment make up the synergy map for each donor experiment (Figure 3.4-14) and individual columns of the synergy matrix (Figure 3.4-15). To demonstrate reproducibility and donor specificity, a simple hierarchical cluster tree was generated using the Euclidean distances between
donor experiments (columns of the synergy matrix). A total of 7 out of the 10 donor pair vectors (Donor pairs D, C, A, H, E, F and I) self clustered, demonstrating that in spite of intra donor variations in synergy, pronounced inter donor variations allow us to distinguish donors. This pattern of clustering was found to be highly significant (p < $8 \times 10^{-7}$) by randomizing observed donor synergies (Figure 3.4-16) considering the fact that 5 donor pairs (the highest number of donor pair clusterings by random chance) self clustered in only 8 out of 10 million random permutation tests.

![Figure 3.4-16 Randomization of donor specific synergisms.](image)

The synergy scores for each donor (values in the columns of the synergy matrix shown in Figure 3.4-15 were randomized to test whether the clustering of the ‘observed’ donor specific synergy vectors was significant. We found that the experimentally observed clustering of 7 donor pairs (red line) out of 10 was highly significant. A P-Value less than $8 \times 10^{-7}$, under the null hypothesis that the donor clustering pattern was by chance alone, was obtained.
Donors separated into at least 2 major subgroups with the cluster of donor experiments D1, D2, J2, C1, C2, B1 and B2 characterized by relative lack of synergy (red) in comparison to other experiments. The cluster of experiments A1, A2, H1, H2, J1, E1, E2, F1, F2, G1, I1, I2 and G2 had marked synergy between moderate doses of SFLLRN and all doses of U46619/ADP, as well as marked synergy for moderate U46619 and high CVX. Mild positive synergisms such as those between low doses of SFLLRN and U46619/ADP, as well as those between ADP and CVX further classify this latter cluster into smaller sub clusters comprising of donors A1, A2, H1, H2 and J1; and donors E1, E2, F1, F2, G1, I1 and I2. PGE2 was found to be antagonistic to all Gq-coupled agonists (ADP, U46619, SFLLRN and AYPGKF) for all donors, in accordance with the known ability of PGE2 to raise cAMP levels [210]. Experiments A1, A2, J1 and I1, I2 were distinguished by positive synergisms at high dose PGE2 and moderate to high dose CVX; positive synergy was also noted between low dose PGE2 and high dose CVX for experiments A1, A2, H1, H2, E1, E2, F2 and G1 possibly via EP3 mediated signals [211] which are known to mediate pro-aggregatory actions.

In a single PAS experiment there are 135 conditions containing 2 agonists. These were tested in duplicate on the same plate giving 2 measurements of the 135 synergy scores (using the mean single agonist integrated areas in a plate to calculate synergy). PAS experiments were repeated twice within two weeks for an individual donor, giving us 4 measurements of an experimental synergy score. The standard errors in synergy scores across all 135 conditions were uncorrelated with the magnitude of synergy and are measures of the experimental uncertainty and day-to-day fluctuations in mean synergy values at these conditions. The mean uncertainty for a representative donor (donor A)
was ± 0.0523 for $S_y$ ranging from –1 to 1 (uncertainties across all 135 conditions are shown in Figure 3.4-17). The mean standard error in synergy scores for all ten donors ranged from ±0.0347 to ±0.0627 (Table 3.4-3).

![Figure 3.4-17 Experimental uncertainty in mean synergy score (Donor A).](image)

Shown above are the mean synergy scores ± standard errors for all 135 conditions for both donor A experiments. The mean standard error across all 135 conditions for each donor was 0.0523. These values were calculated for all the 10 donors studied and are reported in Table 3.4-3.

Since all experiments were performed in diluted platelet rich plasma and not ‘pure’ platelet suspensions, we tested whether the observed clustering patterns were dependent on platelet independent plasma components. We performed two separate PAS scans of platelets from a donor (separate from the donors reported in Figure 3.4-15). In
the first experiment for donor K (experiment K1), a PAS scan was performed identically to the procedure described in the manuscript using autologous plasma (as in Figure 3.4-3). In experiment K2, platelet rich plasma from donor K was supplemented with an equal volume of plasma (platelet free) from donor E (whose PAS scans have been reported in Figure 3.4-15). Both donor K experiments yield very similar PAS scans, and as shown above, both donor K experiments self clustered. Importantly K2 does not cluster with donor E replicates. This demonstrates that the PAS profiles are platelet cell autonomous and not dependent on plasma components.

<table>
<thead>
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<th>Donor</th>
<th>Mean standard error in synergy scores</th>
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<td>B</td>
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<tr>
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</tr>
<tr>
<td>J</td>
<td>0.0469</td>
</tr>
</tbody>
</table>

Table 3.4-3 Mean standard error in synergy scores.

The mean standard error in synergy score was calculated as explained in Figure 3.4-17. These values are a measure of the experimental uncertainty in determining donor specific synergisms.
Figure 3.4-18 Platelet cell autonomous clustering pattern.

The observed pattern of self-clustering was platelet signaling dependent (and not related to donor plasma), as the PAS scans of an individual donor’s platelets with autologous (Experiment K1) or heterologous plasma (Experiment K2) self-clustered.

3.4.9 Dose dependence of binary synergies

To evaluate the dependence of binary synergy scores on the doses of the both of the agonists present in the binary mixture, we plotted the distributions of synergy at each dose combination (Figure 3.4-19). All experimentally observed pairwise synergy scores (4 synergy scores per condition per donor × 10 donors) at the 9 possible binary concentration pairs (at 0.1, 1 and 10 × EC50) were binned according to their binary synergy values.
Figure 3.4-19 Dependence of synergy scores on agonist pair concentrations.

Shown above are the probability distributions of binned synergy scores at each concentration pair. Responses with synergy scores less than -0.1 were classified as antagonistic, those greater than 0.1 were classified as synergistic and scores between -0.1 and 0.1 were classified as additive. Shown in the top left is a summary of the cumulative probabilities of antagonism, additive responsiveness and synergy at each condition. Highest probabilities of synergy were observed when both agonists were at moderate doses. At low doses of both agonists, responses were generally additive. When high doses of both agonists were present the synergy distributions were skewed towards antagonism in accordance with the notion of saturation of signaling pathways.

In general across all conditions and donors, the highest probability of pairwise synergy was observed when moderate doses of both agonists were used. Low doses of both agonists produced additive responses, whereas high doses of both agonists skewed synergy distributions toward antagonism.
Variations in synergy scores both within the same donor and across donors were evaluated. **(A) Intra-donor variability** was calculated as the mean of the standard error in synergy scores across all conditions and **(B) Inter-donor variability** was calculated as the standard error of mean synergy scores across all conditions.
We have conducted an analysis of intra-donor and inter-donor variability (Figure 3.4-20). We found that the source of intra-donor variability was similar, but not identical to the source of inter-donor variability. For example, ADP-CVX interactions were a strong source only of intra-donor variability. In contrast, high PAR4 agonist in combination with ADP, U4619, and SFLLRN were sources of only inter-donor variation.

To calculate intra-donor variability (panel A), the standard errors at each of the 135 conditions containing binary mixtures was first evaluated. Next the means of these standard errors were calculated across all 10 donors. The conditions were plotted on the binary synergy map with the color indicating the magnitude of the relative variability. To calculate inter-donor variability (panel B), the mean synergy at each of the 135 conditions (across all 4 replicates) was first calculated. The standard error of this mean synergy measurement at a particular condition was then calculated across all 10 donors. This gives us an indication of the spread of measured values for a particular condition in the donor cohort. The conditions were again plotted on the binary synergy map.

Conditions containing PGE₂ in general had the lowest intra donor variability. Conditions containing moderate CVX had the highest intra donor variability. Such variability is a measure of the likelihood of experimental noise at a particular condition. Conditions containing PGE₂ had the least inter donor variability as well, thus pointing to highly conserved modes of interaction. Conditions that were identified to have high inter donor variability inspite of low intra donor variability are the most useful in distinguishing the two major donor clusters (Figure 3.4-15). Some of these conditions were (i) high AYPGKF, low/moderate ADP; (ii) high AYPGKF, moderate/high U46619; (iii) moderate SFLLRN, high U46619.
3.4.11 Synergy between thrombin and thromboxane pathways

Figure 3.4-21 Synergism between thrombin and thromboxane.

Noting marked synergism between SFLLRN and U46619, and some synergisms between AYPGKF and U46619, for all donors, we tested for synergism between the physiological agonist thrombin and U46619. A complete binary interaction map spanning 7 concentrations of either agonist was studied, for 2 separate donors. All conditions were tested in replicates of 6 and mean responses were used to calculate synergy scores. Experiments were conducted in EDTA and also included 15 μM Indomethacin, 2 units/ml Apyrase and 100 μM GGACK to prevent autocatalytic amplification by thromboxane, ADP or Xa. 500 μM GPRP was included to prevent fibrin formation with added thrombin. It should be noted that our experiments were conducted in 12% ‘unwashed’ platelet rich plasma in the presence of ~300 nM concentration of the physiological thrombin inhibitor antithrombin III. This might account for the fact that relatively high levels of thrombin (greater than ~10 nM) were required to observe detectable response to thrombin. Such an experimental design has no effect on receptor mediated pathways but produces a right shift in thrombin dose response. As anticipated, we observed synergisms between low and moderate doses of these agonists prior to saturation of their receptor signaling pathways.

All donors showed some synergism between low and moderate doses of SFLLRN and U46619. Except for experiment B2, synergy was also found between AYPGKF and
U46619 for all donors. Moreover, synergistic/additive interactions were noted also between low and moderate doses of SFLLRN and AYPGKF. These results strongly suggested a mechanism of synergy between thrombin and thromboxane. To test this, binary synergy maps of the physiological agonist thrombin and U46619 were constructed for donors A and E (Figure 3.4-21) over 7 doses spanning the active concentration ranges. Marked synergisms were noted at low and moderate doses of these agonists as expected from conserved synergy between SFLLRN/AYPGKF and U46619 (Figure 3.4-15). High doses saturated signaling pathways abolishing synergism. To our knowledge, this is the first report of conserved synergy between thrombin and thromboxane mimetics.

3.4.12 Dominance of pairwise effects in platelets

The PAS approach works because individual and binary interactions dominate, and they are sampled across the full dose range of inputs. We expect the method to break down when ternary interactions in excess of summing binary interactions become strong. We show that the residual ternary synergy \( \Delta(ABC) = S_{ABC} - S_{AB} - S_{BC} - S_{AC} \) was ~0 in each of 27 responses of platelets to different ternary combinations of CVX, ADP and SFLLRN and was minimized in the neural network model training (Figure 3.4-22).

The PAS approach works because unitary and binary interactions dominate and they are no feedbacks, which would otherwise expand the repertoire of participating factors. With these "pure inputs" (which are not broad intracellular perturbations like ionophores or phosphodiesterase inhibitors), the network architecture (Figure 3.2-1) necessitates rapid convergence to a common second messenger. Because of the speed of signaling and the known cell circuitry, it is difficult to envision realization of greater than
second order synergies in such a situation. Thus observed ternary synergies appear to be well approximated by the linear superposition of the individual pairwise synergies. Since residual ternary interactions were undetectable, even higher ordered residuals are likely very small and potentially difficult to evolutionarily select. This exact result of unitary and binary interactions dominating with few detectable ternary interactions was reported for multiple cytokine stimulation of RAW264.7 cells [212].

In general, knowledge of pairwise interactions alone cannot be expected to predict response to several simultaneously present stimuli (>2). However, certain characteristics of platelets and the conditions under which they were studied made such an approach feasible in this instance. These include (i) the relative abundance of binary interactions in signaling systems with minimized ternary interactions (ii) the efficient utilization of system history (Figure 3.4-23); (iii) the dense sampling of interactions across a full dose-response range; (iv) known intracellular wiring that rapidly converges on Ca$^{2+}$, without the possibility of higher order effects from genetic regulation or other interactions on long time scales; and (v) choice of well-characterized extracellular ligands and careful design to avoid autocatalytic feedback.

Thus, the PAS approach is powerful for training NN models of signaling systems that accommodate multiple binary interactions where $\Delta$(ABC) or higher ordered residuals are small. In systems where third order interactions are important “ternary” agonist scanning might be necessary, however this will require added experimental complexity. “Pairwise” agonist scanning is the bare minimum that can be expected to quantify interactions between multiple simultaneously acting signaling pathways over and above the strictly additive interactions between pathways.
Experimentally-measured synergy

Binary synergies between ADP, CVX, and SFLLRN ($S_{AB}^c$, $S_{AC}^c$, $S_{AC}^c$)

$S_{AB} = AB - A - B$

$n = 27$

unique binary interactions

NN-predicted synergy

Binary synergies between ADP, CVX, and SFLLRN ($S_{AB}^c$, $S_{AC}^c$, $S_{AC}^c$)

$S_{AB} = AB - A - B$

$n = 27$

unique binary interactions

Ternary synergies among ADP, CVX, and SFLLRN ($S_{ABC}^c$)

$S_{ABC} = ABC - A - B - C$

$n = 27$

unique ternary interactions

Ternary synergies among ADP, CVX, and SFLLRN ($S_{ABC}^c$)

$S_{ABC} = ABC - A - B - C$

$n = 27$

unique ternary interactions

'Residual' ternary synergy among ADP, CVX, and SFLLRN ($\Delta'ABC$)

$\Delta(ABC) = S_{ABC} - S_{AB} - S_{BC} - S_{AC}$

$n = 27$

unique ternary interactions

'Residual' ternary synergy among ADP, CVX, and SFLLRN ($\Delta'ABC$)

$\Delta(ABC) = S_{ABC} - S_{AB} - S_{BC} - S_{AC}$

$n = 27$

unique ternary interactions
Figure 3.4-22 Minimization of residual ternary synergy.

The PAS trained NN model has complete knowledge about all unitary and binary interactions that occur in the platelet, since these conditions were exhaustively sampled in the experimental training set. However, information about higher order interactions are in general unknown, and thus successful predictions can be made in a system only if such higher order interactions are relatively unimportant.

Our experiment with ADP/CVX/SFLLRN measured platelet response to simultaneous activation of two GPCRs and an immune receptor homologue (GPVI). Above, we investigate the binary synergy score \( S_{AB} = AB - A - B \), the trinary synergy score \( S_{ABC} = ABC - A - B - C \), and the “residual” ternary synergy which measures the synergy in excess of the binary synergies \[ \Delta(ABC) = S_{ABC} - S_{AB} - S_{BC} - S_{AC} \]. We show that the experimentally measured residual ternary synergy \( \Delta(ABC) \) is essentially zero in 27 different tests of platelet response to the 3 agonists used at 0.1, 1, and 10X EC50. By determination of \( S_{AB} \), \( S_{ABC} \), and \( \Delta(ABC) \) from the predicted calcium responses obtained from the neural network, we show that \( \Delta(ABC)_{NN} \) is also minimized during the NN training. If \( \Delta(ABC) \sim 0 \), then it is highly unlikely that higher ordered residuals such as \( \Delta(ABCD..) \) are large. In such a situation, sampling the pairwise interactions experimentally and using this data to train the NN allows the NN to make accurate prediction of n-tuples.

Multiple signals acting simultaneously in vivo is not unique to platelets. Hsueh et. al. recently reported the binary to quintinary interactions between 5 ligands (IFN-β, TGF-β, IL-6, ISO and 8 Br-cAMP) of a single receptor (TLR4), resulting in secretion of 4 cytokines (G-CSF, IL-6, IL-10, and TNF-α) in macrophage like RAW 264.7 cells [212]. They found that almost all pairwise interactions result in non-additive interactions (akin to Figure 3.4-3 and Figure 3.4-15 in the present study). Instances of higher order interactions resulting in unique outcomes that not captured by “binary interactions” were less common (~33% of total combinations with the majority being explicable by ternary interactions). Hsueh et. al. made no consideration of dose dependence of the input signals, nor was any attempt made to distinguish between the responses of the same type originating from different human individuals.

Thus the two actual biological systems tested to date (human platelets and cultured RAW264.7 cells) satisfy the requirement that response to combinatorial agonists is build upon unitary and binary interactions. Since ternary interactions can be easily measured experimentally, this guides the use of PAS in other systems. This approach can be expected to work in systems where residual third or higher order interactions are minimal.
3.4.13 Utilization of system history in making output predictions

The importance of history on predictive ability was evaluated by systematically decreasing the length of system history utilized in making predictions about the current instant.

The NARX model utilizes system history in making predictions of the output value $[Ca^{2+}]_i$ at the current instant. Without any history, there is no notion of “dynamics”, and the system can at best predict outputs proportional to the magnitude of input. However these outputs are constant over the duration of the experiment, unlike the actual calcium transients experimentally observed. With very little history (2 s), the model does not “remember” its unperturbed steady state value (0) and hence calcium traces continually rise to a peak value, but never decrease. The NN performs reasonably well with as little as 8 s of calcium concentration history, although oscillations are produced, as the network progressively utilizes less information about its initial steady state, and heavily relies on just its “short term memory”, repeatedly replicating the initial trend. As additional delay lines were removed from the network, the frequencies of these oscillations were decreased and the network gradually captured experimentally observed dynamics. Thus the long-term system history inputs (e.g., 32, 64, 128 s) dampen these short-term oscillations.
3.5 Discussion

Studying the combinatorial effects of pairs of agonists in low, moderate and high concentrations allowed a rapid, donor-specific phenotypic scan that was predictive of responses to 6 multiple agonists. Importantly, a single 384-well plate of data was sufficient to train a NN model (Figure 3.4-3) that was capable of making accurate predictions of the global 6-dimensional agonist reaction space (Figure 3.4-9), which is difficult to probe experimentally but fundamental to the processes of thrombosis.

Synergies between platelet agonists are dependent not just on agonist pairs and doses, but also vary from donor to donor (Figure 3.4-15). The synergistic effects of epinephrine on other platelet agonists is well documented [213, 214]. Synergy has also been reported between ~EC50 levels of SFLLRN-AYPGKF, AYPGKF-ADP and SFLLRN-ADP [215]. Such unique patterns of synergisms could be used to distinguish donors and be correlated with certain risk factors. In contrast, certain synergisms, such as those observed between U46619 and thrombin receptor agonist peptides, were consistent across donors and suggest conserved modes of interaction amongst these cascades.

We note that in multi-dimensional agonist space emblematic of the thrombotic milieu, highest synergy was observed for combinatorial mixtures containing high U46619 and no PGE2 (Figure 3.4-12). This points to the “high dimensional risk” of large concentrations of thromboxane, in the absence of PGl2, potentiating the effects of other agonists. Aspirin is approximately 170-fold selective to COX-1 mediated thromboxane production, compared to endothelial COX-2 mediated PGl2/PGE2 synthesis. Thus, selective inhibition of thromboxane, without affecting prostacyclin may account for its efficacy in protecting against thrombotic events when diverse platelet activators are
present [216]. In contrast Rofecoxib (Vioxx), by a selective inhibition of COX-2 mediated prostacyclin production, increases cardiovascular risk [217].

This study also introduces the use of neural network models for predicting dynamic cellular signaling data. This application of neural networks offers several unique benefits as a modeling strategy. First, NNs are “dense” modeling structures (few parameters) that do not require a detailed knowledge of the kinetic structure of a system. By comparison, a detailed ordinary differential equation (ODE) model of ADP-stimulated calcium mobilization via P₂Y₁ in the platelet required almost 80 reactions and over 100 kinetic parameters to describe just this one single pathway [168]. We estimate that an ODE model that describes the signaling mechanisms of the 6 agonists (Figure 3.2-1) in this study on a similar level of detail would require over 500 parameters, most of which are currently unavailable. Second, NN models may be trained to match an individual donor’s signaling response. We have demonstrated in Figure 3.4-15 the subtle but consistent variation in the platelet calcium response among healthy donors. This offers a predictive capability that may be used to assess risks associated with an individual’s platelets. A major goal of blood systems biology is to predict patient specific risks and to simulate thrombus formation under realistic hemodynamic conditions of flow [96, 218]. Platelet activation in prior simulations has been course grained by assuming discrete states of activation or arbitrary rules governing transitions between states. The speed of NN calculations of [Ca²⁺]ᵢ will allow large scale, donor-specific simulations with 10³ to 10⁵ activating platelets under conditions of flow. Such a model can be incorporated into lattice kinetic Monte Carlo simulations of particle motion in convective and dispersive flow fields [219] (Section 3.7).
Figure 3.5-1 ODE model of platelet calcium mobilization following an ADP stimulus

An ODE based molecular signaling model (77 reactions, 132 rate constants, 70 species) of platelet phosphoinositide and calcium regulation during homeostasis and P2Y1 activation was developed (top) [168]. This model was able to simulate experimentally obtained calcium dose response to ADP (bottom).
Clinically, we anticipate PAS profiles to depend on such variables as ancestry, age, sex, pharmacology, and cardiovascular state, all of which requires further testing. In contrast to PAS, other current measurements of platelet phenotype provide the coarsest measure of differences among healthy donors. For instance, platelet aggregometry for 359 subjects could classify them as “hypo and hyper” reactive to platelet agonists [220]; and flow cytometry of 26 individuals was able to classify them as high, medium or low responders. A combination of genetic variations may underlie differences in platelet phenotype, however linking genotype (1327 SNPs) to phenotype (flow cytometric measurement of P-Selectin exposure and fibrinogen binding) in 500 individuals [221] demonstrated weak association probabilities.

Platelets are ideal “reduced” cellular systems for quantifying the effects of multiple signaling pathways because they are anucleate, easily obtained from donors, amenable to automated liquid handling, and biochemically well-characterized. In spite of the distinction from the in vivo platelet environment, the simplified in vitro conditions using EDTA were useful for the dissection of signaling pathways ‘decoupled’ from confounding autocrine effects of soluble mediators that are highly dependent on local platelet concentrations and prevailing transport processes. However, the operational advantages of using EDTA prevent direct prediction of important physiologic phenomena like granule release, integrin activation, and outside-in signaling.

Further expansion of the PAS set to include epinephrine, soluble CD40L, serotonin, and nitric oxide donors would essentially map a major portion of the entire platelet response space. The use of PAS with orthogonal pharmacological agents (indomethacin, P$_2$Y$_{12}$ inhibitors, selective PAR antagonists, quanylate cyclase or
adenylate cyclase inhibitors) would allow further assessment of individual clinical risk or sensitivity/resistance to therapy. The PAS method demonstrates that sampling all dual orthogonal "axes" (every agonist pair) can successfully predict the dynamic responses and cross-talks of a higher dimensional system (6 agonists in this case).

3.6 Extension 1: Phenotyping of female donors

All of the donors shown in Figure 3.4-15 were male donors. To test for correlations between the gender of the donor and PAS clustering pattern, we carried out pairwise agonist scans for 10 female donors (donors L to U) and compared the clustering patterns of all donors (male and female) together Figure 3.6-1. All female donors were between the ages of 20 and 26, were from either Asian or Caucasian ancestry; and affirmed before phlebotomy that they had not consumed alcohol for 3 days prior to the blood draw, were not under any medications, and were not currently using birth control hormone. Similar to the experiments with male donors a repeat experiment for every female donor was carried out within a period of two weeks.

All of the experiments beyond donor K were conducted with the same stocks of reagents for the 6 inputs tested. We also tested a male donor (donor V) with these same stocks. Donor V did not consent to a second blood draw, thus only a single experiment (V1) is shown for this donor in Figure 3.6-1.

3 of the 10 female donors self clustered (donors R, M and O). The number of male donors self clustering remain unaltered (7 out of 10). The comparatively reduced magnitude of self clustering for female donors can probably be explained by the fact that the female donors tested did not have as diverse a genetic background as the male donors. Moreover, the likelihood of self clustering can be expected to drop sharply as the number
of donors tested (columns in the synergy matrix) goes on increasing, for an unchanged number of conditions at which phenotypes were tested (the 135 conditions at which synergy between platelet agonists were measured comprising the rows of the synergy matrix). The probability of donor experiment self clustering can be expected to improve if the number of experimental conditions tested increases (either testing of ternary and higher order synergies in addition to the pairwise interactions, or the testing of the binary interactions amongst more signaling pathways). However this will require added experimental complexity, and is limited by technological limitations of current automated liquid handling protocols. The total number of donors self clustering (male and female) still remains highly significant (11 of 21) and to our knowledge PAS shows the most promise in “stratifying” individual donor platelet phenotypes amongst other currently available platelet phenotyping approaches (aggregometry or flow cytometry).

We did not observe a clear pattern in Figure 3.6-1 where female donors clustered separately from male donors. Because hierarchical clustering was inadequate in distinguishing platelet phenotypes on gender, we determined the principal components of the synergy matrix. Principal component analysis (PCA) is a method of dimensionality reduction where an orthogonal basis set of “principal components” is identified (consisting of linear combinations of the original variables), such that much of the variance in the original data is explained by variation along the first few principal components. We determined the principal components of the 135 dimensional synergy matrix and plotted up donor phenotypes along the first two principal components for ease of visualization (Figure 3.6-2). The first 2 principal components account for the most variation in the original data (PC-1 accounts 20.74 % of the variance and PC-2 13.98 %).
Figure 3.6-1 Dependence of synergy clustering pattern on donor gender

10 healthy female donors (donors L to U) were tested twice each within a period of two weeks. Their PAS synergy profiles were arranged in columns of the synergy matrix (in addition to those for all male donors), and the columns of this matrix were clustered.
Figure 3.6-2 Scatter of donor phenotypes along first two principal components of synergy matrix.

The columns of the synergy matrix in Figure 3.6-1 correspond to donors of varying gender and ethnicity. To determine whether the experimentally measured synergy profiles were correlated with any of these factors, we determined the principal components of the 135 dimensional synergy matrix. Donor phenotypes were projected along the first two principal components.

Although the first two principal components account for only about 35% of the variance of the original synergy phenotypes in the synergy matrix, the second principal component showed remarkable promise in distinguishing phenotypes on gender. 15 of the 20 female donor phenotypes had negative values of the second principal component and 21 out of 23 male phenotypes had positive values of the second principal component. The first principal component is correlated with donor ethnicity (phenotypes with large positive values of PC1 were Caucasian, whereas large negative values of PC1 were obtained for donors of African descent).
3.7 Extension 2: Platelet activation module in patient specific multiscale models of thrombosis under flow

The process of thrombosis involves discrete particles (platelets) moving in a convective field. At the site of the growing thrombus these particles can attach to the wall (exposed collagen). Such platelets that attach to the wall will eventually activate fully and release autocatalytic activators like ADP and TXA$_2$, as well as contribute catalytic surface for thrombin formation. This sets up a boundary layer around the growing clot where released species like ADP, TXA$_2$ exist at appreciable concentrations. Unactivated platelets can activate in this layer around the growing thrombus according to the prevailing local concentrations of agonists. Two activated platelets in the vicinity of the growing clot can aggregate together either in solution, or an activated platelet in solution can deposit onto an activated platelet on the growing thrombus (Figure 3.7-1 A).

Flamm et al have developed Lattice Kinetic Monte Carlo (LKMC) algorithm that is capable of simulating convection and diffusion of particles in complex flow fields [219], as well as particulate aggregation for defined rates [222]. Kinetic Monte Carlo algorithms are efficient at predicting system evolution given the rates of governing stochastic processes involved [219]. For the problem at hand, the spatial and temporal growth of the thrombus can be simulated from the rates of convection, diffusion, platelet activation, and activated platelet aggregation/disaggregation. This is the basis for a multiscale model of thrombosis (Figure 3.7-1 B): where (i) the velocity field is obtained by either a Lattice Boltzman solution, or from solution of the full Navier Stokes equation using commercial CFD packages, (ii) the local concentrations of agonists can be obtained from finite element or finite difference solution of the convection-diffusion equation.

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Figure 3.7-1 Multiscale systems model of thrombosis: Contribution of the platelet activation module

(A) The process of thrombosis involves discrete platelet particles moving in a flow field that can attach to the exposed injury, activate in the growing cloud of active agonists, or bind to other active platelets in solution or at the thrombus site.

(B) Multiscale model of thrombosis: An LKMC algorithm is able to simulate the evolution of the growing clot given rates of all events involved in the process (convection, diffusion, activation, aggregation and disaggregation). The rates of platelet activation are supplied by donor specific PAS trained NN models for calcium mobilization. (Image courtesy Matt Flamm)
Given the local concentration of agonists at any position and time, a PAS trained neural network model can predict the level of calcium mobilization in the platelet. Integrated platelet calcium can be used as a measure of the activation status of the platelet and governs the “stickiness” of a given platelet for other platelets, as well as its potential to release autocrine activators. The advantage of this approach is that neural network models can be trained on an individual donor’s PAS dataset, thus yielding donor specific models of thrombosis.

For initial comparisons between *in vitro* experiments studying platelet thrombus formation and their corresponding LKMC simulations, we do not consider the effects of thrombin. In the absence of thrombin, or endothelium produced prostacyclin, the effects of three of the inputs in the original PAS set: the PAR peptides SFLLRN and AYPGKF, and the prostacylin mimic PGE2 can be ignored. For the 3 remaining inputs (ADP, CVX and U46619) complete testing of all single and pairwise mixtures requires testing of 37 conditions. We tested these conditions in replicates of 4, both for regular platelets and for platelets treated with the antiplatelet pharmacological agents MRS2395 (P₂Y₁₂ inhibitor) (*Figure 3.7-2 for donor W*) and Illoprost (prostacyclin analogue) (*Figure 3.7-3 for donor D*). All experiments in this section were conducted in the presence of 2 mM extracellular calcium, and contained 28 µM Indomethacin to prevent autocatalytic amplification by secreted thromboxane. Since the platelet suspensions contained external calcium in these experiments, the level of signal (and background) was higher. This permitted us to use a lower concentration of 6 % PRP as opposed to 12 % PRP used for PAS experiments (*Figure 3.4-3*). Autocatalytic amplification by released ADP at such dilute concentrations of PRP will be minimal (See *Section 3.4.2*).
These datasets were used to train donor specific neural network models of regular platelet phenotypes as well as models of patient specific therapy in response to these drug treatments. The structure of the neural network used for training was identical to the one previously described (2 processing layers with 8 and 4 neurons respectively and an output layer) (Figure 3.3-2). Training was continued until the performance of the trained models (mean squared error between experimental and simulated transients) was \(~ 1 \times 10^{-5}\). (Also see Section 3.3.6 for details about model construction and training)

Maloney et al have developed an 8-channel microfluidic flow assay where blood drawn into PPACK (to inhibit thrombin), and labeled with AlexaFluor 647 conjugated anti-CD41 (\(\alpha_{Ib}\)) antibody to identify platelets, is perfused over focal zones of collagen at an average wall shear of 210 s\(^{-1}\) [223]. For comparison between experiment and LKMC simulation, this assay was used by Tom Colace to simultaneously measure platelet deposition on collagen coated surfaces using the same samples of blood that were used for calcium phenotyping in Figure 3.7-2 and Figure 3.7-3. Either untreated blood; blood treated with indomethacin; MRS2395 (Figure 3.7-2) or Illoprost (Figure 3.7-3); or combinations of Indomethacin and the respective inhibitor were used in each channel of the device and every condition was tested in replicates of two on the same device. Each condition was tested in replicates of 6 (on 3 separate devices). The total fluorescence after 5 minutes of perfusion was used as a measure of platelet accumulation. All values were normalized to the mean value of the control (untreated blood sample) (Figure 3.7-4). The LKMC simulations of thrombosis will attempt to simulate such flow experiments using predictions of each platelet’s activation state (integral calcium levels) from corresponding PAS trained neural network models.
Although P₂Y₁₂ inhibition using MRS2395 has a pronounced effect in decreasing platelet deposition on collagen surfaces (Figure 3.7-4), we note that there was no appreciable effect of P₂Y₁₂ inhibition on calcium mobilization (Figure 3.7-2). The fact that calcium transients were unaffected by P₂Y₁₂ inhibition can be explained by the fact that the P₂Y₁₂ receptor is not G₉ coupled (See Section 1.2), and contributes to aggregation independently of calcium pathways. Thus, neural network model predictions of calcium alone will be inadequate to simulate the effect of this inhibitor. Further experiments using readouts other than calcium mobilization (like flow cytometry), to quantify the diminution of integrin activation by ADP signaling in the presence of this inhibitor are underway. Corrections to activation levels predicted by integral calcium need to be incorporated into the LKMC model to predict the effect of this inhibitor.

In contrast, Illoprost has a pronounced effect in decreasing both calcium mobilization (Figure 3.7-3) as well as platelet deposition onto an exposed collagen surface (Figure 3.7-4). Thus LKMC simulations of platelet deposition without or with Illoprost treatment can be generated using activation state predictions solely obtained from the PAS trained neural network values of integral calcium. We also note a more pronounced effect of thromboxane synthesis inhibition (Indomethacin treatment) for donor W in comparison to donor D for platelet deposition on collagen (Figure 3.7-4). This correlated with heightened levels of calcium synergy between the thromboxane analogue U46619 and ADP for donor W (compare ADP-U46619 pairs between Figure 3.7-2 and Figure 3.7-3). We are currently confirming this correlation for a larger donor sampling. Use of either MRS2395 or Illoprost in conjunction with Indomethacin does not provide any statistically significant improvement in inhibitory capacity (Figure 3.7-4).
Figure 3.7-2 Pairwise scan of Donor W for ADP, U46619 and CVX for platelets treated without or with the P₂Y₁₂ inhibitor MRS2395

Calcium mobilization for all pairwise combinations of ADP, U46619 and CVX were measured for donor W under regular conditions (red) and with the P₂Y₁₂ inhibitor MRS2395 (blue). All conditions were tested in replicates of 4.
Figure 3.7-3 Pairwise scan of Donor D for ADP, U46619 and CVX for platelets treated without or with the prostacyclin mimic Illoprost

Calcium mobilization for all pairwise combinations of ADP, U46619 and CVX were measured for donor D under regular conditions (red) and with the IP receptor stimulant Illoprost (blue). All conditions were tested in replicates of 4.
Figure 3.7-4 Concurrent experiments studying platelet deposition phenotypes on collagen coated surfaces in microfluidic flow devices

Blood was drawn into the thrombin inhibitor PPACK, and platelets were labeled with AlexaFluor 647 conjugated anti-CD41 (αIIb) antibody. These samples were perfused over a strip of patterned collagen in an 8 channel microfluidic device [223]. Each channel contained either regular blood; blood treated with Indomethacin to limit synthesized thromboxane; blood treated with MRS2395 to inhibit ADP signaling via P2Y₁₂ (panel A for donor W); or treated with the antiplatelet drug Illoprost to raise intracellular cAMP levels via IP signaling (panel B for donor D); or with mixtures of Indomethacin and the respective inhibitor. These samples of blood were perfused for 5 minutes over collagen coated surfaces at an average wall shear of 210 s⁻¹ and the level of platelet accumulation was quantified by measuring the final levels of fluorescence. Shown above are the fluorescence intensities scaled to the mean value of the control (untreated blood sample). All conditions were tested in replicates of 6 and blood from the same phlebotomy samples that were used for calcium phenotyping (Figure 3.7-2 for donor W and Figure 3.7-3 for donor D) were used in these flow experiments (panel A and panel B respectively). (Experiments and analyses courtesy Tom Colace)
Chapter 4

Future Work

4.1 Studying the contribution of store operated calcium entry

Calcium exists at concentrations of ~100 nM in the platelet cytoplasm, at concentrations of ~10 µM in the platelet stores (Dense Tubular System (DTS)), and at levels of ~1.5mM outside the cell. Following agonist stimulation, there are 4 fluxes of calcium that regulate net cytoplasmic levels. 1) PLC activation leads to the generation of IP₃ downstream of receptor activation (see Section 1.2). This leads to the opening of IP₃ gated calcium channels within the platelet DTS, and a sharp increase in cytoplasmic calcium levels. 2) The emptying of the intracellular stores is conveyed to the cytoplasmic membrane by the calcium sensor STIM-1 [224] and opens up Orai-1 [225] channels on the plasma membrane causing a dramatic influx of calcium with the steep concentration gradient that exists across the cytoplasm. This component of the signal is called “Store Operated Calcium Entry” (SOCE). There are also pumps that actively transport calcium against concentration gradients back to regions of higher concentration. 3) Sacro Endoplasmic Reticulum Calcium ATPase (SERCA) pumps transport calcium back from the cytosol into the DTS and 4) Plasma Membrane Calcium ATPases transport calcium back from the cytosol to the extracellular milieu. Most of the PAS experiments reported thus far were conducted in the presence of EDTA (except those in Section 3.7). Chelation of extracellular calcium allows us to study the component of the net calcium signal resulting directly from intracellular release from the DTS, but ignores the contribution of SOCE.
4.1.1 Dissecting the calcium signal resulting from agonist stimulation

Separating these components of the net calcium signal (in particular to study the contribution of store operated calcium entry) requires selective recalcification protocols into a medium where activation proceeds initially in the absence of external calcium (using EDTA), as well as judicious use of inhibitors of platelet pumps (Thapsigargin to inhibit SERCA, and Carboxyeosin to inhibit PMCA) (Figure 4.1-1). Some initial experiments utilizing these approaches to isolate individual components of the calcium signal are presented below. These approaches could be explored further.

Figure 4.1-1 The platelet machinery regulating calcium mobilization

Intracellular calcium concentrations downstream of receptor activation are regulated by 1) Release of calcium from IP₃ gated channels on the DTS, 2) SOCE across the Orai-1 channels on the plasma membrane after store emptying has been communicated to the membrane by the intracellular calcium sensor STM-1, 3) Pumping of calcium back into the DTS using SERCA (inhibited by Thapsigargin) pumps and 4) pumping of calcium out of the cell using PMCA pumps (inhibited by Carboxyeosin).
4.1.2 Recalcification protocols to study biphasic calcium signaling

Simultaneous study of the net calcium signal in the absence (using EDTA) or presence of external calcium allows us to determine the contribution of store operated calcium entry. Inclusion of exogenous calcium beyond a certain instant during the time course of calcium mobilization, to a medium that previously contained no external calcium allows us to study the extent of the SOCE component of the signal for a particular duration of the total response. This approach is demonstrated in Figure 4.1-2. Platelet Rich Plasma was treated with EDTA (first addition). CVX (left) or TRAP (right) were then added (second addition) either in the absence of calcium (red) or presence of calcium (blue). The difference in integrated area between the blue and red curves (before the third addition of calcium in the red transient) represents the total difference resulting from the contribution of SOCE in either case. We also note an increase in intracellular calcium levels by mere calcification of agonist untreated platelets at $t=7$ min (green). Calcium can also be added at an intermediate point along the complete transient to gauge the level of SOCE resulting for a given state of “emptiness” of the DTS. This is shown in the recalcification step at $t=7$ min for the red transients. In contrast to the CVX signal at $t=7$ min, where a large amount of calcium has been released from the store (large integrated area below the transient before recalcification), the TRAP stimulus empties a much smaller proportion of the store. This accounts for the relatively minor amount of SOCE observed for TRAP in comparison to CVX.

This experiment can be extended such that recalcification is carried out not just at $t=7$ min, but at several points along the transient. Also, the dependence of SOCE on the concentration of agonist used can be established by carrying out such experiments across
a wide range of agonist concentrations. Moreover, the driving force for SOCE can be controlled by merely adjusting the final level of calcium after recalcification. Finally phenotyping of mice with deletions in either STIM1 or Orai-1 [226] would allow one to quantify the contributions of these molecules to the overall signal. The ODE based model of the platelet developed by Purvis et al [168] does not include the contribution of SOCE to the overall calcium signal resulting from ADP stimulation. Experiments such as these will be useful to calibrate sub-models of SOCE for inclusion in the existing model.

![Figure 4.1-2 Recalcification protocols to isolate Store Operated Calcium Entry](image)

**Figure 4.1-2 Recalcification protocols to isolate Store Operated Calcium Entry**

Calcium mobilization in response to CVX (*left*) and SFLLRN (*right*) were studied in the absence (*red*) or presence (*blue*) of external calcium. For experiments carried out in the absence of external calcium, recalcification at a certain instant during the time course of calcium mobilization allows us to determine the contribution of SOCE beyond that instant. Even addition of external calcium without any prior agonist addition (*green*) causes some increase in cytoplasmic due to basal activity of calcium channels on the plasma membrane.
4.1.3 Use of pharmacological agents to study components of the calcium flux that attenuate the overall signal

The use of the inhibitors for the PMCA and SERCA pumps (Carboxyeosin and Thapsigargin), in various sequential combinations allows us to explore the ability of these two pumps to attenuate the calcium signal. This approach was utilized to identify differences between TRAP (Figure 4.1-3) and CVX (Figure 4.1-4) signaling. These experiments were carried out in EDTA to exclude the possibility of SOCE (and thus simplify the analysis of these transients).

We note that addition of Thapsigargin (Figure 4.1-3) causes calcium levels to increase as the SERCA pumps are gradually inhibited (green transient after the first addition). This is because the ability of SERCA to compensate for constitutively open IP₃ channels on the DTS is lost. However the platelet’s PMCA pumps are able to compensate and the platelet readjusts to a higher steady state of basal calcium. In contrast, inhibition of the PMCA pumps (red transient after the first addition) causes an uncontrolled increase in cytosolic calcium. Thus the PMCA is the most important controller of the constitutive leak out of the DTS, and possible upregulation of SERCA after PMCA inhibition cannot compensate. The effect of constitutively open channels on the plasma membrane could not be explored in this experiment, because there is no calcium outside the cells. Store refilling must constantly occur from extra-cellular calcium to keep the platelet at a steady state of 100 nM calcium.

TRAP treatment after either of these inhibitions causes a spike in calcium. This spike is brought down to basal levels by the unaltered PMCA (green transient after the second addition) or the uninhibited SERCA (red transient after the second addition). We
note however, that the rate of reduction in signal is faster for the red transient in comparison to the green transient after the second addition. This suggests faster kinetics for the SERCA pumps (which remain operational in the red transient after the second addition) in comparison to the PMCA pumps (which remain operational in the green transient after the second addition). The ability of the SERCA pumps is however overwhelmed by the continual leak out of the stores in the absence of PMCA activity and calcium levels continue to steeply rise (red transient after the second addition). When both PMCA and SERCA are inhibited (dark blue transient after the second addition), the subsequent addition of TRAP (third addition), causes a brief spike (barely noticeable at the high concentrations that already exist at the time of TRAP addition because of the continuous leak that has occurred previously). This spike is not diminished at all with both pumps inhibited, thus confirming that PMCA and SERCA mediated pumping are the only mechanisms that attenuate the calcium spike following TRAP stimulation. SERCA or PMCA inhibition after prior TRAP stimulation (pink and light blue transients respectively after the second addition) cause either the platelet to adjust to a higher steady state or an uncontrolled leak out of the DTS as was noted before. Very similar results were also obtained for ADP stimulation (results not shown).

In comparison to the results obtained with TRAP, a CVX signal is regulated differently by the PMCA and SERCA pumps (Figure 4.1-4). The CVX signal is not diminished as the PMCA is inhibited (red transient after the second addition). This is in contrast to the TRAP stimulus where the SERCA is able to compensate atleast to some degree for the loss of PMCA (red transient after the second addition in Figure 4.1-3). In a separate experiment (results not shown) inhibition of SERCA by thapsigargin causes no
change in the rate at which the CVX stimulus is downregulated (Compare this to the green transient after the second addition in Figure 4.1-3, where inhibition of SERCA causes a slower decay of the calcium spike in comparison to uninhibited platelets following TRAP stimulation). These results suggest that the CVX signal is downregulated predominantly by the slower PMCA pumps (and not the faster SERCA pumps). This is in contrast to the $G_q$ coupled ADP or SFLLRN responses which appear to be downregulated by both PMCA and SERCA pumps. This could account for the fact that a $G_q$ coupled response is quickly brought down to baseline levels, whereas a CVX response is long lived.

Figure 4.1-3 Studying the components of the SFLLRN induced calcium signal

Buffer, the SERCA inhibitor Thapsigargin, the PMCA inhibitor Carboxyeosin and the PAR1 activating peptide SFLLRN (TRAP) were added in various sequential combinations as indicated in the legend to study the components of the calcium flux resulting from TRAP stimulation. All of these experiments were carried out in EDTA (0 SOCE) and dispenses were made at the instants indicated (20s, 15 min and 30min). These experiments demonstrated that the TRAP mediated signal is attenuated by both the PMCA and SERCA pumps, and that the PMCA pumps are the predominant controller of a steady leak of calcium out of the stores in the absence of refilling via external calcium.
Buffer, CVX, Thapsigargin and Carboxyeosin were added in varying sequences as indicated in the legend to study the components of the calcium flux resulting from CVX stimulation. These experiments demonstrated that the CVX mediated signal is attenuated predominantly by the slower PMCA pump (in contrast to the TRAP signal which utilizes both PMCA and SERCA pumps).

These experiments can be extended further to aid in the calibration of mechanistic models of platelet metabolism. The experiments reported have utilized only single doses of agonists / pump inhibitors. Studying the effects of different extents of pump inhibition or the responses to different doses of agonists will provide a rich dataset for model calibration as well as mechanistic insight into the process of calcium metabolism. There are reports in literature that suggest the existence of agonist sensitive calcium pools within platelets [227]. Using experiments similar to the ones outlined in this section, it should be possible to distinguish between these different stores. Finally combinations of the selective recalcification protocols (Section 4.1.2) and the use of pump inhibitors would allow one to study how SOCE is regulated by the SERCA and PMCA pumps.
4.2 Downregulation of collagen signaling by prior thrombin stimulation: Differences between thrombin and PAR1+ PAR4 peptides

ADP, thrombin and CVX were added in various sequential combinations where the agonists were either added individually, 2 agonists were added followed by a third or all three agonists were added together (Figure 4.2-1). Many of the conclusions obtained were similar to those in Section 3.4.5 for ADP, the PAR1 peptide (SFLLRN), and CVX. However, differences were noted particularly with regard to inhibition of a CVX signal.

Prior stimulation with ADP, does not affect the ability of thrombin (Figure 4.2-1, panel 1) or CVX (Figure 4.2-1, panel 2) to signal (Similar to the effect of prior ADP stimulation on the SFLLRN or CVX signals in Figure 3.4-7). Both thrombin (Figure 4.2-1, panel 3) and CVX (Figure 4.2-1, panel 5) however cross down regulate the ADP signal (Similar to the effect of prior SFLLRN or CVX signals on subsequent ADP response in Figure 3.4-7). Also prior stimulation with CVX cross-downregulates thrombin response in Figure 4.2-1, panel 6 (Again similar to the ability of CVX to downregulate the SFLLRN response in Figure 3.4-7). One of the most intersting observations in this experiment however, was the fact that prior stimulation with thrombin cross down regulates the CVX response (Figure 4.2-1, panel 4) (In contrast SFLLRN does not inhibit the CVX signal in Figure 3.4-7). These observations were also observed when a third agonist is added to a well that had received two previous agonists in Figure 4.2-1 panels 1-6, or when two agonists are added together followed by a third in Figure 4.2-1 panels 7-9, or when all three agonists are added simultaneously producing a single burst in calcium in Figure 4.2-1 panels 10.
The agonists ADP, thrombin and CVX were added in various sequential combinations to platelet suspensions. In addition to the trends noted in (Figure 3.4-7), we also noticed inhibition of the CVX signal by prior thrombin stimulation. Also the thrombin response was inhibited by prior CVX stimulation.

We expected initially the responses of thrombin to be identical to those of mixtures of the two PAR peptides (SFLLRN and AYPGKF). Indeed several of the conditions involving thrombin which are likely to occur in a realistic thrombotic environment are very well approximated by mixtures of the PAR peptides (Figure 3.4-8). However, to test the distinction between thrombin and mixtures of the PAR peptides over a broad range of conditions we tested the effects of additions of sequential mixtures of ADP, (SFLLRN+AYPGKF) and CVX (Figure 4.2-2); and compared these to the results obtained by adding sequences of ADP, thrombin and CVX (Figure 4.2-2).
To distinguish between the responses to thrombin and mixtures of the thrombin receptor peptides (SFLLRN and AYPGKF), we added various sequential combinations of ADP, (SFLLRN + AYPGKF) and CVX. Trends noted in Figure 3.4-7 remained unchanged, as was the ability of CVX to downregulate the (SFLLRN + AYPGKF) response. In contrast to thrombin however, mixtures of the PAR peptides do not inhibit CVX signaling.

The results obtained with sequential additions of ADP, PAR1+PAR4 peptides and CVX (Figure 4.2-2) were identical to those noted in Figure 3.4-7. However quite unexpectedly, an important difference was noted between thrombin and mixtures of the PAR peptides. The cross down regulation of the CVX stimulus that was obtained by prior thrombin stimulation (Figure 4.2-1, panel 1) was not obtained with mixtures of the PAR peptides (Figure 4.2-2, panel 1). This conclusion was consistently observed throughout all of the conditions tested across Figure 4.2-1 and Figure 4.2-2 (panels 1, 3, 4 and 7).
Thus there is some property of the intact enzyme thrombin that is not reflected by synthetic mimics of PAR ligands (cleaved sections of thrombin receptors). One possibility is that the enzyme thrombin is able to cleave off GPVI from the platelet surface and thus downregulate signaling via this receptor. In contrast synthetic mixtures of PAR peptides that do not contain thrombin’s proteoytic activity will not downregulate the CVX signal. This hypothesis is being explored further by looking for cleaved fragments of the GPVI receptor using flow cytometric detection of this receptor’s levels. Another possibility is that prior thrombin treatment causes the GPVI receptor to be internalized or downregulated via receptor pathways not yet identified. Confirmation of this hypothesis too requires further experimentation.

**4.3 Incorporating donor specific descriptions of platelet function into models of coagulation**

The Platelet-Plasma model developed in Chapter 2, includes a coarse grained description of platelet function where unbinding rates of platelet dependent reactions are decreased by two orders of magnitude via the $\varepsilon$ function as the platelet’s activate with thrombin. As a next step $\varepsilon$ can be obtained from PAS trained donor specific Neural Networks (Chapter 3). In addition to donor specificity, this will allow us to account for the combinatorial effects of the autocatalytically released agonists ADP and thromboxane. Eventually, a heterogenous model of platelet activation dependent enzymatic conversion will be developed; incorporating mass transfer of zymogens from the bulk onto the active platelet surface, coagulation factor assembly on discrete binding sites provided by the platelet, catalytic conversion of zymogens to active enzymes at these sites, and finally the release and diffusion away of these enzymes.
Appendix A: Phenotyping of murine platelets

The high throughput calcium phenotyping protocol that we have developed is useful not just to study human platelets but can also be used to study murine platelets. This is especially useful for murine platelets, because very low volumes of blood can be obtained from mice (about 1 ml from a cardiac puncture for each mouse). Our assay allows the simultaneous testing of phenotypes across multiple conditions with several replicates in the same plate, using very small volumes of blood. Moreover experiments can be designed to compare both wildtype and genetically modified murine platelet phenotypes side by side on the same plate, and this allows for the rapid identification of genotype-phenotype linkages.

A.1 Experimental protocol for murine platelet phenotyping

Blood was drawn from the inferior vena cava of anesthetized mice (100 mg/kg Nembutal) using a heparinized syringe (1000 U/ml, 1:100 dilution with blood), diluted 1:1 with HEPES-Tyrodes buffer, and spun at 800 rpm for 6 min to isolate platelet rich plasma (PRP). PRP was diluted with HEN buffer (10 mM HEPES pH 6.5, 1 mM EDTA, 150 mM NaCl). The HEN buffer was supplemented with 1 µM PGE$_1$ during the wash steps to prevent unwarranted platelet activation during rapid sedimentation. Platelets were sedimented at 1300 rpm for 15 min and then resuspended in HEPES-Tyrode’s buffer to the desired platelet counts.

Washed platelets pooled from two mice of each genotype (either regular or genetically modified) were resuspended in 1.5 ml of HEPES-Tyrode’s buffer containing 1 U/ml apyrase and 1 µM PGE$_1$ and incubated with 25 µl of Fura-2AM in DMSO (1
mg/ml) for 45 min in the dark. Afterwards the platelets were washed with HEN buffer and resuspended in HEPES-Tyrod’s buffer at $2 \times 10^8$ platelets/ml. A 384 well microtiter plate containing platelet agonists was prepared on a Perkin Elmer Janus. A separate microtiter plate containing diluted platelet suspension ($1 \times 10^6$ platelets/well) was prepared on a Perkin Elmer Evolution. Agonists were dispensed on a Molecular Devices FlexStation. Fura 2 fluorescence was measured at excitation 340/380 nm and emission 510 nm for 4 minutes in every column of the plate. The 340/380 fluorescence ratio $R(t)$ was scaled to the mean baseline values for each well $R_0(t)$ and relative calcium concentrations were quantified as $R(t)/R_0(t)$. All conditions were tested in replicates of 8.

A.2 Mice with substitution in $G_{i2\alpha}$ rendering resistance to RGS signaling

This study explored one of the intrinsic mechanisms that prevent overly robust platelet activation following vascular injury (in contrast to extrinsic regulators of platelet function such as NO and PGI$_2$). To study the role of RGS (Regulators of G Protein Signaling) proteins in platelets, mice with a G184S substitution in $G_{i2\alpha}$ that blocks RGS/$G_{i2}$ interactions were phenotyped. This allows us to examine the consequences of lifting constraints on $G_{i2}$ dependent signaling without altering receptor effector coupling. The results showed that the $G_{i2\alpha}$ (G184S) allele enhances platelet aggregation in vitro and increases platelet accumulation following vascular injury. These changes occur in concert with an attenuated rise in cAMP levels in response to PGI$_2$ and a substantial increase in basal Akt activation.

Because of suggestions in literature that $G_i$ derived $G_{\beta\gamma}$ can activate phospholipase C$\beta$, we explored the effect of the $G_{i2\alpha}$ (G184S) substitution on changes in cytosolic Ca++
concentration when $G_{\text{i2}} (+/G184S)$ and matched control platelets were incubated with ADP, AYPGKF, U46619 or convulxin. Each agonist tested caused an increase in cytosolic $\text{Ca}^{++}$, but the extent of the increase was unaffected by the presence of the $G_{\text{i2}} (G184S)$ substitution (Figure A.2-1). This finding was consistent with the unperturbed timing and extent of Rap1b activation in response to ADP (one of the critical steps leading to $\alpha_{IIb}\beta_3$ activation in platelets downstream of $G_q$ mediated increases in cytosolic calcium). It suggests that in this context, there is no crosstalk between $G_{i2}$ and $G_q$ mediated pathways in the presence of the G184S mutation. (The calcium phenotyping experiments in this section were done in collaboration with Rachel Signarvic).

These experiments collectively 1) demonstrated an active role for RGS proteins in regulating platelet responsiveness, and 2) suggested that RGS proteins help to prevent unwarranted platelet activation and limit the magnitude of haemostasis [228].

![Figure A.2-1 Comparison between platelet calcium mobilization phenotypes of wildtype and $G_{\text{i2}}$ knockin mice](image)

$G_{i2} (+/G184S)$ and matched control platelets were loaded with Fura-2 and stimulated with several concentrations of each of the indicated agonists. $\text{Ca}^{++}$ dependent Fura-2 fluorescence over time for each agonist was measured for wild type and $G_{i2} (+/G184S)$ platelets. A comparison of average maximal fluorescence over the range of agonist concentrations studied for each genotype is shown above. Each curve represents the average of 8 replicates. The experiment was performed twice with similar results obtained. Blood obtained from 2 mice of each genotype was used for each experiment.
A.3 ESAM<sup>−/−</sup> mice

Endothelial cell Specific Adhesion Molecule (ESAM), normally found in the alpha granules of quiescent platelets, is expressed in abundance on the cell surface following activation. ESAM localizes to the junctions between adjacent platelets following platelet activation, suggesting a role for this protein in contact dependent events that regulate thrombus formation. This study explored the role of ESAM by phenotyping ESAM deleted platelets.

In vivo, ESAM<sup>−/−</sup> mice achieved more stable hemostasis than wild-type mice following laser injury of cremaster muscle arterioles. In vitro, ESAM<sup>−/−</sup> platelets aggregated at lower concentrations of G protein-dependent agonists than wild type platelets, and were more resistant to disaggregation.

To examine whether ESAM regulates early platelet signaling events downstream of agonist stimulation, calcium mobilization was measured in ESAM null platelets and this phenotype was compared to that of wildtype platelets. Calcium mobilization in response to either a PAR-4 agonist peptide or ADP was the same in ESAM<sup>−/−</sup> platelets as in wild type controls (Figure A.3-1). (The calcium phenotyping experiments in this section were done in collaboration with Tim Stalker).

Similarly integrin activation measured by flow cytometry using an antibody selective for the active conformation of α<sub>IIb</sub>β<sub>3</sub> (or by measuring FITC-labeled fibrinogen binding) did not differ between ESAM<sup>−/−</sup> and wild type platelets. Neither was there any appreciable difference in platelet spreading or alpha granule secretion phenotypes between wildtype and ESAM<sup>−/−</sup> platelets. These observations suggest that the gain of function observed in the absence of ESAM for aggregation or in vivo thrombosis were
not because of inside out signaling events but were rather dependent on post-aggregation signaling.

To understand the molecular mechanism by which ESAM regulates platelet activity, a PDZ domain array was used to identify the scaffold protein NHERF-1 as an ESAM binding protein, and further experiments were carried out to demonstrate that this protein associates with ESAM in both resting and activated platelets.

Taken together, these findings support a model in which ESAM localizes to platelet contacts following platelet activation in order to limit thrombus growth and stability so that the optimal hemostatic response occurs following vascular injury [33].

![Figure A.3-1 Comparison between platelet calcium mobilization phenotypes of wildtype and ESAM knockout mice](image)

**Figure A.3-1 Comparison between platelet calcium mobilization phenotypes of wildtype and ESAM knockout mice**

Calcium mobilization in response to ADP (left panel) and the PAR4 agonist AYPGKF (right panel) were measured using the fluorescent calcium indicator Fura-2. Values are mean ± SD and are representative of two independent experiments. ESAM did not have any effect in regulating calcium mobilization in isolated mouse platelets.
Bibliography


