Kastrup *et al.* 10.1073/pnas.0605560103.

Supporting Information

Files in this Data Supplement:

Supporting Figure 5

Fig. 5. Schematic drawing of the set-up for experiments with the chemical model. A PDMS gasket (gray) was sealed to a siliconized glass coverslip. The chemical model reaction mixture (30 μl, purple) was placed in the chamber. A photoacid layer (20-30 μm) of a dispersion of 2-nitrobenzaldehyde (50% by weight) in dimethylsiloxane ethylene oxide block copolymer was placed on top of the PDMS and in contact with the chemical model reaction mixture. A photomask was placed on top, allowing UV light (300-400 nm) to pass only in specific locations (green).

Supporting Figure 6

Fig. 6. Rate plots of the rate equations incorporated in the numerical simulation of the modular mechanism (see text above for details). (*A*) Two rate equations representing (*i*) the module of autocatalytic production of *C* (green curved line), and (*ii*) the module of the linear consumption of *C* (black straight line). The crossing points between these two lines represent steady states. The steady state at $[C] = 1.1 \times 10^{-9}$ M is stable. However, the steady state at $[C] = 8.9 \times 10^{-9}$ M is unstable and represents C_{thresh}, the threshold $[C]$. When $[C] > C_{\text{thresh}}$, the rate of production is greater than the rate of consumption, and rapid amplification of $[C]$ occurs. (*B*) Two additional equations representing (*i*) the reactions involved in production of *C* at the surface of the patch (blue horizontal line), and (*ii*) the module of precipitation that occurs at high $[C]$ (red, dashed line). The precipitation module was not incorporated in the simulation (although it was incorporated in the experimental chemical model) and has been schematically included here for clarity.

Supporting Figure 7

Fig. 7. Numerical simulation indicated that the probability of initiating "clotting" in the model exhibits a threshold response to patch size. In simulation, patches $P \leq 50$ μm never initiated "clotting," but patches $P \geq 60$ μm always initiated "clotting."

Supporting Figure 8

Fig. 8. Set-up for experiments with blood plasma and patterned phospholipid bilayer substrates. (*A*) Schematic of a PDMS microfluidic chamber (gray) used to contain a glass coverslip coated with a patterned phospholipid bilayer. Clot-promoting negatively charged phospholipids with reconstituted tissue factor (TF) (red) were patterned in a background of inert neutral lipids (green). The chamber contained blood plasma (light brown) and was sealed with a siliconized glass coverslip on top. (*B*) Cross-section of the chamber.

Supporting Figure 9

Fig. 9. The amount of acid generated is dependent on the total surface area of the patches. In absence of the model reaction mixture, the H^+ production was monitored

with an acid sensitive dye, 5-(and-6)-carboxy-seminaphthofluorescein-1 (SNAFL, a dye with dual emission, dual excitation properties). First, a calibration curve of fluorescence intensity vs. H^+ concentration was determined for SNAFL, by titration with HCl (data not shown). Then, the change in green and red fluorescence intensity of SNAFL was measured every 2 min following a 20 s pulse of UV light through the photomask and photoacid layer. Using the fluorescence intensity data, the measured calibration curve, and the known volume of the sample, the amount of H^+ produced was determined. The H^+ production was measured for different arrays of patches with the same total surface area, *a*, of patches but with different patch sizes, *p*. The H^+ production was approximately the same for arrays with the same total surface area (within a factor of two). The H^+ production was also measured for a single 400 μm patch, which had a surface area four times smaller than the arrays and produced 2.4-4.8 times less H^+.

Supporting Figure 10

Fig. 10. Quantifying fluorescence intensity profile of pH-sensitive dye in the chemical model on the photoacid surface. Original (not false colored and not processed) images are shown and analyzed. (*A*) Time-lapse fluorescent micrographs and line scans (dashed lines) of initiation of "clotting" in the chemical model on a 400 μm patch. Line scans show that at 22 sec "clotting" was initiated and quenched the fluorescence. (*B*) Time- lapse fluorescent micrographs and line scans of the chemical model on an array of 200 μm patches. Line scans show that "clotting" did not initiate on these patches, as the fluorescence intensity did not significantly decreases. Modifications and false-coloring of images did not distort the information, and analysis of false-colored images gave analogous intensity profiles.

Supporting Figure 11

Fig. 11. Quantifying initiation of clotting of blood plasma. (*A* and *B*) A 61 μm patch of TF-reconstituted bilayer containing a red lipid dye (*B*) was patterned in a background of inert bilayer containing a green lipid dye (*A*). (*C* and *D*) No large increase in fluorescence intensity due to MCA was observed within 20 min on the 61 μm patch. No formation of cross-linked fibrin strands or platelet aggregation was observed on the 61 μm patch. (*E*) Line scans (dashed lines in *C*) quantifying the fluorescence intensity in *C*. (*F* and *G*) A 137 μm patch of TF-reconstituted bilayer containing a red lipid dye (*G*) was patterned in a background of an inert bilayer containing a green lipid dye (*F*). (*H* and *I*) A large increase in fluorescence

intensity due to release of MCA by thrombin was seen within 2 min on the 137 μm patch. Formation of crosslinked fibrin strands and aggregation of platelets (solid white arrow) was observed on the 137 μm patch. The open white arrows point to imperfections in the PDMS chamber underneath the coverslip. (*J*) Line scans (dashed lines in *H*) quantifying the fluorescence intensity in *H*.

Supporting Figure 12

Fig. 12. Quantifying initiation of clotting of blood plasma on arrays presented in Fig. 2*D* (main text). (*A* and *B*) For arrays of 50 μm patches, clotting did not initiate on the patch within 43 min. No large increase in fluorescence due to MCA was observed. (*C* and *D*) For arrays of 400 μm patches, clotting initiated on the patches within 3 min. A large increase in fluorescence due to release of MCA by thrombin was observed. *The background line scan shown for the 400 μm patches (*D*) was taken from an early time point (30 sec) in a different experiment done with a 200 μm patch. It is shown here to demonstrate the change in fluorescence intensity that occurs during the first few minutes of clotting. For all fluorescent images of MCA shown in the main text, monitoring of clotting was started at 3 min after the NPP was added to the PDMS chamber.

Supporting Text

Materials

Chemicals and Reagents. All solvents and salts used in buffers were purchased from commercial sources and used as received unless otherwise stated. Poly (dimethylsiloxane) (PDMS) (Sylgard Brand 184 Silicone Elastomer kit) was purchased from Dow-Corning. 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC), L-α-phosphatidylserine from porcine brain (PS), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were purchased from Avanti Polar Lipids. Texas red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Texas red DHPE), Oregon green 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Oregon green DHPE), *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (NBD-DHPE), 5-(and-6)-carboxy SNAFL-1 (SNAFL), rhodamine 110, bis-(*p*-tosyl-L-glycyl-L-prolyl-L-arginine amide) and FluoSpheres (sulfate microspheres, 1.0 μm, yellow-green fluorescent (505/515), 2% solids) were purchased from Molecular Probes/Invitrogen. Normal pooled plasma (human) (NPP) was purchased from George King Bio-Medical. *t*-butyloxycarbonyl-β-benzyl-L-aspartyl-L-prolyl-L-arginine-4-methyl-coumaryl-7-amide (Boc-Asp(OBzl)-Pro-Arg-MCA) was purchased from Peptides International. Albumin (BSA), and medium

viscosity alginic acid were purchased from Sigma. Human recombinant tissue factor (TF) and corn trypsin inhibitor (CTI) were purchased from Calbiochem. Argatroban was manufactured by Abbot Laboratories. Bromophenol blue and sodium chlorite ($NaClO_2$, 80% purity) were purchased from Acros Organics. Krytox fluorinated grease is a product of DuPont. Siliconized glass coverslips were purchased from Hampton Research. Anhydrous hexadecane, 2-nitrobenzaldehyde, and *n*-octadecyltrichlorosilane (OTS) were purchased from Aldrich. Sodium thiosulfate ($Na_2S_2O_3$, 99.9% purity) and anhydrous methyl sulfoxide (DMSO, 99.7% purity) were purchased from Fisher Scientific.

Methods

Preparing the Reagents of the Chemical Model. The reagents of the chemical model consisted of solution-phase reagents (the model reaction mixture) and a solid-phase patterned substrate. The model reaction mixture was a solution containing $NaClO_2$, $Na_2S_2O_3$, alginic acid, and bromophenol blue [Runyon MK, Johnson-Kerner BL, Ismagilov RF (2004) *Angew Chem Int Ed* **43,** 1531-1536]. A solution containing $NaClO_2$ and $Na_2S_2O_3$ was metastable and could be triggered by addition of a threshold concentration of acid (H_3O^+) to react rapidly and autocatalytically to produce more acid [Nagypal I, Epstein IR (1986) *J Phys Chem* **90,** 6285-6292]. Alginic acid, under basic conditions, is present as sodium alginate and is water-soluble. However, under acidic conditions, alginic acid produces an insoluble gel. Bromophenol blue was the pH indicator used to monitor the time that the reaction mixture reacts and initiates "clotting." The reaction mixture was monitored by fluorescence (λ_{ex} = 535-585 nm, λ_{em} = 600-680) of bromophenol blue. When "clotting" was initiated, the basic reaction mixture became acidic, which resulted in the quenching of the red fluorescence and a visible color change from purple to yellow. The solid-phase patterned substrate consisted of a coverslip coated with a thin layer (20-30 μm) of a dispersion of 2-nitrobenzaldehyde in dimethylsiloxane-ethylene oxide block copolymer. UV-irradiation through a photomask photoisomerized 2-nitrobenzaldehyde (not acidic) to 2-nitrosobenzoic acid (acidic, pK_a <4).

Preparing two stable solutions as precursors to the metastable model reaction mixture. Two stable solutions were prepared. When these two solutions were combined, the resulting solution constituted the metastable model reaction mixture. Solution 1 was an aqueous solution of $Na_2S_2O_3$, alginic acid, and bromophenol blue. Solution 2 was an aqueous solution of $NaClO_2$.

Preparation of solution 1: The stock alginic acid solution was made by adding alginic acid (0.290 g, medium viscosity) to a solution of NaOH (50 ml, pH 10.8) and was dissolved by heating at ~90°C for 45 min. The stock $Na_2S_2O_3$/alginic acid/bromophenol blue solution was made by combining $Na_2S_2O_3$?/B>5H_2O (0.122 g, 0.492 mmol) and bromophenol blue (sodium salt) (12.5 μl of 0.17 M solution in aqueous NaOH, pH 11.6) in 5 ml of the stock alginic acid solution. This procedure resulted

in a $Na_2S_2O_3$/alginic acid/bromophenol blue solution with a final pH of ~7.

Preparation of solution 2: The stock $NaClO_2$ solution was made by dissolving $NaClO_2$ (0.270 g, 2.99 mmol) in 10 ml of Millipore-filtered H_2O (final pH ~ 10.7). This solution was used within 12 h.

Combining the reagents to form the metastable reaction mixture used in the chemical model. The model reaction mixture was prepared by combining the stock $Na_2S_2O_3$/alginic acid/bromophenol and the stock $NaClO_2$ solutions 1:1 by volume. This procedure resulted in a solution that was initially purple and fluoresced in red. Addition of one drop of 1N HCl initiated the "clotting" reaction, turning the solution visibly yellow and quenching the red fluorescence. Without addition of acid, spontaneous initiation (usually within 20 min) resulted in the same purple to yellow transition due to the stochastic nature of the chlorite/thiosulfate reaction [Nagypal I, Epstein IR (1986) *J Phys Chem* 90, 6285-6292].

Preparing the photoacid-coated substrate. The photoacid 2-nitrobenzaldehyde was kept in the dark at all times. The photoacid was dissolved into dimethylsiloxane-ethylene oxide block copolymer (1:1 by weight) by heating to 60°C with stirring. This mixture was maintained at 60°C until spin-coated. The homogeneous photoacid/siloxane mixture was spin-coated by placing 50 μl of warm mixture in the center of a siliconized coverslip (22 mm diameter) at room temperature. The substrate was immediately spun at 500 rpm for 10 sec, then at 1,500 rpm for 15 sec. Within 5 min, 2-nitrobenzaldehyde solidified out of the siloxane fluid, producing a thin gel-like layer (20-30 μm thick) over the coverslip. The photoacid-coated substrates were kept in the dark and used within 12 h.

Measuring Initiation of "Clotting" in the Chemical Model in a Microfluidic Chamber. *Designing and assembling the chamber.* The microfluidic chamber (Fig. 5) used in the chemical model experiments was constructed by sealing a PDMS gasket to a siliconized coverslip. The disposable chamber had an inner diameter of 10 mm, an outer diameter of 20 mm, and a depth of 1 mm. A 30 μl drop of the model reaction mixture was placed in the chamber. The glass coverslip coated with photoacid substrate was placed on top.

Creating acidic patches by UV irradiation. A 100 W Hg lamp was used to irradiate the sample from above. Light passed through a heat absorbing filter (50 mm diameter Tech Spec heat absorbing glass) then a short-pass filter (chroma no. D350), allowing primarily 300-400 nm wavelengths to reach the sample. Light then passed through a condenser which was defocused to yield a uniform illumination area of ~6 mm in diameter on the sample. UV light was illuminated through a "silver on Mylar" photomask (CAD/Art Services) placed directly on top of the glass coverslip coated with the photoacid dispersion.

Imaging the model reaction mixture using epi-fluorescence microscopy. A 150 W Xenon light source was used to monitor the model reaction mixture from below the sample. Light passed through a filter cube (λ_{ex} = 535-585 nm, λ_{em} = 600-680) and a 5 × 0.15

N.A. objective. Exposure times of 10 ms were taken every 180 ms, with the camera set at bin = 2 × 2 and gain = 255. The quenching of red fluorescence indicated that the model reaction mixture had reacted and initiated "clotting." Significant photobleaching was not seen for the pH sensitive dye. When the initiation of "clotting" occurred (after ~22 s of irradiation for large patches), quenching of fluorescence intensity occurred rapidly, decreasing by a factor of ~10 in <1 sec (see example in Fig. 10). This is NOT consistent with simple photobleaching.

The images of the acidic patches in the chemical model system (here we are not referring to monitoring of "clotting") were obtained by filtering the "UV irradiation source" through a green-pass filter (HOYA). Green light passed through the photomask and the experimental setup to an objective below. Images of the patches were taken from below the sample (see Fig. 5). An image taken from below shows patches that appear "fuzzy" due to the distortion of light as it passed through the thin layer of the solid suspension of the photoacid.

Analyzing images of initiation of "clotting" in the chemical model system. For the model reaction mixture, the original grayscale time-lapse fluorescence images showed a quenching of fluorescence (transition from high fluorescence to low fluorescence) when "clotting" was initiated (see *Additional Control Experiments* and Fig. 10 for images). In MetaMorph these images were uniformly false colored yellow and thresholded for dark objects. This procedure resulted in an inversion of light yellow and dark areas in all images. The end result was images going from dark to light yellow when "clotting" was initiated. This procedure allowed us to use more sensitive fluorescent imaging and obtain the yellow color visually observed upon "clotting."

The original images of the acidic patches were false colored to green and the levels were adjusted in MetaMorph.

The processed MetaMorph images were opened in a new Adobe Photoshop document set to RGB mode. An overlaid image was created consisting of two layers: the top layer was the green image of the patch and bottom layer was the yellow image of the "clotting" solution. The blending options for the top layer were set to blend only if green.

Quantifying Acid Production from Patches in the Chemical Model Using 5-(and 6)-Carboxy-seminaphthofluorescein-1 (SNAFL). *Fabricating an experimental setup to quantify acid production.* An experimental set-up similar to that described above for the chemical model was used (same illumination and imaging settings). The following differences were applied: 1) a different chamber was used, 2) a 40 × 0.85 *N.A.* objective was used, and 3) the model reaction mixture was replaced by a SNAFL solution. For these experiments, the chamber consisted of a 100 μm diameter silver wire wound in a circle ~3 mm in diameter and placed on top of a siliconized coverslip (22 mm). Silicon grease was applied around the wire. A 2 μl drop of 10 μM SNAFL (red fluorescence = basic, green fluorescence = acidic) in 10 mM Tris (hydroxymethyl)aminomethane (Tris, pH 9.7) was placed in the silver wire circle, but did not directly contact the wire. The photoacid substrate was placed on top of

the silver wire and sealed down with the silicon grease. The photomask was placed on top of the photoacid substrate.

Generating an acid calibration curve with SNAFL. A calibration curve was generated for fluorescence intensity of SNAFL vs. concentration of acid added. SNAFL/Tris solutions were prepared with varying amounts of HCl added. The final pH of the solutions ranged from 6.5 to 9.7. The green and red fluorescence intensities were measured for the SNAFL/Tris + HCl solutions in the chamber. The calibration curve (ratio green/red intensity vs. $[H_3O^+]$) for this acid titration was fitted with a sigmoidal curve.

Quantifying acid production for different patch sizes. The acid production of arrays and single patches was measured using the experimental set-up described for the SNAFL solution. Samples were irradiated with a UV pulse for 20 sec, allowed to equilibrate for 2 min, and then the green and red fluorescence intensities were measured. The amount of acid produced was determined using the fluorescence intensity data, the measured calibration curve, and the known volume of the sample (see *Additional Control Experiments* and Fig. 9 for results).

Numerical Simulations of the Modular Mechanism of Initiation of Clotting on Surfaces Presenting Clotting Stimuli. We used numerical simulations to illustrate that a threshold patch size can exist for the proposed modular mechanism, using a single rate equation to represent the kinetics of each module.

We had three goals: (*i*) test if competition between two modules, one producing an activator (autocatalytically) and one consuming the activator (linearly), could produce a threshold response to concentration of the activator; (*ii*) test if a simulation incorporating diffusion, these two modules, and a surface patch that produced activators, could produce a threshold response to the size of the patch; and (*iii*) test if reasonable parameters for biochemical reactions of blood clotting could produce a threshold patch size of the same magnitude as the experimentally measured value. The purpose was not to predict the exact size of the threshold patch. We believe that the time scale of reaction, t_R, a single experimentally determined parameter, is a simpler and more reliable predictor of the size of the threshold patch.

Choosing parameters used in numerical simulations. In the modular mechanism, the diffusion and reactions occurring at a patch presenting "clotting" stimuli were numerically simulated using a commercial finite element package FEMLAB version 3.1 (Comsol, Stockholm, Sweden). The surface consisted of patches presenting "clotting" stimuli and a 1 mm "inert" vicinity around the patch. The effect of varying patch size on concentration profiles and "clot time" was determined.

To numerically simulate the change in concentration of activator, "C," we considered diffusion in solution and reactions occurring in solution and on a surface patch, all in the x, y dimensions. C may be compared to the set of clot-promoting molecules present in blood. The mass transport of C was modeled with the standard convection-diffusion equation. We used a diffusion coefficient 5×10^{-11}

m^2s^{-1}(approximate value for a solution-phase protease in blood clotting, such as thrombin) [Krasotkina YV, Sinauridze EI, Ataullakhanov FI (2000) *Biochim Biophys Acta* 1474, 337-345]. Convective flow was not used in the simulation. In this pseudo-2D simulation, the z-dimension was modeled by a single layer of 1 μm-thick mesh elements (1 μm boundary layer). For this boundary layer thickness, we can assume that lateral diffusion through the layer is fast and that the solution is homogeneous in the z-dimension. The size of the boundary layer is rather arbitrary, and a range of thicknesses may be used, as long as diffusion through the thickness of the boundary layer is much faster than the rate of reactions and the rate of diffusion across the smallest patch. The boundary layer is used to simplify 3D simulation to a computationally more efficient pseudo-2D simulation. This boundary layer thickness has been used by others [Kuharsky AL, Fogelson AL (2001) *Biophys J* 80, 1050-1074]. In the x, y dimensions, a boundary condition of insulation/symmetry was used at the outer edge of the "inert" vicinity.

Three rate equations were incorporated into the simulation: (*i*) production of C at the surface of the patch, rate = k_{patch},; (*ii*) autocatalytic production of C in solution, rate = $k_{prod}[C]^2 + b$; and (*iii*) linear consumption of C in solution, rate = $-k_{consum}[C]$. The values used were $[C]initial = 1 \times 10^{-9}$ M, $k_{patch} = 1 \times 10^{-9}$ M s^{-1}, $k_{prod} = 2 \times 10^7$ $M^{-1}s^{-1}$, $b = 2 \times 10^{-10}$ M s^{-1}, and $k_{consum} = 0.2$ s^{-1}. These values were selected based on approximate values [Kuharsky AL, Fogelson AL (2001) *Biophys J* 80, 1050-1074] for representative reactions in blood clotting. Using these values, two steady states were present, one at $[C] = 1.1 \times 10^{-9}$ M and one at 8.9×10^{-9} M. The existence of these steady states may be understood by considering the rate plots for the reaction rate equations (Fig. 6) (for a review describing rate plots, see Tyson JJ, Chen KC, and Novak B [(2003) *Curr Opin Cell Biol* 15, 221-231]. The steady state at $[C] = 1.1 \times 10^{-9}$ M was stable. The steady state at $[C] = 8.9 \times 10^{-9}$ M was unstable and represented the threshold concentration of C, C_{thresh}. When $[C] > C_{thresh}$, rapid amplification occurred, which lead to the production of a sufficient $[C]$ to initiate precipitation (formation of the solid "clot"). In simulations that did not have a patch (where the patch size, p, was zero), $[C]$ remained at the stable steady state value of $[C] = 1.1 \times 10^{-9}$ M. When a large patch was incorporated into the simulation the combined production of C in solution and on the patch resulted in $[C]$ exceeding $[C]_{tr}$ in 10 s.

Results of the simulation. The concentration profiles obtained by numerical simulation indicated that "clotting" in the simulations displayed a threshold response to the patch size, p (Fig. 7). Using the parameters above for patches P = 50 μm, $[C]$ never increased to C_{thresh}. However, when P = 100, $[C]$ increased to C_{thresh} in 10 s. The threshold patch size, p_{tr}, (smallest p that will initiate clotting) was between 50 and 60 μm. The value of p_{tr} increased as k_{patch} was decreased, indicating that the rate of production at the surface of the patch will affect p_{tr}. This change in p_{tr} is consistent with preliminary experimental results that showed that when the TF concentration was decreased, t_R increased and p_{tr}

increased. In the numerical simulations, the value of p_{tr} also increased as D was increased.

We emphasize that the quantitative agreement of the simulation with the experiment is likely to be coincidental. We believe that the time scale of reaction, t_R, a single experimentally determined parameter, is a simpler and more reliable predictor of the size of the threshold patch for different blood plasma samples.

The effect of curvature should also be important for patches in small capillaries. Three-dimensional simulations are needed take this effect into account.

Numerical simulation for "clotting" on tight clusters of subthreshold patches. The effect of changing the distance between subthreshold patches on the concentration profile of C and on "clot time" was determined. A cluster of subthreshold patches P = 40 μm generated $[C] > C_{thresh}$ only when positioned sufficiently close together. When 40 μm patches were separated by 80 μm, C_{thresh} was never reached. However, if patches were separated by only 20 μm, C_{thresh} was rapidly reached and "clotting" initiated.

Preparing the PDMS Microfluidic Chamber for Experiments with Blood Plasma.
Designing and fabricating the chamber. The microfluidic chambers (Fig. 8) used in the blood plasma and whole blood experiments were constructed primarily from poly (dimethylsiloxane) (PDMS) and fabricated from multilevel, machine-milled, brass masters. The disposable PDMS chamber had an inner diameter of 13 mm, an outer diameter of 20 mm, and a depth of 1 mm.

Eliminating convective flow and background clotting in the chamber. To reduce convective flow in the solution, the PDMS chamber was soaked in a solution of NaCl (150 mM) for 4–8 h. To further reduce convective flow and background clotting on the PDMS surface, the chamber was soaked in a 1% BSA (in PBS solution, pH 7.3) for 1–2 h. Before the blood plasma or whole blood experiment, the chamber was rinsed thoroughly with a solution of NaCl (150 mM). To allow a good seal to form between the PDMS and the siliconized glass coverslip, a portion of BSA was removed from the top outer surface of the chamber by wiping with a dust free wipe.

Assembling the chamber for clotting experiments. The soaked chamber was placed in a 35 × 10 mm Petri dish (BD Biosciences). The substrate (patterned coverslip) was placed in the chamber. A thin layer of Krytox fluorinated grease was applied on top of the chamber. The appropriate blood plasma or whole blood sample (see below) was then placed in the chamber. A siliconized glass coverslip was pressed down lightly, pushing out excess blood plasma, making contact with the grease, and sealing the chamber. The Petri dish was then filled with a solution of NaCl (150 mM), keeping the chamber submerged to eliminate evaporation through the PDMS. The chamber was maintained at either 23–24°C or 37°C.

Measuring convective flow inside the chamber. In control experiments, the flow inside the PDMS chamber was measured by taking time-lapse fluorescent micrographs

of fluorescent microspheres (FluoSpheres) in normal pooled blood plasma. The distance traveled by individual FluoSpheres was measured and divided by the elapsed time. The stock solution of FluoSpheres [sulfate microspheres, 1.0 μm diameter, yellow-green fluorescent (505/515), 2% solids] was diluted (25 μl to 5 ml) with a solution of NaCl (150 mM). The diluted FluoSpheres solution was vortexed for 30 s and sonicated for 1 min to break up aggregates of FluoSpheres. This FluoSpheres solution (70 μl) was added to citrated, normal pooled blood plasma (210 μl). The FluoSpheres/plasma mixture was added to the chamber, and the chamber was sealed. Images were taken every 1 min at up to 10 positions throughout the chamber (see *Additional Control Experiments* for values).

Preparing Patterned Supported Phospholipid Bilayers to Spatially Control the Initiation of Clotting Via the Tissue Factor (TF) Pathway. *Cleaning coverslips to reduce contamination and to generate a hydrophilic surface.* To obtain reproducible results in clotting experiments with phospholipid bilayers, it was essential to eliminate contaminants such as large glass particles and dust. The cleaning process of coverslips consisted of the following steps: 1) applying 3M Scotch tape (# 810) to remove large glass particles, 2) sonicating using the solution cycle [(*i*) EtOH, (*ii*) H_2O, (*iii*) 10% ES 7X detergent, (*iv*) EtOH, (*v*) Millipore filtered water] with H_2O and EtOH rinses between steps to further eliminate loose glass particles, 3) soaking in a freshly made "piranha" solution (H_2SO_4:H_2O_2, 3:1, by volume, DANGER: this mixture reacts violently with organic materials and must be handled with care) for ≈20 min, and 4) rinsing thoroughly with Millipore filtered water and drying in a stream of N_2. The cleaned coverslips were used immediately after drying.

Preparing solutions of lipid-vesicles. The preparation of unilamellar vesicles has been described elsewhere [Yee CK, Amweg ML, Parikh AN (2004) *J Am Chem Soc* 126, 13962-13972, and references therein]. Our procedure will be briefly described. In a piranha cleaned glass vial, the appropriate chloroform solutions of lipids were mixed to the desired concentration and mole ratios. The chloroform was evaporated with a stream of N_2 (gas), and then the lipid cake was dried under a vacuum [50 millitorr (1 torr = 133 Pa)] for at least three hours. The dry lipids were suspended in Millipore filtered water (10 mg/ml) by vortexing and then hydrated overnight at 4°C. The hydrated vesicles were subjected to five freeze-thaw cycles. They were frozen in a dry ice/acetone bath and thawed in an oven set at a temperature above the lipid transition temperature. These vesicles were extruded (Lipex Extruder, Northern Lipids) ten times through a Whatman Nuclepore Track-Etch membrane (100 nm pore size) at a temperature above the lipid transition temperature. The extruded vesicles were diluted to the stock concentration (5 mg/ml) using Millipore filtered water and stored at 4°C. All vesicle solutions were used within two weeks.

Reconstituting tissue factor (TF) to obtain clot-promoting vesicles. TF was reconstituted into mixed vesicles [van 't Veer C, Mann KG (1997) *J Biol Chem* 272, 4367-4377; Smith SA, Morrissey JH (2004) *J Thromb Haemostasis* 2, 1155-1162] of DLPC/PS/Texas red DHPE (79.5/20/0.5 mole percents) at a concentration of 1.25 mg/ml in 1X Hepes-buffered saline/Ca^{2+} buffer. For experiments in Figs. 1-3 (main text)

the TF concentration in the vesicle solution was 0.40 nM (TF:lipid ratio of 2.5×10^{-7}). Assuming that all of the TF was incorporated into the vesicles, the calculated surface concentration would be 0.08 fmol/cm^2. After addition of TF to the vesicle solution, the solution was incubated at 37°C for 30 min and then stored at 12°C. The vesicles were used within 18 h.

Forming an inert bilayer. The inert supported phospholipid bilayers consisted of DPPC (97%) and green fluorescent dye (3% of either Oregon green DHPE or NBD-DHPE) [Jung SY, Holden MA, Cremer PS, Collier CP (2005) *ChemPhysChem* 6, 423-426]. Bilayers were made by adding 215 μl of the DPPC vesicle solution (0.34 mg/ml vesicles in PBS) to a freshly cleaned coverslip in a hydrophilic PDMS chamber at 50° C. PDMS was made hydrophilic by oxidation with a plasma cleaner (SPI Plasma Prep) before adding the coverslip. The microfluidic chamber containing the vesicle solution was incubated at 50°C for 10 min and then cooled to room temperature. Excess vesicles were removed by repeated rinsing with a solution of NaCl (150 mM). The bilayers were stored in the dark at room temperature and used within 24 h.

Backfilling into the inert bilayer to remove any areas of exposed glass. To ensure that there were no areas of exposed glass substrate caused by imperfections in the DPPC bilayers, all bilayers were backfilled with 30 μl of the DLPC vesicle solution (2.5 mg/ml vesicles in PBS buffer) and allowed to incubate in the dark at room temperature for 40 min. The excess vesicles were removed by extensive rinsing with a solution of NaCl (150 mM). These bilayers were photopatterned within a few hours.

Photopatterning to selectively remove patch regions of the inert bilayer. The DPPC bilayers that had been backfilled with DLPC were photopatterned using previously published methods (Yee, C. K., Amweg, M. L. & Parikh, A. N. *J. Am. Chem. Soc.* 126, 13962-13972 (2004), Yu, C. H., Parikh, A. N. & Groves, J. T. *Adv. Mater.* 17, 1477-1480 (2005)). Our procedure will be briefly described. The bilayer coated coverslip was positioned on an aluminum alignment tray under a photomask (chrome on quartz, Photo Sciences, Inc.). This set-up was placed on a chilling plate (Echo therm, Torrey Pines Scientific) set to 0°C to maintain a temperature of the sample at 20-30°C during irradiation. Bilayers were irradiated for 7 min with deep UV light (Hanovia medium pressure 450 W Hg immersion lamp in a double walled cooled quartz immersion well) and then rinsed thoroughly with a solution of NaCl (150 mM). Patterned bilayers were backfilled within 2 h.

Generating patches by backfilling clot-promoting lipids into the photo-removed regions of bilayer. To generate the clot-promoting patches, the patterned bilayers (see above) were backfilled with 30 μl of the TF-reconstituted vesicle solution (1.25 mg/ml vesicles in PBS buffer) and allowed to incubate for 4 min at room temperature. Phospholipid bilayers containing active TF have been prepared previously [Contino PB, Hasselbacher CA, Ross JB, Nemerson Y (1994) *Biophys J* 67, 1113-1116]. Excess vesicles were removed by vigorous rinsing with a solution of NaCl (150 mM). Patterned bilayers were used immediately in clotting experiments.

Preparing Patterned Hydrophilic Patches on Silanized Glass Coverslips to Spatially Control the Initiation of Clotting Via the Factor XII Pathway. *Forming an inert*

silanized surface on glass coverslips. A detailed procedure for silanization of glass coverslips has been previously described [Howland MC, *et al.* (2005) *J Am Chem Soc* 127, 6752-6765]. Our procedure will be briefly described. Freshly piranha cleaned glass coverslips (see above) were placed in a clean glass dish. Anhydrous hexadecane (10 ml) and *n*-octadecyltrichlorosilane (OTS) (40 μl) were added to the coverslips in a N_2(g) environment. This solution was incubated for 30 min. Then, a second 40 μl aliquot of OTS was added to the solution and incubated for an additional 45 min. Excess OTS was removed by rinsing six times with anhydrous hexadecane followed by several rinses with EtOH. The silanized coverslips were stored under a vacuum and used within 48 h.

Photopatterning to selectively generate hydrophilic glass patches in the inert silanized layer. Hydrophilic patches were generated using the photopatterning set-up described above and in the literature [Howland MC, *et al.* (2005) *J Am Chem Soc* 127, 6752-6765]. The silanized coverslips were irradiated under a photomask for 2 h. After irradiation, the coverslips were rinsed with EtOH and Millipore filtered water. The patterned coverslips were used with 30 min.

Detecting hydrophilic patches using a wetting test. Hydrophilic regions were detected using a glycerol wetting test [Wu MH, Whitesides GM (2002) *J Micromech Microeng* 12, 747-758]. The patterned coverslips were coated with glycerol, and the excess glycerol was removed using a gentle vacuum. This process left droplets of glycerol only on the areas of the coverslip that were exposed to UV light (hydrophilic regions). After imaging and before addition of normal pooled plasma, the glycerol was removed by vigorous rinsing with a solution of NaCl (150 mM).

Preparing Human Blood Samples for Experiments. *Preparing whole blood, and platelet rich plasma from donor blood.* Blood samples were obtained from individual healthy donors in accordance with the guidelines set by the Institutional Review Board (protocol # 12502A) at the University of Chicago. Whole blood was collected in Vacutainer tubes containing 3.2% sodium citrate (9:1 by volume). Platelet rich plasma (PRP) was obtained by centrifugation at 300 × *g* for 10 min.

Preparing normal pooled plasma. Citrated normal pooled plasma (NPP) (human) [Butenas S, Bouchard BA, Brummel-Ziedins KE, Parhami-Seren B, Mann KG (2005) *Blood* 105, 2764-2770] was purchased from George King Bio-Medical and was stored in 1 ml aliquots at -80°C until needed. When needed, the plasma was thawed by incubating at 18°C.

Recalcifying blood plasma samples and adding the thrombin-sensitive dye. All blood plasma samples were recalcified by adding a solution of $CaCl_2$ containing the thrombin-sensitive fluorescent dye Boc-Asp(OBzl)-Pro-Arg-MCA, ($CaCl_2$, 40 mM; NaCl, 90 mM; and Boc-Asp(OBzl)-Pro-Arg-MCA, 0.4 mM). At the start of each experiment, the plasma and the solution containing $CaCl_2$ were mixed 3:1 by volume. This recalcified plasma solution (400 μl) was added to the experimental set-up shown in Fig. 5 with gentle mixing. Clotting was detected by the appearance of fibrin using bright field microscopy and by the appearance of fluorescence signal generated when 4-Methyl-

Coumaryl-7-Amine (MCA) was cleaved from Boc-Asp(OBzl)-Pro-Arg-MCA by thrombin.

Recalcifying whole blood samples and adding the thrombin-sensitive dye. Whole blood samples were recalcified [Rivard GE, Brummel-Ziedins KE, Mann KG, Fan L, Hofer A, Cohen E (2005) *J Thromb Haemostasis* 3, 2039-2043] first by mixing the whole blood (376 μl) with a thrombin-sensitive fluorescent dye, rhodamine 110-bis-(*p*-tosyl-L-glycyl-L-prolyl-L-arginine amide) (2 μl, 10 mM in DMSO) and then with a solution of $CaCl_2$ (23.5 μl, 200 mM). This recalcified whole blood solution was added to the experimental set-up shown in Fig. 8. Clotting was detected by the appearance of fluorescence signal generated when rhodamine 110 was cleaved from rhodamine 110-bis-(*p*-tosyl-L-glycyl-L-prolyl-L-arginine amide) by thrombin. The Rhodamine 110 dye was used for thrombin detection in the whole blood experiments instead of the MCA dye, because red blood cells have a lower absorbance coefficient at the maximum excitation and emission wavelengths of rhodamine 110 than for MCA [Gandjbakhche AH, Bonner RF, Arai AE, Balaban RS (1999) *Am J Physiol* 277, H698-H704].

Inhibiting the factor XII pathway with corn trypsin inhibitor. For experiments measuring clot times for the TF pathway (all experiments using phospholipid bilayers and reconstituted TF), the factor XII (contact) pathway was inhibited with corn trypsin inhibitor (CTI). A stock solution of CTI (6.27 mg/ml) was added to the blood plasma, either immediately after the plasma was thawed (for NPP) or after centrifugation (for PRP), to a final concentration of 100 μg/ml and incubated for ≈10 h at 18°C before each experiment. For whole blood, CTI was added to a final concentration of 100 μg/ml after collection. For experiments measuring clot times for the factor XII (contact) pathway (all experiments with hydrophilic glass patches or gelatin), CTI was not added. Instead, the NPP was thawed and stored at 18°C for 4 h before each experiment.

Imaging the Initiation of Clotting of Blood Plasma. *Detecting clotting and fluorescent lipids with fluorescence microscopy.* Images were acquired using a Leica DMI 6000B epi-fluorescence microscope with a 10 × 0.4 *N.A.* objective coupled to a cooled CCD camera ORCA ERG 1394 (12-bit, 1344 × 1024 resolution) (Hamamatsu Photonics) with a 0.65x coupler. Lighting was provided by a 75W Xe light source. Three filter cubes were used: 1) DAPI/Hoechst/AMCA (λ_{ex} = 320-400 nm, λ_{em} = 435-495) (chroma no. 31000v2) to detect MCA, 2) Texas red (λ_{ex} = 530-590 nm, λ_{em} = 600-680) (chroma no. 41004) to detect the Texas red DHPE lipid dye, and 3) FITC/Bodipy/Fluo3/DiO (λ_{ex} = 455-505 nm, λ_{em} = 510-565) (chroma no. 41001) to detect the Oregon green DHPE lipid dye, NBD-DHPE lipid dye, and rhodamine 110. Bright field microscopy (illumination from halogen lamp) was also used to detect formation of fibrin during clotting (see Fig. 11 for an example). MetaMorph imaging system (Universal Imaging) was used to collect images. Images were processed using MetaMorph Imaging System and Adobe Photoshop. All image adjustments were applied uniformly to the entire image and to all sets of acquired images.

Analyzing images of initiation of clotting. The original grayscale fluorescence images of clotting and the phospholipid bilayers were false colored in MetaMorph. The color was set by the emission wavelength of the filter cube. For all

fluorescence images of clotting, the levels were adjusted to the same values. These images were copied and pasted directly from MetaMorph into a new Adobe Photoshop document set to RGB mode. In Adobe Photoshop, the blue fluorescence images from MCA and representative red fluorescence images of the lipid bilayers were overlaid by screening the red images. All transformations were applied uniformly to every image, and all images were processed in an identical fashion.

Additional Control Experiments

Establishing that Initiation of "Clotting" in the Chemical Model Was Due to Photo-Induced Acid Generation at the Patch Only. *Ruling out heating and photochemistry as sources of initiation of model reaction mixture.* To minimize heating of the photomask, we used short-pass and IR filters to remove light with $\lambda < 300$ nm and $\lambda > 400$ nm. Irradiation of a photomask with no open patches did not initiate the reaction, indicating that the reaction is not triggered by heating of the mask. Irradiation in the absence of 2-nitrobenzaldehyde did not initiate the reaction, indicating that photochemistry of the chemical model itself [Horvath AK, Nagypal I, Epstein IR (2002) *J Am Chem Soc* 124, 10956-10957] does not induce initiation under our conditions. In the absence of irradiation, the model reaction mixture was also stable for 500-1,200 s.

Establishing that acid generation is dependent on patch area. To measure the amount of acid produced by the acidic patches (H^+ production), the model system was replaced by a solution of an acid sensitive fluorescent dye, 5-(and 6)-carboxy-seminaphthofluorescein-1 (SNAFL) (see above for preparation of this solution). The H^+ production was measured for various arrays of acidic patches by measuring the fluorescence intensity of SNAFL (Fig. 9). The H^+ production was measured to establish that different arrays with the same total surface area, a, of acidic patches but different sizes of individual patches, p, produced approximately the same amount of acid. Each array had the same total surface area of patches ($a = 5.03 \times 10^5$ μm^2), and each array produced approximately the same amount of acid (within a factor of two). A single 800 μm patch ($a = 5.03 \times 10^5$ μm^2) produced H^+ at a rate of 2.9×10^{-2} nmol/s, an array of 4 × 400 μm patches ($a = 5.03 \times 10^5$ μm^2) produced 3.4×10^{-2} nmol/s, an array of 16 × 200 μm patches ($a = 5.03 \times 10^5$ μm^2) produced 2.6×10^{-2} nmol/s, and an array of 64 × 100 μm patches ($a = 5.03 \times 10^5$ μm^2) produced 1.7×10^{-2} nmol/s. A single 400 μm patch ($a = 1.26 \times 10^5$ μm^2) produced 7×10^{-3} nmol/s. The rates were determined by measuring the slopes of the H^+ production lines (Fig. 9). This single 400 μm patch had an area four times smaller than the $p \leq 200$ arrays and produced approximately four times less acid but was able to initiate "clotting" of the chemical model. The arrays of patches $p \leq 200$ did not initiate "clotting." These results support the argument that the threshold was determined not simply by the total amount of acid produced but also by the size of the patch producing acid.

Quantifying the Fluorescence Intensity Profile of a pH-Sensitive Dye in the Chemical Model on the Photoacid Surface. Initiation of "clotting" in the chemical model caused a change from basic to acidic conditions and the quenching of red

fluorescence from the dye bromophenol blue. For the model reaction mixture, the original grayscale time-lapse fluorescence images showed quenching of fluorescence (a shift from high fluorescence to low fluorescence) when "clotting" was initiated. The detailed procedure for image analysis used for images presented in the main text is described above. In Figs. 1-3 (main text), images of the chemical model were uniformly false-colored yellow and thresholded for dark objects. This procedure resulted in an inversion of light yellow and dark areas in all images. In these images, initiation of "clotting" appears yellow, whereas in the original images, initiation of "clotting" appears black (Fig. 10*A*). The adjustments to images in the main text were done to clarify comparison between initiation of the chemical model and initiation of blood plasma. We quantified the fluorescence intensity of the original (unmodified) images to determine "clot" time in all experiments with the chemical model (examples in Fig. 10). Line scans show that when "clotting" was initiated there was a dramatic decrease in fluorescence intensity. A single 400 μm patch initiated "clotting" in 22 sec (Fig. 10*A*). The "clot" propagated away from the patch as a reactive front, quenching the fluorescence as it propagated. An array of 200 μm patches did not initiate "clotting" within 220 sec (Fig. 10*B*). The increased intensity at the patch was due to a small amount of red and green light passing through the clear patch of photomask from the light source above (see schematic of model system in Fig. 5). The fluorescence intensity appeared lower at the edges of the images due to normal non-uniform illumination at the low magnification used to measure fluorescence (in contrast, UV illumination from the top of the sample was defocused to yield a uniform illumination area of ~6 mm in diameter). As a control experiment, a uniform solution of a fluorescent dye was imaged, and it showed the same degree of nonuniformity and decreased intensity at the edges.

Quantifying the Fluorescence Intensity Profile of a Thrombin-Sensitive Dye in Blood Plasma on Patterned Supported Phospholipid Bilayers. Initiation of clotting of blood plasma results in a burst of thrombin generation, accompanied by the onset of fibrin formation. To detect the initiation of clotting in blood plasma, we used both fluorescence microscopy to detect the thrombin-induced cleavage of a peptide-modified coumarin dye which releases 4-methyl-coumaryl-7-amide (MCA, blue fluorescence) (Fig. 11*H*), and bright-field microscopy to detect the formation of fibrin (Fig. 11*I*). For a 61 μm patch (Fig. 11 *A*–*E*), clotting of platelet rich plasma (PRP) did not initiate on the patch within 45 min. No large increase in fluorescence due to release of MCA by thrombin was observed (Fig. 11 *C* and *E*), and no formation of cross-linked fibrin strands or aggregation of platelets was observed (Fig. 11*D*). This general response was seen for all patches that did not initiate clotting. For a 137 μm patch (Fig. 11 *F*–*J*), the clotting of PRP initiated on the patch within 2 min. A large increase in fluorescence due to release of MCA by thrombin was observed. (Fig. 11 *H* and *J*). Formation of cross-linked fibrin strands and the aggregation of platelets were also observed (Fig. 11*I*). This general response was seen for all patches that initiated clotting.

In the arrays of patches presented in Fig. 2 *C* and *D* of the main text, the same general responses were observed (Fig. 12). For arrays of 50 μm patches, clotting of normal pooled plasma (NPP) did not initiate on the patch within 43 min. No large increase in fluorescence due to release of MCA by thrombin was observed (Fig. 12 *A*

and *B*), and no formation of cross-linked fibrin strands was observed. For arrays of 400 μm patches, clotting of NPP initiated on the patches within 3 min. A large increase in fluorescence due to release of MCA by thrombin was observed (Fig. 12 *C* and *D*). Formation of cross-linked fibrin strands was also observed.

Measuring and Eliminating Convective Flow in the Chamber Containing Blood Plasma. The flow inside the blood plasma chamber (Fig. 8) was measured by taking time-lapse fluorescent micrographs of fluorescent microspheres (FluoSpheres) in normal pooled blood plasma. The distances traveled by individual FluoSpheres were measured and divided by the elapsed time (see above for preparation of this solution). After the chamber was optimized to eliminate flow, the flow rate was typically less than 3 μm/min at 10 μm above the substrate and less than 10 μm/min at 100 μm above the substrate. A flow rate of 3 μm/min is ten times smaller than the rate of spreading of initiated clotting (25–35 μm/min).

Steps Taken to Eliminate Flow. The steps taken to eliminate flow included (*i*) using a sealed PDMS chamber to eliminate convective flow generated at the air/plasma interface (Marangoni flow) and evaporation, (*ii*) soaking the PDMS chamber in a solution of NaCl (150 mM) for 4–8 h to eliminate evaporation through the PDMS and to maintain a constant osmotic pressure, (*iii*) then soaking the chamber in a 1% BSA in PBS (pH 7.3) for 1 h to eliminate Marangoni flow generated at the PDMS/plasma interface due to possible gradients in surface tension, (*iv*) submerging the chamber in a solution of NaCl (150 mM) after plasma was sealed inside, (*v*) minimizing the amount of irradiation during microscopy, and (*vi*) minimizing stage movement.