METHODS

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, (Norwalk, CT). 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, 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).

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 prior to phlebotomy. Following centrifugation at 120g for 12 minutes to obtain platelet rich plasma, 2ml of platelet rich plasma was incubated with each vial of Fluo4-NW dye mixture reconstituted into 8 ml of buffer, for 30 minutes.

High throughput experimentation

An ‘agonist plate’ containing varying combinatorial concentrations of platelet agonists was prepared on a Perkin Elmer Janus (PerkinElmer Life and Analytical Sciences, Boston, MA) using 10x stock solutions of ADP, CVX, SFLLRN, AYPGKF and U46619. A separate ‘platelet plate’ containing dye loaded platelets was prepared on a Perkin Elmer Evolution. Final 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 second baseline read from columns of the ‘agonist plate’ onto the

corresponding columns of the 'platelet plate' on a Molecular Devices (Sunnyvale, CA) FlexStation III. Fluo4 fluorescence was measured at excitation 485nm and emission 535nm for 4 minutes 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 minutes.

Neural network model construction, training, and simulation. Neural network (NN) modeling and analysis was performed using the Neural Network Toolbox for MATLAB (The MathWorks™, Natick, MA). Training data consisted of: (1) the dynamic inputs, which represent the combination of agonist concentrations present at each time point for a particular experiment. Since the concentration of agonists remains essentially constant throughout each experiment, these values were generally a constant vector of concentration values repeated at 1-second intervals; (2) the dynamic outputs, which represent the experimentally measured calcium concentrations, also interpolated at 1-second intervals. To normalize the input data, agonist concentrations of 0, 0.1, 1, and 10 × EC50 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 400 possible 1- and 2-layer NNs with between 1 and 20 nodes in each processing, or "hidden", layer and testing each network for accuracy, a final NN topology with a 6-node input layer (representing the 6 agonists), 2 processing layers (8 nodes / 4 nodes), and a single-node output layer (representing the intracellular calcium concentration)[27] was most optimal and thus selected to predict successive time points from all 154 Ca^{2+} release curves gathered experimentally (**Fig. 2**). NARX (*N*onlinear *A*uto*R*egressive network with e*X*ogenous inputs) models are well-suited for predicting time series data[28] because they process inputs sequentially, i.e., at successive time points. Calcium outputs prior to the current instant were fed back to hidden layers utilizing a delay line spanning 128s. Initial states of the delay line were set to 0, corresponding to the steady state of the platelet preceding agonist stimulation. Such a structure allows the network output to progress over time,

utilizing the "memory" of the previous 128s in calculating the current output. Training was performed using Levenberg-Marquardt backpropagation until the performance of the model (mean squared error) did not improve more than 1×10^{-5}. It should be noted that each trained NN model produces a deterministic prediction of platelet activation. Experimental variations are inherent in replicates of donor specific training data (**Supplementary Fig. 9**), and the tightness of the measured mean will determine the predictive quality of such a donor specific NN model.

Busch *et al.*[29] 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 HGF treatment. Utilizing the NN weights for a signaling network, they define the modulation and control elements of the response[29]. Also, previous studies have used Partial Least Squares Regression Analysis (PLSR) to understand the interplay of molecular mechanisms during signaling [30, 31]. 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 ($\sim 10^2$) physiologically relevant conditions.