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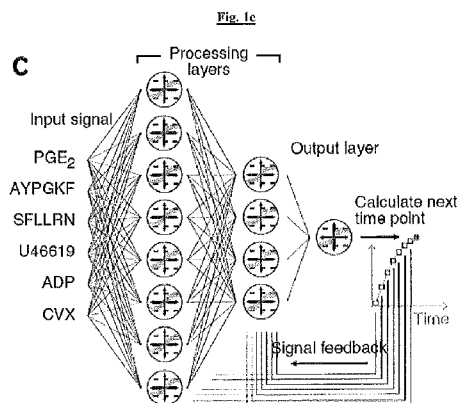
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(54) Title: METHODS FOR PREDICTING CELLULAR SIGNALING RESPONSES TO COMBINATORIAL STIMULI



(57) Abstract: The invention includes a method of predicting a signaling response of at least one cell to a combination of given stimuli, wherein the at least one cell comprises a primary cell from an individual. The method comprises contacting at least one cell with stimuli, and measuring an output response from the at least one cell to the contacting, to generate a cellular response learning set. A learning set is then used to generate a dynamic cellular signaling model to predict the signaling response of the at least one cell to the combination of the given stimuli. The cell is obtained from a biopsy or blood sample from a human mammal.

TITLE OF THE INVENTION

Methods for Predicting Cellular Signaling Responses to Combinatorial Stimuli

5

CROSS REFERENCE TO RELATED APPLICATIONS

The present application is entitled to priority under 35 U.S.C. § 119(e) to U.S. Provisional Applications No. 61/355,842, filed June 17, 2011, which application is hereby incorporated by reference herein in its entirety.

10

BACKGROUND OF THE INVENTION

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.

Because cells produce integrated responses to dose-dependent combinations of numerous external signals, efficient methods are needed to survey such high-dimensional systems. Predicting tissue function based upon an individual's unique cells requires a multiscale systems biology approach to understand the coupling of intracellular signaling with spatiotemporal gradients of extracellular biochemicals controlled by convective-diffusive transport. Primary human tissues such as blood, marrow or biopsies provide a limited number of cells, generally allowing only $\sim 10^2$ or fewer phenotypic tests. Evaluating the cellular 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.

For example, detailed phenotyping of platelets may be helpful to predict cardiovascular risk. Platelets are critical to human life through their maintenance of hemostatic integrity. 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 (1). Moreover, platelets are ideal "reduced" cellular systems for quantifying the effects of multiple signaling pathways because

they are anucleate, easily obtained from donors, and amenable to automated liquid handling.

During clotting, platelets experience diverse signaling cues simultaneously. Collagen activates glycoprotein VI (GPVI)-dependent tyrosine kinase signaling. ADP is released from dense granules to activate the G protein-coupled receptors P₂Y₁ and P₂Y₁₂. Thromboxane A₂ (TxA₂) is synthesized by platelet cyclooxygenase (COX1) and binds thromboxane-prostanoid (TP) receptors. Tissue factor at the damaged vasculature leads to the production of thrombin, which cleaves the protease-activated receptors PAR1 and PAR4. These activating signals occur in the context of inhibitory signals from endothelial nitric oxide and prostacyclin (PGI₂).

While these diverse signaling events occur simultaneously *in vivo* during thrombosis, most *in vitro* studies examine a single agonist in isolation. ADP (48), thrombin (49), and collagen (50) have been investigated in detail in the context of thrombosis, yet these studies do not capture the highly heterogeneous and dynamic thrombotic environment in flowing blood. Platelet signaling varies spatially and temporally in growing thrombi (2). Few experimental or computational tools are available for building a global understanding of how a cell integrates multiple stimuli present at varying levels.

In addition to platelet biochemistry, the overall function of blood at the molecular and cellular level is highly dependent upon hemodynamic forces. Examples include: shear induced platelet activation (SIPA) at $>5000\text{ s}^{-1}$ shear rate, requirement of von Willebrand Factor (vWF) in arterial thrombosis, shear effects on vWF structure and ADAMTS13 function and GPIb-vWF A1 domain bonding dynamics, RBC-dependent platelet migration toward the wall, and mass transfer to and from local zones of clotting or bleeding. Human blood function can be studied in the laboratory under flow conditions using aggregometry, cone-and-plate viscometry, capillaries, parallel plate chambers, and most recently microfluidic devices. Unfortunately, clinical laboratory assays such as aggregometry have chaotic “stir-bar” flows that have no *in vivo* correlate, while the Platelet Function Analyzer (PFA) requires relatively large blood samples and cannot be used in a high throughput mode for testing numerous clotting/bleeding scenarios. The need for advanced functional phenotyping motivates a combined experimental and computational approach to predict blood function under flow conditions.

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There is a need in the art to identify novel methods for predicting cellular signaling responses to combinatorial stimuli, especially when such stimuli may occur at distinct concentrations and/or at varying moments in time. Such methods would represent a novel and efficient approach for understanding how a cell integrates multiple signals. Such methods would also allow one to predict complex signal integration in a patient-specific disease milieu with respect to predicting disease progression or response to a therapeutic drug or treatment. The present invention fulfills this need.

BRIEF SUMMARY OF THE INVENTION

The invention includes a method of predicting a signaling response of at least one cell to a combination of given stimuli, wherein the at least one cell comprises a primary cell from an individual. The method comprises the step of providing the at least one cell. The method further comprises the step of contacting the at least one cell with a subset of the given stimuli, wherein the subset comprises at least two given stimuli. The method further comprises the step of measuring an output response from the at least one cell to the contacting, to generate a cellular response learning set. The method further comprises the step of using the learning set to generate a dynamic cellular signaling model. The method further comprises the step of using the signaling model to predict the signaling response of the at least one cell to the combination of the given stimuli.

In one embodiment, the cell is obtained from a biopsy of the individual. In another embodiment, the cell is obtained from a blood sample of the individual. In yet another embodiment, the individual is a mammal. In yet another embodiment, the mammal is a human.

In one embodiment, the given stimuli in the subset are provided in at least three distinct concentrations. In another embodiment, the at least three distinct concentrations comprise a value selected from the group consisting of a concentration below the EC_{50} of the stimulus in the cell, a concentration equal to about the EC_{50} of the stimulus in the cell, and a concentration above the EC_{50} of the stimulus in the cell. In yet another embodiment, the concentration below the EC_{50} of the stimulus in the cell is about $0.1 \times EC_{50}$. In yet another embodiment, the concentration above the EC_{50} of the stimulus in the cell is about $10 \times EC_{50}$.

In one embodiment, measuring the output response comprises using a high-throughput screening method. In another embodiment, generating the dynamic cellular signaling model comprises using a neural network model.

In one embodiment, the cell comprises a platelet. In another embodiment, at least one of the stimuli binds to a receptor selected from the group consisting of GPVI, PAR1, PAR4, P₂Y₁, P₂Y₁₂, TP, IP, EP, alpha2 receptor of epinephrine, histamine receptor, serotonin receptor, and nitric oxide receptor. In yet another embodiment, at least one of the stimuli is selected from the group consisting of iloprost, ADP, convulxin, U46619, SFLLRN (SEQ ID NO:3), AYPGKF (SEQ ID NO:4), PGE₂, a salt thereof, and a combination thereof. In yet another embodiment, the output response comprises a response selected from the group consisting of intracellular calcium release, cAMP level elevation or reduction, cGMP level elevation or reduction, phosphorylation or dephosphorylation of a protein or lipid, integrin activation, and granule release.

In one embodiment, the signaling response of the cell is used to predict disease progression in the individual. In another embodiment, the signaling response is used to predict response of the individual to a therapeutic drug or treatment. In yet another embodiment, the signaling response comprises sensitivity to IP receptor activation in the individual. In yet another embodiment, at least one of the stimuli is an agonist of the IP receptor. In yet another embodiment, the agonist is iloprost or a salt thereof.

In one embodiment, the signaling response predicts clotting under flow for the individual. In another embodiment, the signaling response predicts drug sensitivity under flow for the individual. In yet another embodiment, the signaling response identifies a defective cellular function or pathway related to a specific gene mutation in the individual. In yet another embodiment, the mutation occurs in a TP receptor. In yet another embodiment, identification of the specific gene mutation indicates that the individual is unresponsive to a therapeutic drug or therapy. In yet another embodiment, the therapeutic drug or therapy comprises indomethacin or a salt thereof. In yet another embodiment, the neural network is trained from the at least one cell obtained from the individual, and wherein the network is embedded in a larger calculational model that predicts cellular response as a function of space or time. In yet another embodiment, the neural network is used to predict disease

progression rates in the individual or used to predict responses to a therapeutic drug or treatment in the individual.

BRIEF DESCRIPTION OF THE DRAWINGS

5 For the purpose of illustrating the invention, there are depicted in the drawings certain embodiments of the invention. However, the invention is not limited to the precise arrangements and instrumentalities of the embodiments depicted in the drawings.

Figure 1, comprising Figures 1a-1c, illustrates experimental and
 10 computational methods to study platelet signaling. Figure 1a is a schematic illustration of signaling pathways in platelets, which converge on intracellular calcium release. Figure 1b is a schematic illustration of the high-throughput experimental procedure. An agonist plate containing combinatorial agonist combinations and a platelet plate containing dye-loaded platelets were separately assembled. Agonists
 15 were dispensed onto platelet suspensions and fluorescence changes were measured to quantify platelet calcium concentrations $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ transients may be represented as overlapping plots (lower right panel) or parallel heat maps (lower left panel). RFU, relative fluorescent units. Figure 1c is a schematic illustration of a dynamic neural network used to train platelet response to combinatorial agonist activation. A
 20 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 $[Ca^{2+}]_i$ at the next time point.

Figure 2, comprising Figures 2a-2b, illustrates the PAS methodology. Figure 2a is a schematic illustration of the experiment wherein all 154 binary
 25 combinations of the agonists CVX (convulxin, a snake venom that activates GPVI; alpha subunit: SEQ ID NO:1; beta subunit: SEQ ID NO:2), ADP, U46619, SFLLRN (a PAR1 activating peptide, SEQ ID NO:3), AYPGKF (a PAR4 activating peptide, SEQ ID NO:4) and PGE2 at concentrations of 0, 0.1, 1 and $10 \times EC_{50}$ were combined on the same plate (in replicates of 2) and the dynamic response of the platelet to each
 30 combination was recorded. The neural network model was trained on this dataset. Figure 2b is a schematic representation of pairwise agonist synergy scores. These 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. EC_{50} : 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, comprising Figures 3a-3d, illustrates how the neural network model reveals the global platelet response to all agonist combinations. Figure 3a is a schematic illustration of the measurement and prediction of the platelet response to all 64 ternary combinations of ADP, SFLLRN and CVX at 0, 0.1, 1 and $10 \times \text{EC}_{50}$. The neural network model was trained only on pairwise interactions but successfully predicted ternary interactions. The experimental curves are labeled "a." Figure 3b is a schematic illustration of the measurement and prediction of the platelet response to 45 predictions in the full combinatorial agonist space. Figure 3c is a schematic illustration of the predicted versus measured synergy scores for the 45 conditions in Figure 3b (upper left). Distribution of calculated 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 marked with "****" denotes the three most highly synergistic conditions, which all contained high U46619, no PGE_2 and low levels of other agonists. Figure 3d is a series of graphs illustrating measured and predicted platelet responses to sequential additions of ADP, SFLLRN and CVX.

Figure 4 is a set of plots illustrating donor-specific synergy maps. 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.

Figure 5, comprising Figures 5a-5b, is a set of graphs illustrating single agonist dose response curves. The curves were constructed for platelet responses to the agonists ADP, CVX, U46619, SFLLRN, AYPGKF and the antagonist PGE_2 . All conditions are for a single donor with 8 replicates per agonist dose. EC_{50} levels were determined by fitting a 4 parameter curve (dashed lines) to the peak calcium signal. EC_{50} levels were almost unchanged in 1.5 mM extracellular calcium (Figure 5a; with 15 μM indomethacin to prevent autocrine amplification via TXA_2) or 5 mM EDTA (Figure 5b). Values shown are mean $\pm$ standard deviation.

Figure 6, comprising Figures 6a-6b, is a set of bar graphs illustrating 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 (Figure 6a) the peak calcium signal or (Figure 6b) the integrated calcium signal was noted upon stimulation with either 0.1, 1, or 10x EC₅₀ levels of CVX, U46619, SFLLRN, AYPGKF or PGE₂. *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 Tables 1 and 2. Values shown are mean ± standard deviations. For Figures 6a-6b, each four-bar cluster represents, from left to right: no added inhibitors; 15 μM added indomethacin; 2 Units/mL added apyrase; both inhibitors added.

Figure 7 is a graph illustrating predicted versus measured synergy scores for the 135 conditions in a PAS experiment that contained binary agonist pairs (Figure 2). This fit was a measure of the “adequacy” of training of the NN on the binary interaction dataset. The strong correlation showed that the NN was suitably trained by the data, but does not yet demonstrate the predictive capability of the model.

Figure 8 is a graph illustrating predicted versus measured synergy scores for the 27 conditions in the ternary experiment (Figure 3a) 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.

Figure 9 is a graphical representation illustrating predicted distribution of synergies scores. 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 10, comprising Figures 10A-10B, is a series of graphs that illustrate the process of probing the complete agonist space. Experimentally observed mean (lines with error bars) and NN predicted (lines without error bars) [Ca²⁺]_i time courses for 45 out of the 4077 possibilities (illustrated in Figure 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.

Figure 11 is a graphical representation illustrating the effect of input silencing on predictive ability.

Figure 12, comprising Figures 12a-12b, illustrates sequential additions mimicking the *in-vivo* thrombotic environment. Figure 12a is a scheme illustrating conditions whereby 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. Figure 12b illustrates the results of mimicking these 4 conditions by making selective sequential additions (similar to Figure 3d) of the collagen analogue CVX, the physiological agonist thrombin as well as preassembled mixtures of SFLLRN and AYPGKF peptides all at 10x EC₅₀.

Figure 13, comprising Figures 13a-13b, is a series of graphs illustrating donor specific platelet agonist synergisms. PAS was performed twice for 10 healthy male donors to construct 135 parameter donor specific synergy maps.

Figure 14 is a graph illustrating experimental uncertainty in mean synergy score. 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 4 measurements of an experimental synergy score. Illustrated are the mean synergy scores $\pm$ 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.

Figure 15 is a graph illustrating the randomization of donor specific synergisms. The synergy scores for each donor (values in the columns of the synergy matrix shown in Figure 4) were randomized to test whether the clustering of the “observed” donor specific synergy vectors was significant. The experimentally observed clustering of 7 donor pairs (vertical line) out of 10 was highly significant. A P-Value less than 8×10^{-7} , under the null hypothesis that the donor clustering pattern was by chance alone, was obtained 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 16 is a graphical representation of platelet cell autonomous clustering pattern. Since all experiments were performed in diluted platelet rich plasma and not “pure” platelet suspensions, the possible dependence of the observed clustering patterns on platelet independent plasma components was evaluated. Two separate PAS scans of platelets from a donor were performed (separate from the donors reported elsewhere herein). In the first experiment for donor K (experiment K1), a PAS scan was performed identically to the procedure described herein using autologous plasma (as in Figure 2). 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 the manuscript). Both donor K experiments yielded very similar PAS scans, and both donor K experiments self clustered. Importantly K2 did not cluster with donor E replicates. This demonstrated that the PAS profiles were platelet cell autonomous.

Figure 17 is a series of graphs illustrating the probabilities of synergy scores at different agonist pair concentrations.

Figure 18 is a graphical illustration of synergism between thrombin and thromboxane.

Figure 19, comprising Figures 19a-19c, is a series of bar graphs illustrating the minimization of residual ternary synergy.

Figure 20, comprising Figures 20a-20b, is a series of graphs illustrating the use of system history in making output predictions.

Figure 21, comprising Figures 21a-21d, illustrates the multiscale model of combinatorial platelet activation and thrombus formation under flow. Platelet agonists used individually or in pairs to activate GPVI or G-protein coupled receptors (thromboxane receptor, TP; purinergic receptors P2Y₁ and P2Y₁₂; and the prostacyclin receptor, IP) resulted in modulation of intracellular calcium from intracellular stores distal of phospholipase C (PLC) activation or from store operated calcium entry via Stim1-Orai1 activation. Inhibitors such as acetylsalicylic acid (ASA) or indomethacin inhibited cyclooxygenase 1 (COX1), while indomethacin at high dose could inhibit nitric oxide synthase (NOS). Autocrine pathways include release of TXA₂ and ADP (Figure 21a). A 2-layer (8-node/4-node) neural network (NN) with feedback is trained with 74 measured calcium traces to predict $[Ca]_i$ for

each patient-specific platelet Pairwise Agonist Scan (PAS) (Figure 21b). The multiscale simulation of platelet deposition under flow requires simultaneous solution of the instantaneous velocity field over a complex and evolving platelet boundary $\Omega(t)$ by Lattice Boltzmann (LB), concentration fields of ADP and TXA₂ by finite element method (FEM), individual intracellular platelet state ($[Ca]_i$) and release reactions R for ADP and TXA₂ by NN, and all platelet positions and adhesion/detachment by lattice Kinetic Monte Carlo (LKMC) (Figures 21c-21d).

Figure 22, comprising Figures 22a-22c, illustrates pairwise agonist scanning (PAS) for platelet calcium mobilization for 3 separate donors. For donors 1 to 3, calcium traces were measured in the presence of low, medium, and high doses of ADP, U46619, convulxin (CVX) in the presence or absence of iloprost and simulated by NN (Figure 22a). The normalized synergy parameters ($-1 \leq S \leq 1$) for all measured pairs ($\pm$ iloprost) was calculated for each donor as well as for the donor-specific NN-simulated calcium responses under identical conditions (Figure 22b). Correlation between measured and NN-simulated synergy values were high correlated indicating successful training of the NN models (Figure 22c).

Figure 23, comprising Figures 23a-23b, is a series of graphs illustrating PAS calcium measurements and NN prediction of calcium measurements for Donor 1. NN prediction of calcium measurements was identical to measured values.

Figure 24 is a bar graph illustrating measured clot size for 3 donors at 200 s⁻¹ (5 min) and sensitivity to aspirin (ASA) or indomethacin (Indo) or P2Y₁ inhibitor MRS2179. Donor 1 made larger clots under flow conditions. Donor 3 was aspirin-insensitive compared to Donor 1 and 2.

Figure 25 is a series of graphs illustrating the finding that, in PAS scanning tests of calcium response with time, Donor 3 was not responsive to TP activation using increasing doses of U46619, consistent with a mutation in the TP receptor. Donor 1 displayed the largest response to U46619, consistent with larger measured clots. Donor 3 had normal TP receptor levels as measured by flow cytometry.

Figure 26 is a graph illustrating the finding that Donor 3 had a V221G mutation (heterozygote) in TP receptor. A heterozygous T→G mutation was found at position 11,921 in Exon 2 (NCBI ref# NG_013363.1). This mutation causes the codon change GTG to GGG, corresponding to a newly described Val221Gly mutation

associated with U46619-insensitivity and ASA/indomethacin resistance in Donor 3. Only 2 other mutations have been described for the TP receptor.

Figure 27 is an illustration of the multiscale simulation of patient-specific platelet deposition under flow using PAS/NN model of platelet calcium regulation. Platelet activation (white = 100% activated for $F(\xi) = 1$; black = 0 % activated for $F(\xi) = \alpha$) (inlet wall shear rate, 200 s^{-1}) in the presence of released ADP (top) and TXA_2 (middle) where local shear rate near the platelet deposit varies markedly from $< 50 \text{ s}^{-1}$ to greater than 1000 s^{-1} (bottom).

Figure 28, comprising Figures 28a-28b, is a series of graphs illustrating the multiscale simulation of clot size with time using PAS/NN platelet models for 3 separate donors for clotting of whole blood (PPACK-treated whole blood without pharmacological agent) or treated with indomethacin or iloprost. Donor 1 was predicted to generate the largest clots. Donor 3 was predicted to be unresponsive to indomethacin. In experimental measurements, Donor 1 produced the largest clots under flow and Donor 3 was unresponsive to high dose indomethacin.

DETAILED DESCRIPTION OF THE INVENTION

This invention includes the unexpected discovery of a novel method, named "pairwise agonist scanning" (PAS), which allows the generation of straightforward and effective predictions about cellular states. In a non-limiting aspect, PAS may provide data for training a neural network to make predictions about cellular states.

If one wants to determine how a cell behaves towards a collection of stimuli, the testing of all stimuli at all doses and combinations against the cell would require tens of thousands or millions of individual tests. PAS greatly reduces the number of tests needed and may make accurate predictions of cellular response to all combinations of stimuli. PAS may thus be used to understand how cells respond to multiple physiological and drug "stimuli". In one embodiment, PAS may be used to predict how cancer cells respond to multiple drug regimen or how blood responds to multiple drug regimen. In another embodiment, PAS may be applied to primary human cells obtained fresh from the human body (blood draw, biopsy) for making patient-specific predictions of disease risk, progression, or response to pharmacological intervention. The neural network model of a patient's cells may be

embedded into large scale models of disease (a heart attack or stroke, for example),
allowing for more efficient and rational intervention in the disease state of the patient.
In one aspect, training neural networks with pairs of stimuli across the dose-response
regime represents an efficient approach for predicting complex signal integration in a
5 patient-specific disease milieu.

Definitions

As used herein, each of the following terms has the meaning associated
with it in this section.

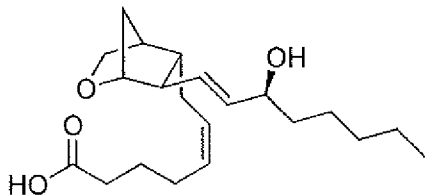
10 Unless defined otherwise, all technical and scientific terms used herein
generally have the same meaning as commonly understood by one of ordinary skill in
the art to which this invention belongs. Generally, the nomenclature used herein and
the laboratory procedures in biochemistry, analytical chemistry and organic chemistry
are those well-known and commonly employed in the art. Standard techniques or
15 modifications thereof are used for chemical syntheses and chemical analyses.

The articles “a” and “an” are used herein to refer to one or to more
than one (i.e. to at least one) of the grammatical object of the article. By way of
example, “an element” means one element or more than one element.

As used herein, the term “about” will be understood by persons of
20 ordinary skill in the art and will vary to some extent on the context in which it is used.
As used herein when referring to a measurable value such as an amount, a temporal
duration, and the like, the term “about” is meant to encompass variations of $\pm 20\%$ or
 $\pm 10\%$, more preferably $\pm 5\%$, even more preferably $\pm 1\%$, and still more preferably
 $\pm 0.1\%$ from the specified value, as such variations are appropriate to perform the
25 disclosed methods.

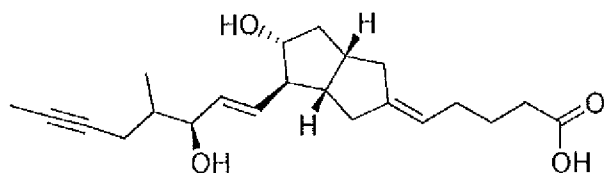
As used herein, the term “PAS” refers to pairwise agonist scanning.

As used herein, the term “U46619” refers to (Z)-7-[(1*S*,4*R*,5*R*,6*S*)-5-
[(*E*,3*S*)-3-hydroxyoct-1-enyl]-3-oxabicyclo[2.2.1]heptan-6-yl]hept-5-enoic acid or a
salt thereof:

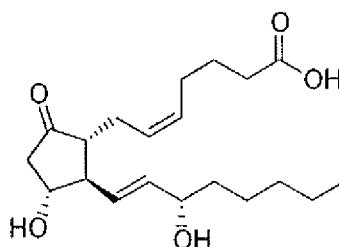


30

As used herein, the term “iloprost” refers to 5-[(E)-(1S,5S,6R,7R)-7-hydroxy-6[(E)-(3S, 4RS)-3-hydroxy-4-methyl-1-octen-6-ynyl]-bi-cyclo[3.3.0]octan-3-ylidene]pentanoic acid or a salt thereof:

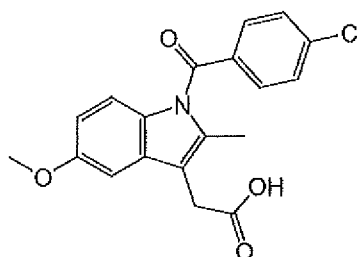


5 As used herein, the term “PGE₂” refers to 7-[3-hydroxy-2-(3-hydroxyoct-1-enyl)-5-oxo-cyclopentyl] hept-5-enoic acid or a salt thereof:



As used herein, the term “indomethacin” or “indomethacin” refers to 2-{1-[(4-chlorophenyl)carbonyl]-5-methoxy-2-methyl-1H-indol-3-yl}acetic acid or a salt thereof:

10



As used herein, the term “GPVI” refers to glycoprotein VI.

As used herein, the term “PAR1” refers to proteinase-activated receptor 1 or coagulation factor II (thrombin) receptor.

15 As used herein, the term “PAR4” refers to protease-activated receptor 4 (PAR4), or coagulation factor II (thrombin) receptor-like 4.

As used herein, the term “TP” refers to thromboxane receptor.

As used herein, the term “IP” refers to prostacyclin receptor, or prostaglandin I₂ receptor.

20 As used herein, the term “EP” refers to prostaglandin E₂ receptor.

As used herein, the term “P₂Y₁” receptor refers to P2Y purinoceptor 1.

As used herein, the term "P₂Y₁₂" refers to an ADP chemoreceptor, preferentially, purinergic receptor P₂Y, G-protein coupled, ₁₂.

As used herein, the term "ADP" refers to adenosine diphosphate or a salt thereof.

5 As used herein, the term "convulxin" refers a snake venom toxin ,made up of an α -subunit made of 142 amino acids (SEQ ID NO:1) and a β -subunit made of 123 amino acids (SEQ ID NO:2). It is a tetramer with an oligomeric structure (84).

As used herein, the term "RFU" refers to relative fluorescent units.

10 An "amino acid" as used herein is meant to include both natural and synthetic amino acids, and both D and L amino acids. "Standard amino acid" means any of the twenty L-amino acids commonly found in naturally occurring peptides. A "nonstandard" or "synthetic" amino acid residue means any amino acid, other than the standard amino acids, regardless of whether it is prepared synthetically or derived from a natural source.

15 As used herein, the terms "protein", "peptide" and "polypeptide" are used interchangeably, and refer to a compound comprised of amino acid residues covalently linked by peptide bonds. The term "peptide bond" means a covalent amide linkage formed by loss of a molecule of water between the carboxyl group of one amino acid and the amino group of a second amino acid. A protein or peptide must
20 contain at least two amino acids, and no limitation is placed on the maximum number of amino acids that may comprise the sequence of a protein or peptide. Polypeptides include any peptide or protein comprising two or more amino acids joined to each other by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for
25 example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types. "Proteins" include, for example, biologically active fragments, substantially homologous proteins, oligopeptides, homodimers, heterodimers, protein variants, modified proteins, derivatives, analogs, and fusion proteins, among others. The proteins include natural proteins, recombinant proteins,
30 synthetic proteins, or a combination thereof. A protein may be a receptor or a non-receptor.

By "nucleic acid" is meant any nucleic acid, whether composed of deoxyribonucleosides or ribonucleosides, and whether composed of phosphodiester linkages or modified linkages such as phosphotriester, phosphoramidate, siloxane,

carbonate, carboxymethylester, acetamdate, carbamate, thioether, bridged phosphoramidate, bridged methylene phosphonate, phosphorothioate, methylphosphonate, phosphorodithioate, bridged phosphorothioate or sulfone linkages, and combinations of such linkages. The term nucleic acid also specifically includes nucleic acids composed of bases other than the five biologically occurring bases (adenine, guanine, thymine, cytosine and uracil). The term "nucleic acid" typically refers to large polynucleotides. The term "DNA" as used herein is defined as deoxyribonucleic acid. The term "RNA" as used herein is defined as ribonucleic acid. The term "recombinant DNA" as used herein is defined as DNA produced by joining pieces of DNA from different sources.

By the term "specifically binds," as used herein, is meant a molecule, such as an antibody or a small molecule, which recognizes and binds to another molecule or feature, but does not substantially recognize or bind other molecules or features in a sample.

The phrase "inhibit," as used herein, means to reduce a molecule, a reaction, an interaction, a gene, an mRNA, and/or a protein's expression, stability, function or activity by a measurable amount or to prevent entirely. Inhibitors are compounds that, e.g., bind to, partially or totally block stimulation, decrease, prevent, delay activation, inactivate, desensitize, or down regulate a protein, a gene, and an mRNA stability, expression, function and activity, e.g., antagonists.

"Effective amount" or "therapeutically effective amount" are used interchangeably herein, and refer to an amount of a compound, formulation, material, or composition, as described herein effective to achieve a particular biological result. Such results may include, but are not limited to, the treatment of a disease or condition as determined by any means suitable in the art.

An "individual", "patient" or "subject", as that term is used herein, includes a member of any animal species including, but are not limited to, birds, humans and other primates, and other mammals including commercially relevant mammals such as cattle, pigs, horses, sheep, cats, and dogs. Preferably, the subject is a human.

The term "treat" or "treating", as used herein, means reducing the frequency with which symptoms are experienced by a subject or administering an agent or compound to reduce the frequency and/or severity with which symptoms are experienced. As used herein, "alleviate" is used interchangeably with the term

“treat.” Treating a disease, disorder or condition may or may not include complete eradication or elimination of the symptom. The term “therapeutic” as used herein means a treatment and/or prophylaxis.

As used herein, the term “container” includes any receptacle for
5 holding the pharmaceutical composition. For example, in one embodiment, the container is the packaging that contains the pharmaceutical composition. In other embodiments, the container is not the packaging that contains the pharmaceutical composition, i.e., the container is a receptacle, such as a box or vial that contains the packaged pharmaceutical composition or unpackaged pharmaceutical composition
10 and the instructions for use of the pharmaceutical composition. Moreover, packaging techniques are well known in the art. It should be understood that the instructions for use of the pharmaceutical composition may be contained on the packaging containing the pharmaceutical composition, and as such the instructions form an increased functional relationship to the packaged product. However, it should be understood
15 that the instructions may contain information pertaining to the compound’s ability to perform its intended function, e.g., treating or preventing a disease in a subject.

“Instructional material,” as that term is used herein, includes a publication, a recording, a diagram, or any other medium of expression which can be used to communicate the usefulness of the composition and/or compound of the
20 invention in a kit. The instructional material of the kit may, for example, be affixed to a container that contains the compound and/or composition of the invention or be shipped together with a container which contains the compound and/or composition. Alternatively, the instructional material may be shipped separately from the container with the intention that the recipient uses the instructional material and the compound
25 cooperatively. Delivery of the instructional material may be, for example, by physical delivery of the publication or other medium of expression communicating the usefulness of the kit, or may alternatively be achieved by electronic transmission, for example by means of a computer, such as by electronic mail, or download from a website.

30 Throughout this disclosure, various aspects of the invention can be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible sub-ranges

as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed sub-ranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 2.7, 3, 4, 5, 5.3, and 6. This applies regardless of the breadth of the range.

Description

To predict cellular responses to multiple stimuli, pairwise agonist scanning (PAS) was developed (Figure 1). This strategy comprises selecting stimuli molecules based on prior knowledge (Figure 1a), measuring cellular responses to all pairwise combinations of stimuli in a high-throughput manner (Figure 1b), and then training a two-layer, nonlinear, autoregressive neural network with the cellular responses to exogenous inputs (Figure 1c). Neural networks are remarkable in learning patterns of inputs and predicting outputs by optimizing intermediate connection weights, akin to a platelet's ability to respond to multiple thrombotic signals through coupled biochemical reactions.

"Neural networks" and "artificial neural networks" are defined as equivalent identities. Neural networks can be formulated with a wide variety of choices of processing layers, number of nodes per processing layer, connectivity among nodes, feedback between nodes, and choice of response function for each node. Data for training of neural networks can be normalized between specific ranges (for example, 0 to 1, -1 to +1, etc) or unnormalized.

In one aspect, a living cell is essentially a neural network which connection weights have been selectively adjusted during evolution (3). A "top-down" approach (4) was thus adopted to model platelet signaling. The application of neural networks for predicting dynamic cellular signaling is beneficial because neural networks are "dense" modeling structures — meaning that they do not require detailed knowledge of the kinetic structure of a system. By comparison, an ordinary differential equation model of ADP-stimulated calcium mobilization through P_2Y_1 required almost 80 reactions and over 100 kinetic parameters to describe just this one single pathway (5). As a non-limiting example, an ordinary differential equation model that describes the signaling mechanisms of the six agonists (Figure 1a) in this study on a similar level of detail could require >500 parameters, many of which are currently unavailable.

Platelet Signaling and PAS Scanning

Excessive thrombus formation following plaque rupture remains difficult to predict and can be linked to hyperactive platelet function. Inter-individual
5 variation in platelet reactivity, even in a healthy population, has been associated with a number of factors including: female gender, fibrinogen level, ethnicity, inherited variations, and polymorphisms. Similarly, platelet dysfunction or anti-platelet therapy can be associated with bleeding risks. However, defining, quantifying, and linking a patient's unique platelet phenotype and coagulation or bleeding phenotype to clinical
10 risk remains a diagnostic challenge.

In the presence of multiple activating stimuli, platelet signal transduction results in calcium mobilization, leading to inside-out signaling, including dense granule release (of ADP, ATP, serotonin, epinephrine), alpha granule release (of von Willebrand factor and thrombospondin), phospholipase A₂/(COX-1)-
15 dependent thromboxane synthesis, integrin activation, and shape change (Figure 21a). The present studies indicate an efficient way to study this combinatorial signaling by testing pairs of agonists at low, medium, and high concentrations. Then, neural network models (NN) can be trained that interpolate between the tested doses and extrapolate to predict responses to ternary stimulation, sequential exposures, and
20 exposure to up to 6 simultaneous agonists. The NN model of calcium regulation (Figure 21B) is ideal for use with multi-scale models (Figures 21c-d) where platelets convect through boundary layers of ADP and TXA₂ while depositing on collagen or on other platelets where they are exposed to high concentrations of stimulating molecules.

25 In addition to platelet biochemistry, the overall function of blood at the molecular and cellular level is highly dependent upon hemodynamic forces. Examples include: shear induced platelet activation (SIPA) at $> 5000 \text{ s}^{-1}$ shear rate, requirement of von Willebrand Factor (vWF) in arterial thrombosis, shear effects on vWF structure and ADAMTS13 function and GPIb-vWF A1 domain bonding
30 dynamics, RBC-dependent platelet migration toward the wall, and mass transfer to and from local zones of clotting or bleeding. Human blood function can be studied in the laboratory under flow conditions using aggregometry, cone-and-plate viscometry, capillaries, parallel plate chambers, and most recently microfluidic devices. Unfortunately, clinical laboratory assays such as aggregometry have chaotic "stir-bar"

flows that have no *in vivo* correlate, while the Platelet Function Analyzer (PFA) requires relatively large blood samples and cannot be used in a high throughput mode for testing numerous clotting/bleeding scenarios. The need for advanced functional phenotyping motivates a combined experimental and computational approach to
5 predict blood function under flow conditions.

Platelet Function and Flow

During thrombotic or hemostatic episodes, platelets bind collagen and release ADP and thromboxane- A_2 (TXA $_2$) to facilitate the recruitment of additional
10 platelets to a growing deposit that distorts the flow field. To develop an integrated view of platelet and blood function in response to multiple stimuli encountered during thrombosis and hemostasis, in the experiments described herein PAS was employed to measure calcium mobilization in platelets obtained from 3 individuals. Simultaneously, whole blood from each of these 3 individuals was perfused over
15 collagen using microfluidic devices where the blood was pre-treated with inhibitors of cyclooxygenase (indomethacin or aspirin), P $_2$ Y $_1$ (MRS-2179), or IP receptor activator (iloprost). Neural network models of platelet signaling in response to any time-dependent exposure to collagen, ADP, and thromboxane A_2 were embedded in a multiscale lattice kinetic Monte Carlo (LKMC) model of platelet deposition where
20 soluble species were computed by finite element method (FEM) and the instantaneous flow field was computed by Lattice Boltzmann (LB) calculation (Figure 21D). The results of these experiments indicated that *in silico* representations of an individual's platelet phenotype allows prediction of blood function, essential to prioritizing patient-specific cardiovascular risk and drug response or to identify unsuspected gene
25 mutations.

Methods of the Invention

The invention includes a method of predicting a signaling response of at least one cell to a combination of given stimuli, wherein the at least one cell
30 comprises a primary cell from an individual. The method comprises the step of providing the at least one cell. The method further comprises the step of contacting the at least one cell with a subset of the given stimuli, wherein the subset comprises at least two given stimuli. The method further comprises the step of measuring an output response from the at least one cell to the contacting, to generate a cellular

response learning set. The method further comprises the step of using the learning set to generate a dynamic cellular signaling model. The method further comprises the step of using the signaling model to predict the signaling response of the at least one cell to the combination of the given stimuli.

5 In one embodiment, the at least one cell comprises a single cell, such as but not limited to a platelet. In another embodiment, the at least one cell comprises one or more cells belonging to the same cell type, such as but not limited to platelets. In yet another embodiment, the at least one cell comprises one or more cells belonging to one or more distinct cell types, such as but not limited to whole blood
10 cells, comprising white cells, red cells and platelets.

 In one embodiment, the cell is obtained from a biopsy of the individual. In another embodiment, the cell is obtained from a blood sample of the individual. In yet another embodiment, the individual is a mammal. In yet another embodiment, the mammal is a human.

15 In one embodiment, the given stimuli in the subset are provided in at least three distinct concentrations. In another embodiment, the at least three distinct concentrations comprise a value selected from the group consisting of a concentration below the EC_{50} of the stimulus in the cell, a concentration equal to about the EC_{50} of the stimulus in the cell, and a concentration above the EC_{50} of the stimulus in the cell.
20 In yet another embodiment, the concentration below the EC_{50} of the stimulus in the cell is about $0.1 \times EC_{50}$. In yet another embodiment, the concentration above the EC_{50} of the stimulus in the cell is about $10 \times EC_{50}$.

 In one embodiment, measuring the output response comprises using a high-through screening method. In another embodiment, generating the dynamic
25 cellular signaling model comprises using a neural network model.

 In one embodiment, the cell comprises a platelet. In another embodiment, at least one of the stimuli binds to a receptor selected from the group consisting of GPVI, PAR1, PAR4, P_2Y_1 , P_2Y_{12} , TP, IP, EP, alpha2 receptor of epinephrine, histamine receptor, serotonin receptor, and nitric oxide receptor. In yet
30 another embodiment, at least one of the stimuli is selected from the group consisting of iloprost, ADP, convulxin, U46619, SFLLRN (SEQ ID NO:3), AYPGKF (SEQ ID NO:4), PGE_2 , a salt thereof, and a combination thereof. In yet another embodiment, the output response comprises a response selected from the group consisting of intracellular calcium release, cAMP level elevation or reduction, cGMP level

elevation or reduction, phosphorylation or dephosphorylation of a protein or a lipid, integrin activation, and granule release.

In one embodiment, the signaling response of the cell is used to predict disease progression in the individual. In another embodiment, the signaling response is used to predict response of the individual to a therapeutic drug or treatment. In yet another embodiment, the signaling response comprises sensitivity to IP receptor activation in the individual. In yet another embodiment, at least one of the stimuli is an agonist of the IP receptor. In yet another embodiment, the agonist is iloprost or a salt thereof. In yet another embodiment, the signaling response predicts clotting under flow for the individual. In yet another embodiment, the signaling response predicts drug sensitivity under flow for the individual. In yet another embodiment, the signaling response identifies a defective cellular function or pathway related to a specific gene mutation in the individual. In yet another embodiment, the mutation occurs in a TP receptor. In another embodiment, identification of the specific gene mutation indicates that the individual is unresponsive to a therapeutic drug or therapy. In yet another embodiment, the therapeutic drug or therapy comprises indomethacin or a salt thereof. In yet another embodiment, the neural network is trained from the at least one cell obtained from the individual. In yet another embodiment, the neural network is embedded in a larger calculational model predicting cellular response as a function of space or time. In yet another embodiment, the neural network is used to predict disease progression rates in the individual or used to predict responses to a therapeutic drug or treatment in the individual.

The present invention includes a neural engine for use in generating a cellular response learning set. As used herein, the term "neural network" or "neural engine" or "artificial neural network" refers to a numerical or mathematical engine or network having resident therein a plurality of rules, wherein, upon access to information to which such rules may be applied, the rules may be updated, modified, or varied as to which next set of those rules is applied. A neural engine or network would include, in brief, computer models, algorithms, comparisons, calculations and the like designed to simulate the behavior of human reasoning and learning, but additionally capable of such simulation with data quantities, breadth of calculations and the like that are beyond human capabilities, such as in pattern recognition, data accumulation, language processing, and comparative problem solving. The goal of neural networking is learning by the network, such that self-directed information

processing may occur. Such an engine may be, for example, a business rules engine that is associated with one or more processors, which may be resident locally and/or at one or more servers. General requirements for construction of generic architectures for neural engines and/or networks as understood by those skilled in the art may be
5 used in conjunction with the present invention to perform the novel functionalities as describe hereinthroughout.

Kits of the Invention

The invention also includes a kit comprising reagents and/or materials
10 useful within the methods of the invention and an instructional material that describes, for instance, screening a cell according to the methods described elsewhere herein. In an embodiment, the kit further comprises a device that may be used to remove the cell from the individual under testing. In another embodiment, the kit further includes software for implementing calculations within the methods of the invention on a local
15 computer. In yet another embodiment, the kit further includes instructions for using a web platform to remotely run calculations within the methods of the invention.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, numerous equivalents to the specific procedures, embodiments, claims, and examples described herein. Such equivalents were
20 considered to be within the scope of this invention and covered by the claims appended hereto. For example, it should be understood, that modifications in reaction conditions, including but not limited to reaction times, reaction size/volume, and experimental reagents, such as solvents, catalysts, pressures, atmospheric conditions, e.g., nitrogen atmosphere, and reducing/oxidizing agents, with art-recognized
25 alternatives and using no more than routine experimentation, are within the scope of the present application.

It is to be understood that wherever values and ranges are provided herein, all values and ranges encompassed by these values and ranges, are meant to be encompassed within the scope of the present invention. Moreover, all values that fall
30 within these ranges, as well as the upper or lower limits of a range of values, are also contemplated by the present application.

The following examples further illustrate aspects of the present invention. However, they are in no way a limitation of the teachings or disclosure of the present invention as set forth herein.

EXAMPLES

The invention is now described with reference to the following Examples. These Examples are provided for the purpose of illustration only, and the invention is not limited to these Examples, but rather encompasses all variations that are evident as a result of the teachings provided herein.

The materials and methods employed in the experiments and the results of the experiments presented in this Example are now described.

10 Materials

PAR1-agonist peptide SFLLRN (thrombin receptor agonist peptide, TRAP, SEQ ID NO:3) and the PAR4-agonist peptide AYPGKF (SEQ ID NO:4) were obtained from Bachem (King of Prussia, PA, USA). Convulxin (CVX, SEQ ID NO:1 and SEQ ID NO:2) was obtained from Centerchem (Norwalk, CT, USA). Thrombin (SEQ ID NO:5) and GGACK (SEQ ID NO:6) were obtained from Haematologic Technologies (Essex Junction, VT, USA).

Clear, flat-bottom, black 384-well plates were obtained from Corning (Corning, NY, USA). ADP, U46619, PGE₂, EDTA, HEPES, the fibrin polymerization inhibitor Gly-Pro-Arg-Pro (GPRP, SEQ ID NO:7), NaCl, NaOH, apyrase, indomethacin and sodium citrate were all from Sigma (St. Louis, MO, USA). Fluo-4 NW Calcium assay kits were obtained from Invitrogen (Carlsbad, CA, USA). 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).

25 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 stated not to have taken any medications for the past 10 days and not to have consumed 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.

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. 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.

Agonist Selection

The number of agonists tested in a PAS experiment is limited to six by the need of testing all the 154 conditions in duplicate in a single 384-well plate. Agonists were selected to be representative of physiological signaling cascades.

Convulxin is a selective GPVI activator (11A) and under static conditions this receptor is the predominant determinant of collagen-induced signal strength (20A).

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 (21A, 22S). “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 a minor effect on calcium mobilization (23), allowing the use of 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 (24,25). This prompted the use of selective PAR agonist peptides (SFLLRN – SEQ ID NO:3 and

AYPGKF – SEQ ID NO:4) to distinguish the separate signal contribution of both these receptor pathways. Moreover, thrombin stimulation of unwashed PRP requires inhibition of fibrin and coagulation factor Xa (FXa) formation (Figure 18). Washing or gel-filtering platelets are processing steps that decrease throughput in a large-scale experiment and often cause residual platelet activation in the absence of PGE₂ or other PGI₂ analogs. The use of a short-lived prostaglandin like PGI₂ (26A) 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 the use of PGE₂ as an agonist causing elevation in intracellular cAMP.

Similarly, for reasons of stability during the course of the experiment, the thromboxane analog U46619 was used instead of its physiological equivalent TxA₂ (27A).

Definition of Synergy Score

To quantify cross-talk 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 (28). The use of a ratio rather than a difference is prone to numerical errors for small values of the predicted additive response.

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) and (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 $10 \times \text{EC}_{50}$ 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) (29A) was most optimal (best predicted the ‘net’ output response $[\text{Ca}^{2+}]_i$ 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 2). For the sake of simplicity and because reasonably accurate time series predictions are already obtained for $[\text{Ca}^{2+}]_i$, more processing layers or >20 neurons in each layer were not tested. 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 1c) does not correspond to an actual signaling network (Figure 1a) 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 (30) 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 become better than $>1 \times 10^{-5}$. 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 I of the concentrations of the six inputs species can be compactly described by the following formula:

$$y(t) = f \left[\begin{array}{c} \overline{L3}_{(1 \times 4)} \times f \left[\overline{H2}_{(4 \times 8)} \times \overline{y_h}_{(8 \times 1)} + \overline{L2}_{(4 \times 8)} \times f \left[\overline{H1}_{(8 \times 8)} \times \overline{y_h}_{(8 \times 1)} + \overline{IW}_{(8 \times 6)} \times \overline{I}_{(6 \times 1)} + \overline{b1}_{(8 \times 1)} \right] + \overline{b2}_{(4 \times 1)} + \overline{b3}_{(1 \times 1)} \right] \end{array} \right]$$

(4×1) (1×1)

where IW is the matrix of input weights, $L2$ and $L3$ are the weight matrices that operate on the ‘inputs’ coming from the first and second processing layers respectively. $H1$ and $H2$ are matrices that contain history coefficients that weigh the history vector yh (containing the output of the system 1, 2, 4, 8, 16, 32, 64 and 128 s prior to the current instant). $bb12$, and $b3$ 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 14), and the tightness of the measured mean will determine the predictive quality of such a donor-specific neural network model.

The fold-expression kinetics of nine “top-ranked” genes involved in the sustained migration of keratinocytes after hepatocyte growth factor (HGF) treatment has been described by means of a continuous-time recurrent neural network, and the neural network weights were used to define the modulation and control elements of the response (31A). Also, previous studies have used partial least-squares regression analysis (PLSR) to understand the interplay of molecular mechanisms during signaling (32A, 33A). 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-h experiment) and efficient prediction of dynamic input-output relationships at numerous ($\sim 10^2$) physiologically relevant conditions.

Blood Collection

In experiments requiring extracellular calcium, the blood was drawn from healthy volunteers who self-reported as being free of any medication into H-D-PhePro-Arg-chloromethylketone (100 μ M PPACK final concentration, Calbiochem) to inhibit thrombin production. A 15 mL blood sample was divided for simultaneous microfluidic studies of platelet deposition under flow and for preparation of 4 mL of platelet rich plasma (PRP) for platelet calcium studies. All volunteers provided informed consent in accordance with IRB approval and the Declaration of Helsinki.

10 Pairwise Agonist Scanning (PAS) of Platelet Calcium Signaling

Diluted PPACK-treated PRP (final concentration in assay, 12 %) was treated with indomethacin (28 μ M) to eliminate autocrine signaling via thromboxane and loaded with Fluo4NW dye (Life Technology) for 30 min prior to activation in a 384-well plate assay with all pairwise mixtures of ADP, U46619, and convulxin using a FlexStation III (Molecular Devices) at final concentrations of 0, 0.1, 1, and 10 x EC_{50} levels (EC_{50} : ADP, 1 μ M, U46619, 1 μ M; convulxin, 5 nM). All wells contained normal extracellular calcium. This results in 74 traces (averaged from 4 replicates of each condition) for neural network (NN) training [(3 pairs x 9 conditions x 2 ($\pm$ iloprost), 3 single agonists x 3 conditions x 2 ($\pm$ iloprost), 2 null conditions ($\pm$ iloprost)], as described previously (72) using the Neural Network Toolbox for MATLAB. Input concentrations were mapped onto the values -1, -0.333, +0.333 and +1 corresponding to 0, 0.1, 1 and 10x EC_{50} levels of each input. Output concentrations were mapped between 0 (resting calcium levels) and 1 (maximal response). The structure of the NARX (Nonlinear AutoRegressive network with eXogenous inputs) model had 2 processing layers having 8 and 4 hyperbolic tangent transfer function nodes, and a tapped delay line with 128 s of feedback to each layer (Figure 21b), as previously described (51). Normalized pairwise synergy between agonists ("A" and "B" and their combined use "AB") was calculated from experimental data or NN simulations of integrated calcium curves by:

$$S_{AB} = \frac{\int AB - \left(\int A + \int B \right)}{\max \left[\int AB - \left(\int A + \int B \right) \right]} \quad (1)$$

30 *Pairwise Synergy Score,*

Microfluidic Phenotyping of Platelets

Microfluidic devices were fabricated in poly(dimethyl)siloxane as previously described (51) with 8 parallel channels (250 μm wide by 60 μm high) that converge to a common outlet. The channels run perpendicularly over a 250 μm wide stripe of pre-patterned equine fibrillar collagen type 1 (Chronopar, Chronolog), allowing 8 separate platelet deposition events per device to be imaged simultaneously by epifluorescence microscopy (4X; 520 nm EX/700 nm EM) every 15 sec (ORCA-ER CCD camera, Hamamatsu).

Prior to blood perfusion, channels were blocked with 0.5% bovine serum albumin (BSA) in HEPES buffered saline (HBS, 20 mM HEPES, 160 mM NaCl, pH 7.5) for 30 min. Blood was treated with vehicle (0.01% DMSO final concentration) or indicated concentrations of indomethacin (Tocris Bioscience) or acetyl salicylic acid (Sigma, St. Louis, MO, USA) 40 min prior to perfusion or with iloprost or MRS 2179 (Tocris Bioscience) for 5 min prior to perfusion. All samples were treated with a 1:50 by vol. Alexa-conjugated anti-CD41 (Clone PM6/248, AbD Serotec, Raleigh, NC, USA) for 3 min prior to perfusion. Samples were perfused at a wall shear rate of 200 s^{-1} (2 $\mu\text{L}/\text{min}$ per channel; PHD2000 Harvard Syringe pump) for 8 min.

Multiscale Simulation of Platelet Deposition under Flow

For a 2D rectangular domain with depositing platelets, the blood velocity field at any instant was solved by LB for constant parabolic inlet flow rate, Newtonian blood viscosity (3 cP), no-slip boundary condition, and constant pressure at the outlet. A D2Q9 lattice was employed with Zou-He boundary conditions (73) and with the same lattice spacing as the LKMC ($h_{\text{LKMC}} = h_{\text{LB}}$).

To follow platelet positions in the blood flow and adhesive interactions with the surface (250 μm long patch of collagen at $t \geq 0$), the rates, Γ , of all possible transport events for a platelet (radius, R) on a lattice spacing h_{LKMC} were calculated including:

$$\text{platelet diffusion, } \Gamma_D = D_{\text{platelet}}/h_{\text{LKMC}}^2,$$

where D_{platelet} is the effective platelet diffusion coefficient due to Brownian motion and RBC motion (74) and

$$\text{platelet convection, } \Gamma_C = v_i/h_{\text{LKMC}},$$

for a lattice direction $\mathbf{e}_i$ with corresponding velocity component v_i . The rate of motion for each platelet is given by $\Gamma_{motion} = \Gamma_D + \Gamma_C$. Rates of blocked particles were passed forward in the direction of flow to correct for biasing artifacts on the lattice (75, 76). RBC motion creates an inhomogeneous radial platelet distribution, where the platelet concentration is highest near the walls. This effect was modeled as a radial drift velocity (77) that was superimposed on the velocity field obtained from LB. LKMC naturally creates a 3-fold wall excess of platelets at wall shear rate of 200 s^{-1} , and the radially-varying inlet concentration profile was set to the steady-state profile.

- 10 To calculate activation-dependent rates of attachment and detachment to and from collagen or deposited platelets, the internal activation state ξ of the i^{th} platelet at time t was defined as the integral calcium above the basal concentration,

$$\xi_i(t) = \int ([Ca]_i - [Ca]_0) dt$$

- $[Ca]_0$ was defined as 100 nM, and the maximal calcium concentration was set to 1 μM . The donor-specific NN gives the instantaneous calcium concentration $[Ca]_i$ for each platelet as it moves along its trajectory and experiences collagen (constant on the surface and set equal to $0.3 \times \text{EC}_{50}$ of convulxin for surface vs. soluble activation of GPVI), ADP, and/or TXA_2 (set equal to $10 \times [\text{U46619}]$ based on relative potency on TP). The integrated calcium concentration $\xi(t)$ for each platelet was then used to define a time-dependent extent of inside-out signaling $F(\xi)$ for $\alpha_2\beta_1$ and $\alpha_{2b}\beta_3$ integrin activation which varies from α (resting) to 1 (fully activated):

$$F(\xi) = \alpha + (1 - \alpha) \frac{\xi^n}{\xi^n + \xi_{50}^n} \quad (2)$$

- where n controls the sharpness of the response and ξ_{50} is the critical value for 50% activation. In the present work, the values of $n = 0.75$ and $\xi_{50} = 9 \mu\text{M}\cdot\text{sec}$ were set identical for $\alpha_2\beta_1$ and $\alpha_{2b}\beta_3$ integrin activation. The overall rate constant of attachment for a fully activated platelet to collagen is given by $k_{att}^{\text{collagen}}$ which coarse-grains receptor and ligand copy number and single bond kinetics. The rate of binding for a platelet with no activation ($\xi = 0$) to collagen is $\alpha_{\text{collagen}} k_{att}^{\text{collagen}}$ for $\alpha \ll 1$ (with α set to 0.001). The rate of binding for a platelet to another platelet via fibrinogen

depends on the activation states of both platelets. Thus, to model the two-body interaction, the rate of binding is given by the geometric mean of the two platelets, so for two platelets i and j . The overall rate of attachment for each platelet to collagen or to another platelet via fibrinogen (fbg) is then given by:

$$5 \quad \Gamma_{att}^{collagen} = k_{att}^{collagen} F(\xi_i) \quad \text{and} \quad \Gamma_{att}^{fbg} = k_{att}^{fbg} \sqrt{F(\xi_i)F(\xi_j)}. \quad (3)$$

As platelet calcium rises leading to integrin activation, the strength of the platelet-surface or platelet-platelet adhesion increases, whereby the unbinding rate is inversely proportional to the activation state, $F(\xi)$. Using a Bell model exponential (78) to characterize the cell detachment due to the force-dependent breakage of an ensemble of bonds, the overall detachment rates of platelets from collagen or from another platelet are:

$$10 \quad \Gamma_{det}^{collagen} = k_{det}^{collagen} F(\xi_i)^{-1} \exp\left(\frac{\gamma_i}{\gamma_c}\right) \quad \text{and} \quad \Gamma_{det}^{fbg} = \frac{k_{det}^{fbg}}{\sqrt{F(\xi_i)F(\xi_j)}} \exp\left(\frac{(\gamma_i + \gamma_j)}{2\gamma_c}\right), \quad (4)$$

where again the geometric mean of the platelet activation states is taken and the algebraic mean of the local shear rate (from LB) is used. The parameter γ_c is a scaling parameter that determines the level of shear at which the detachment rate increases.

The concentration field $C_j(x,y,t)$ for $j = \text{ADP}$ and TXA_2 was determined by finite element method (FEM solution of the convection-diffusion-reaction equation:

$$\frac{\partial C_j}{\partial t} + \nabla \cdot (\mathbf{v} C_j) = D_j \nabla^2 C_j + R_j \quad (5)$$

where D is the Brownian diffusion coefficient (augmentation effects for small solutes in blood flow are relatively small (79)), and R is the rate of release or generation, and $\mathbf{v}$ is the velocity field (from LB). The release rate of soluble species ADP and TXA_2 depends on the activation states of the platelets (NN and ξ_{crit}). A platelet only releases ADP and TXA_2 if its integral calcium is larger than the critical threshold, $\xi_{crit} = 9 \mu\text{M}$ -sec). The time at which a platelet crosses this threshold is denoted $t_{release}$, and the rate of release is modeled as an exponential decay of the form:

$$R_j(t) = \frac{M_j}{\tau_j} \exp\left(-\frac{t - t_{release}}{\tau_j}\right), \quad (6)$$

where R_j is averaged over the entire platelet volume, M_j is the total amount of ADP or TXA_2 in a platelet, and τ_j is the characteristic time of release. Once activated,

each platelet releases 50% of its 3×10^{-8} nmoles of ADP in 3.5 seconds (80, 81) and can release 50% of its 4×10^{-10} nmoles of TXA₂ over 69 seconds (82). All variables and parameters are defined in Table 4.

5 Example 1:

Study of Platelet Signaling

Six major agonists of human platelets were selected: convulxin (CVX; GPVI activator), ADP, the thromboxane analog U46619, PAR1 agonist peptide (SFLLRN), PAR4 agonist peptide (AYPGKF) and prostaglandin E2 (PGE₂) (activator
10 of the prostacyclin receptor IP and the E series prostanoid receptors EP 1-4).

These agonists activate platelet signaling pathways that converge on the release of intracellular calcium (Ca²⁺) (Figure 1a), which may be measured using a fluorescent calcium dye. Calcium mobilization is critical to physiologically important platelet responses needed for aggregation and clotting, including granule release,
15 exposure of phosphatidylserine, actin polymerization, shape change and integrin activation (6A). To determine appropriate dynamic ranges and the effective concentration for half-maximum response (EC₅₀) values for the six agonists, each compound was first tested individually to determine dose-response relationships (Figure 5). The inhibitory response of PGE₂ was studied by concomitantly
20 stimulating the platelet with 60 μM SFLLRN.

All experiments were conducted in 5 mM EDTA, which chelates extracellular calcium, to minimize the sensitivity of cells to confounding autocrine effects of soluble mediators dependent on platelet concentrations and transport processes. The removal of external calcium does not affect the ability of the studied
25 receptors to signal, as no appreciable difference in EC₅₀s were noted with or without external calcium (Figures 5a-5b). Although this experimental design did not capture the contribution of store-operated calcium entry, it offered several operational advantages by (i) lowering background fluorescence without extensive platelet washing, (ii) preventing thrombin production, (iii) inhibiting granule release (7A,8A)
30 as well as TxA₂ formation (9) and (iv) inhibiting integrin-mediated signaling downstream of Ca²⁺ release (10). The operational advantages of using EDTA, however, prevented prediction of important physiologic phenomena like granule release, integrin activation and outside-in signaling.

To test whether the intracellular Ca^{2+} signal detected was being influenced by endogenously released agonists, the effects of 2 units/ml apyrase (which hydrolyzes released ADP) or 15 μM indomethacin (which inhibits production of TxA_2) were studied.

5 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
10 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 (45). Also assuming release of 75% of platelet
15 dense granule ADP content of $1.74 \mu\text{moles}/10^{11}$ platelets (46) 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 EC_{50} 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 (47). Furthermore, during thrombosis,
20 platelet concentrations in deposited aggregates can increase 10 to 100-fold over platelet-rich plasma levels. At such close confinement during thrombosis, autocatalytic amplifications caused by secreted ADP and TxA_2 becomes significant. However in the controlled, dilute (12% PRP), and unstirred environment inside the well plate containing EDTA which attenuates secretion, only primary effects of receptor
25 mediated calcium release from cell stores were observed.

Both of these inhibitors had no effect on individual responses (Figure 6 and Tables 1 and 2), suggesting that endogenous autocrine activators have no effect on the Ca^{2+} signal. This confirms that the resulting traces of Ca^{2+} are directly dependent only on receptor-mediated release from intracellular stores.

30 The PAS method was applied by first measuring platelet responses to all 135 pairwise combinations of low ($0.1 \times \text{EC}_{50}$), moderate ($1 \times \text{EC}_{50}$) and high ($10 \times \text{EC}_{50}$) agonist concentrations (Figure 2a). Then, a neural network model was trained on 154 time-course traces (135 pairwise responses, 18 single-agonist

responses, 1 null control response). A pairwise agonist synergy (S_{ij}) score was defined as to be the scaled difference between the integrated transient (area under the curve) for the combined response and the integrated area for the individual responses (Figure 2b) ($S_{ij} > 0$, synergism; $S_{ij} = 0$, additivity; $S_{ij} < 0$, antagonism). The trained
 5 network accurately reproduced 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 (Figures 2a-2b and 7).

Example 2:

10 Testing of Trained Network

As an initial test of the trained network, the response of platelets to all 64 ternary combinations of the agonists ADP, SFLLRN and CVX at 0, 0.1, 1 and $10 \times EC_{50}$ concentrations was predicted and then compared the predictions to experimentally measured responses (Figure 3a). A CVX response required GPVI
 15 multimerization (11A) and was characterized by a slow rise to a large peak signal followed by a slow decline. G_q -coupled responses (ADP or SFLLRN) produced rapid bursts that were quickly brought down to baseline. Increasing CVX for a fixed ADP level resulted in a steady increase in Ca^{2+} on longer timescales. In contrast, increasing ADP for a fixed CVX level bolstered early Ca^{2+} release. A moderate dose of both
 20 ADP and CVX (for 0 and low SFLLRN) produced a response that almost instantaneously plateaued at a steady level above baseline. Both the time-course behavior ($R = 0.844$) and ternary agonist synergy scores ($R = 0.881$) (Figure 8) were accurately reproduced for the 27 unique ternary conditions in this experiment that were not present in the training set.

25 To fully test and utilize the predictive power of the neural network, time-course and synergy predictions were made *in silico* for the complete six-dimensional agonist space consisting of 4,077 unique agonist combinations of two to six agonists at 0, 1, 1 or $10 \times EC_{50}$ concentrations (Figure 9). Based on these predictions, 45 combinations of four, five or six agonists that displayed a range of
 30 predicted synergy scores from synergy to strong antagonism were selected and then tested experimentally in addition to no agonist and 18 single-agonist controls (Figure 3b). To prevent any bias in the selection, conditions that had maximal dissimilarity in the types and concentrations of agonists were selected. Strong agreement between both predicted and measured transient shapes ($R = 0.845$) (Figure 3b and Figure 10a)

was observed, as well as between predicted and measured S_{ij} scores ($R = 0.883$, slope = 1.08) (Figure 3c). For comparison, the full distribution of synergy predictions for all 4,077 agonist combinations is shown as a vertical heat map in Figure 3c.

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 each input to the model was systematically silenced (Figure 11). 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 in Figure 11. 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 showed that the NN model utilizes information from all inputs to gain accuracy. Any suppression of 1, 2, 3, 4, or more inputs drove 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 reduced the predictive power severely to zero correlation between experiment and the NN. This data demonstrated that no single pair embedded in the experiment that predicts all the tested outputs better than the full input test. In other words, the neural network did not exclusively rely on smaller subsets of input.

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) did 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), the NN integrates information from all input species.

Conditions containing high levels of all agonists showed especially low synergy due to saturation of Ca^{2+} release. The highest synergy was observed for agonist combinations that contained high levels of the thromboxane analog U46619 with no PGE_2 present (Figure 3c, bar marked with "****"). Given that only 8 of 45 conditions had maximal U46619/ PGE_2 ratio, this ordering of the top three conditions was highly significant ($P < 0.004$), considering there are 14,190 possible ways to

order the first three conditions of which only 56 combinations would contain high U46619 and low PGE₂. Thus, the neural network model trained on pairwise data facilitated discovery of a high-dimensional synergy that occurs at a 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 PGI₂ without affecting platelet production of thromboxane (12). This result suggested a “high-dimensional” COX2 inhibition risk of high concentrations of thromboxane, in the absence of PGI₂, potentiating the effects of other agonists.

The effect of adding the agonists ADP, SFLLRN and CVX in various sequential combinations was also explored (Figure 3d). Several notable behaviors were accurately predicted by the neural network model despite the network being trained on purely synchronous interactions. For instance, the temporal sequence ADP-SFLLRN-CVX (Figure 3d, panel 1) produced three distinct Ca²⁺ bursts, whereas the ADP response was completely abolished in the sequence SFLLRN-ADP-CVX (Figure 3d, panel 3).

Example 3:

Effects of Sequential Addition of ADP, SFLLRN and CVX

The results obtained above suggest mechanisms of cross-downregulation of ADP signaling by component(s) of the PAR1 cascade. This hypothesis was further explored as illustrated herein. Preceding a high dose of ADP with prior treatment with low dose of ADP (Figure 3d, panel 12) desensitized the signal expected from a high dose of ADP (as in Figure 3d, panel 1). This behavior has been observed previously (34, 35) and is attributed to the internalization of P₂Y₁. Prior addition of CVX abolished responsiveness to both ADP (Figure 3d, panel 5) and SFLLRN (Figure 3d, panel 6) again suggesting a mechanism where components of the GPVI signal are able to down regulate the G_q-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 3d, panel 9) and SFLLRN (Figure 3d, panel 8). CVX-mediated calcium mobilization events were unaffected by pretreatment with either ADP (Figure 3d, panel 2) or SFLLRN (Figure 3d, panels 4 and 3), or a binary combination of these agonists (Figure 3d, panel 7). Activation of GPVI or thrombin

receptors phosphorylates the ITIM domain of platelet PECAM (36). ITIM phosphorylation inhibits response via phosphatases like SHP-2 (37). Such mechanisms, or even agonist selective stores (38), 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 ~260 s (Figure 3d, panels 2, 4-10). This limitation was expected because the NN was trained on measurements spanning only 260 s (Figure 2a) and not the entire duration (up to 900 s). Importantly, the NN captured cross-talks of sequential additions despite being trained on purely synchronous interactions.

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Example 4:

Feasibility of Using Time-Dependent Agonist Forcing Functions and Simulating Realistic Hemodynamic Flow Conditions

During the *in vivo* response to injury, the first adhering platelets experience strong GPVI signaling while subsequent depositing platelets contribute to the rising ADP and serotonin levels, followed by rising thromboxane levels and thrombin formation. While no single combination of agonists may replicate the dynamics of this *in vivo* situation, PAS-trained NN models offer the potential of using time-dependent agonist forcing functions as demonstrated in Figure 3d (and Figure 12 for tests with thrombin compared to SFLLRN+AYPGKF). The ability to simulate thrombus formation under realistic hemodynamic conditions of flow (39, 40) requires calculations of $[Ca^{2+}]_i$ for platelets experiencing spatial and temporal exposures to stimuli. NN models are especially well suited for incorporation into multiscale models of patient-specific cell function in convective, reactive and dispersive flow fields (41).

25

Example 5:

Sequential Additions Mimicking the *In-vivo* Thrombotic Environment

In a realistic thrombotic setting, the platelet is likely to get activated upon an exposed collagen surface and subsequently 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

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aggregates in solution. These conditions are illustrated in Figure 12a. We mimic these 4 conditions by making selective sequential additions (similar to Figure 3d) 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 5 12b. The signals generated by thrombin for these selective additions were similar to those generated by a mixture of high dose PAR1+PAR4 peptides. A PAS trained NN for donor A, qualitatively reproduced these sequential additions of PAR1+PAR4 peptides (to approximate the function of thrombin). Prior GPVI activation abolished detectable responsiveness to ADP or thrombin. Thrombin (or PAR1+PAR4) 10 abolished subsequent response to ADP (presumably by cross down regulation of the ADP cascade by PAR1). In contrast, prior ADP stimulation did not abolish subsequent detectable responsiveness to thrombin (or PAR1+PAR4). 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 15 the PARs.

Example 6:

Further Investigation of PAS Procedure

To investigate the reproducibility of the PAS procedure and to 20 investigate the potential for using it to stratify individuals' platelet responses, PAS was performed twice in a 2-week period for ten healthy male donors (Figure 4). The 135 conditions containing pairs of agonists in a single PAS experiment make up the synergy map for each donor experiment (Figure 13) and individual columns of the synergy matrix (Figure 4). The standard errors in synergy scores across all 135 25 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_{ij} ranging from -1 to +1 (uncertainties across all 135 conditions are shown in Figure 14). The mean standard error in synergy scores for all ten donors ranged from 30 ± 0.0347 to ± 0.0627 (Table 3).

A hierarchical cluster tree was generated using the Euclidean distances between donor experiments. Seven of the ten donor pair vectors (donor pairs D, C, A, H, E, F and I) self-clustered, demonstrating that despite variation between samples from the same donor, pronounced inter-donor variations allow distinguishing among

donors. This pattern of clustering was found to be highly significant ($P < 8 \times 10^{-7}$) by randomizing observed donor synergies (Figure 15). 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 or heterologous plasma self-clustered (Figure 16). 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 (Figure 17).

Donors separated into at least two major subgroups with the cluster of donor experiments D1, D2, J2, C1, C2, B1 and B2 characterized by relative lack of synergy 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 or ADP, as well as marked synergy for moderate U46619 and high CVX. All donors showed some synergism between low and moderate doses of SFLLRN and U46619. Synergy was also typically observed between AYPGKF and U46619. Moreover, synergistic or additive interactions were noted also between low and moderate doses of SFLLRN and AYPGKF. These results 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 18) over seven doses spanning the active concentration ranges. This appears to be the first report of conserved synergy between thrombin and thromboxane mimetics.

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 multiple agonists. Importantly, a single 384-well plate of data was sufficient to train a neural network model (Figure 2) capable of making accurate predictions of the global six-dimensional agonist reaction space (Figure 3), which is difficult to probe experimentally but fundamental to the processes of thrombosis. Synergies between platelet agonists were dependent not just on agonist pairs and doses, but also varied from donor to donor (Figure 4). In contrast to PAS, current measurements of platelet phenotype can only coarsely stratify healthy donors. For instance, platelet aggregometry has been used (13A) to classify 359 individuals as "hypo- or hyper-" reactive to platelet agonists; and flow cytometry was used (14) to classify 26 individuals as high, medium or low responders. Previous

studies have reported synergistic aggregation responses of platelets to combinations of multiple agonists (15--17). Such unique patterns of synergisms could be used to distinguish donors and be correlated with certain risk factors. Clinically, PAS profiles may depend on variables such as ancestry, age, sex, pharmacology and cardiovascular state - all of which require further testing - although linking genotype (1,327 single nucleotide polymorphisms) to phenotype (flow cytometric measurement of P-selectin exposure and fibrinogen binding) in 500 individuals (18) demonstrated only weak association probabilities.

10 Example 7:

Donor-Specific Platelet Responses

Mild positive synergisms such as those between low doses of SFLLRN and U46619/ADP, as well as those between ADP and CVX further classify the cluster of experiments A1, A2, H1, H2, J1, E1, E2, F1, F2, G1, I1 and I2 into smaller sub clusters comprising of donors A1, A2, H1, H2 and J1; and donors E1, E2, F1, F2, G1, I1 and I2 (Figure 4). PGE₂ was found to be antagonistic to all G_q-coupled agonists (ADP, U46619, SFLLRN and AYPGKF) for all donors, in accordance with the known ability of PGE₂ to raise cAMP levels (42). 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 (43) which are known to mediate pro-aggregatory actions.

Example 8:

25 Probabilities of Synergy Scores at Different Agonist Pair Concentrations

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 the synergy values. Illustrated in Figure 17 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. Illustrated in the top left of Figure 17 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.

5

Example 9:

Synergism between Thrombin and Thromboxane

Noting marked synergism between SFLLRN and U46619, and some synergisms between AYPGKF and U46619, for all donors, synergism between the physiological agonist thrombin and U46619 was evaluated. 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 μ M Indomethacin, 2 units/ml Apyrase and 100 μ M GGACK to prevent autocatalytic amplification by thromboxane, ADP or Xa. 500 μ M GPRP was included to prevent fibrin formation with added thrombin. It should be noted that the 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, as illustrated in Figure 18, synergisms between low and moderate doses of these agonists prior to saturation of their receptor signaling pathways were observed.

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Example 10:

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 may be made in a system only if such higher order interactions are relatively unimportant.

The experiment with ADP/CVX/SFLLRN measured platelet response to simultaneous activation of two GPCRs and an immune receptor homologue

(GPVI). In Figure 19, the binary synergy score ($S_{AB}=AB-A-B$), the ternary synergy score ($S_{ABC} = ABC-A-B-C$), and the “residual” ternary synergy which measures the synergy *in excess* of the binary synergies [$\Delta(ABC) = S_{ABC}-S_{AB}-S_{BC}-S_{AC}$] were investigated. 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 EC_{50} . By determination of S_{AB} , S_{ABC} , and $\Delta(ABC)$ from the predicted calcium responses obtained from the neural network, $\Delta(ABC)_{NN}$ was found to be 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 (44). They found that almost all pairwise interactions result in non-additive interactions (akin to Figures 2 and 4 herein). 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 built 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. In systems where third order interactions are important “ternary” agonist scanning might be necessary, however this may require added experimental combinatorial complexity. “Pairwise” agonist scanning is the bare minimum that can be expected to quantify interactions between multiple simultaneously acting signaling pathways.

Example 11:PAS Approach

In a non-limiting aspect, the PAS approach provides useful results because individual and binary interactions dominate, and they are sampled across the full dose range of inputs. The method may break down when ternary interactions in excess of summing binary interactions become strong. As demonstrated in these studies, 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 19).

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 1a) 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 (44). 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 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 (Figure 19) (19); (ii) the efficient utilization of system history (Figure 20); (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.

Further, application of PAS to stimuli including epinephrine, soluble CD40L, serotonin, histamine, and nitric oxide would map a major portion of the entire platelet response space. The use of PAS with orthogonal pharmacological agents (indomethacin, P₂Y₁₂ inhibitors, selective PAR antagonists, guanylate cyclase or adenylyate cyclase inhibitors) would allow further assessment of individual clinical risk or sensitivity to therapy. The studies of the PAS method reported herein have demonstrated that sampling all dual orthogonal “axes” (every agonist pair) can successfully predict the dynamic responses and cross-talk of a system receiving complex combinations of inputs.

Example 12:

Use of Pairwise Agonist Scanning/Neural Networks for Predicting Human Blood

Function in Hemodynamic Environments

In this experiment, calcium dye-loaded platelets in PPACK and indomethacin-treated plasma (thus lacking thrombin and TXA₂) from 3 healthy donors were subjected to Pairwise Agonist Scanning (PAS) where platelets were exposed to all pairwise combinations of ADP, U46619, and convulxin (at 0, 0.1, 1, 10 x EC₅₀) to activate P₂Y₁/P₂Y₁₂, TP, and GPVI receptors, respectively, in the presence or absence of the IP receptor agonist, iloprost. A set of 74 calcium responses allowed training of a neural network (NN) model of platelet calcium mobilization for each donor. Each NN model was then embedded into a kinetic Monte Carlo/finite element/lattice Boltzmann simulation of platelet deposition under flow.

Simulations predicted the measured clot buildup dynamics for each donor for PPACK-treated whole blood flowing over collagen at 200 s⁻¹ wall shear rate in the presence of indomethacin, iloprost, or MRS-2917 (a P₂Y₁ inhibitor). Consistent with measurement and simulation, one donor displayed a gain of function phenotype (larger clots), while another donor was distinguished by combined indomethacin-resistance and U46619-insensitivity (confirmed as a point mutation in the TP receptor).

All combinations of the 3 agonists ADP, U46619, CVX at 0, 0.1, 1 and 10x EC₅₀ levels were tested both in the absence and presence of the platelet inhibitor Iloprost. All conditions were tested in replicates of 4 on a single plate. These

experiments were carried out for three separate donors on a single day, and were repeated within a week to ensure reproducibility. Similar to the approach reported in Chatterjee et al. (72), a NARX neural network was trained on the mean of each of the 74 calcium transients obtained in a single experiment. These “patient-specific” models were further used in the LKMC simulations of platelet deposition. Representative results comparing experiment and simulations, on the first day for each of the 3 donors are shown in Figure 22. Excellent agreement was obtained between experimental and simulated calcium transients (Figure 22A). “Synergy scores” quantified by the scaled difference between the integral response of the combinatorial and individual responses have been shown to be compact representations of the interactions amongst multiple signaling pathways (72). Negative values of this synergy metric signify antagonistic interactions; values close to 0 represent purely additive interactions; and positive values imply synergism between the interacting pathways. Most interactions tested were found to be additive or weakly synergistic in the absence of the platelet inhibitor Iloprost, whereas in its presence interactions were strongly antagonistic (Figure 22B). The synergy scores at each of the 63 conditions, containing two or three inputs (agonists with or without iloprost) were highly correlated ($R=0.9543$) between experiment and simulation (Figure 22C). This fit is a measure of the accuracy of Neural Network training. Results obtained on the second repeat experiment for each of the 3 donors were very similar to those reported.

In summary, *in silico* representations of an individual’s platelet phenotype allows prediction of blood function, essential to prioritizing patient-specific cardiovascular risk and drug response or to identify unsuspected gene mutations.

Table 1:

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

5 conditions tested.

Agonist	Dose (×EC50)	15μM Indomethacin	2 Units/ml Apyrase	Both Inhibitors added
CVX	0.1	0.6039	0.6504	0.6504
	1	0.3312	0.4686	0.4091
	10	0.3961	0.5314	0.3496
U46619	0.1	0.7857	0.0325*	0.4091
	1	0.6039	0.9794	0.9794
	10	0.3961	0.9535	0.9535
SFLLRN	0.1	0.3961	0.6504	0.5314
	1	0.8355	0.9924	0.9957
	10	0.5346	0.5314	0.7576
AYPGKF	0.1	0.0087*	0.0898	0.2944
	1	0.2684	0.5909	0.2944
	10	0.6039	0.9102	0.8799
PGE ₂ +60μM SFLLRN	0.1	0.6039	0.9102	0.8452
	1	0.6039	0.803	0.3496
	10	0.3312	0.7056	0.5909

Table 2:

10 *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.

Agonist	Dose (×EC50)	15μM Indomethacin	2 Units/ml Apyrase	Both Inhibitors added
CVX	0.1	0.5346	0.6504	0.7056
	1	0.2684	0.5314	0.4091
	10	0.3961	0.5314	0.4091
U46619	0.1	0.1234	0.0325*	0.5314
	1	0.2684	0.8799	0.934
	10	0.1234	0.987	0.987
SFLLRN	0.1	0.1645	0.5314	0.4686
	1	0.8355	0.9957	0.9957
	10	0.1645	0.6504	0.5314
AYPGKF	0.1	0.0152*	0.0076*	0.0898
	1	0.4654	0.8452	0.2424
	10	0.7316	0.9102	0.8799
PGE ₂ +60μM SFLLRN	0.1	0.6039	0.9102	0.934
	1	0.4654	0.8799	0.2424
	10	0.5346	0.5909	0.5909

Table 3:

Mean standard error in synergy scores. The mean standard error in synergy score was calculated as explained in Figure 14. These values are a measure of the experimental uncertainty in determining donor specific synergisms.

Donor	Mean standard error in synergy scores
A	0.0523
B	0.0627
C	0.0331
D	0.0432
E	0.0347
F	0.0356
G	0.0498
H	0.0494
I	0.0443
J	0.0469

5

Table 4:

Model parameters for Example 12.

Symbol	Name	Value	Reference or Comment
ρ_{blood}	Density of blood	1 g/cm ³	
μ_{blood}	Viscosity of blood	0.03 g/cm s	
D_{platelet}	Diffusion (dispersion) Coefficient of platelet in flowing blood of Hc = 0.4	1.25×10^{-7} cm ² /s	79
R_{platelet}	Radius of platelet	1.5×10^{-4} cm	
α	Resting level of integrin activation	0.001	<0.1 % active on resting platelet
n	Sharpness of activation function	0.75	
ξ_{50}	Critical value for 50% activation and platelet release	9 μ M-s	

$k_{att}^{collagen}$	Attachment rate constant for collagen binding	1000 s^{-1}	
k_{att}^{fbg}	Attachment rate constant for fibrinogen-mediated binding	50 s^{-1}	
$k_{det}^{collagen}$	Detachment rate constant for collagen binding	$1 \times 10^{-5} \text{ s}^{-1}$	
k_{det}^{fbg}	Attachment rate constant for fibrinogen-mediated binding	$1 \times 10^{-4} \text{ s}^{-1}$	
γ_c	Critical shear rate	200 s^{-1}	
M_{ADP}	Total amount of ADP in dense granules	3×10^{-11} moles/platelet	81
M_{TXA}	Total amount of TXA_2 generated upon activation	4×10^{-13} moles/platelet	82
τ_{ADP}	Characteristic release time for ADP	5 s	80
τ_{TXA_2}	Characteristic release time for TXA_2	100 s	82
C_{CVX}	Effective concentration of surface collagen in units of CVX ($EC_{50} = 5.3 \text{ nM}$)	$0.3 \times EC_{50}$	
θ	Relative potency of $TXA_2/U46619$	10	

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10 The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety.

While the invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The appended claims are intended to be construed to include
15 all such embodiments and equivalent variations.

CLAIMS

What is claimed is:

- 5 1. A method of predicting a signaling response of at least one cell to a combination of given stimuli, wherein said at least one cell comprises a primary cell from an individual, wherein said method comprises the steps of:
- providing said at least one cell;
- contacting said at least one cell with a subset of said given stimuli,
- 10 wherein said subset comprises at least two given stimuli;
- measuring an output response from said at least one cell to said contacting, to generate a cellular response learning set;
- using said learning set to generate a dynamic cellular signaling model;
- and,
- 15 using said signaling model to predict said signaling response of said at least one cell to said combination of said given stimuli.
2. The method of claim 1, wherein said cell is obtained from a biopsy of said individual.
3. The method of claim 1, wherein said cell is obtained from a
- 20 blood sample of said individual.
4. The method of claim 1, wherein said individual is a mammal.
5. The method of claim 4, wherein said mammal is a human.
6. The method of claim 1, wherein said given stimuli in said subset are provided in at least three distinct concentrations.
- 25 7. The method of claim 6, wherein said at least three distinct concentrations comprise a value selected from the group consisting of a concentration below the EC_{50} of said stimulus in said cell, a concentration equal to about the EC_{50} of

said stimulus in said cell, and a concentration above the EC_{50} of said stimulus in said cell.

8. The method of claim 7, wherein said concentration below the EC_{50} of said stimulus in said cell is about $0.1 \times EC_{50}$.

5 9. The method of claim 7, wherein said concentration above the EC_{50} of said stimulus in said cell is about $10 \times EC_{50}$.

10. The method of claim 1, wherein measuring said output response comprises using a high-through screening method.

10 11. The method of claim 1, wherein generating said dynamic cellular signaling model comprises using a neural network model.

12. The method of claim 3, wherein said cell comprises a platelet.

13. The method of claim 12, wherein at least one of said stimuli binds to a receptor selected from the group consisting of GPVI, PAR1, PAR4, P_2Y_1 , P_2Y_{12} , TP, IP, EP, alpha2 receptor of epinephrine, histamine receptor, serotonin
15 receptor, and nitric oxide receptor.

14. The method of claim 12, wherein at least one of said stimuli is selected from the group consisting of iloprost, ADP, convulxin, U46619, SFLLRN (SEQ ID NO:3), AYPGKF (SEQ ID NO:4), PGE_2 , a salt thereof, and a combination thereof.

20 15. The method of claim 12, wherein said output response comprises a response selected from the group consisting of intracellular calcium release, cAMP level elevation or reduction, cGMP level elevation or reduction, phosphorylation or dephosphorylation of a protein or lipid, integrin activation, and granule release.

25 16. The method of claim 1, wherein said signaling response of said cell is used to predict disease progression in said individual.

17. The method of claim 1, wherein said signaling response is used to predict response of said individual to a therapeutic drug or treatment.

18. The method of claim 1, wherein said signaling response comprises sensitivity to IP receptor activation in said individual.

5 19. The method of claim 18, wherein at least one of said stimuli is an agonist of said IP receptor.

20. The method of claim 19, wherein said agonist is iloprost or a salt thereof.

10 21. The method of claim 18, wherein said signaling response predicts clotting under flow for said individual.

22. The method of claim 18, wherein said signaling response predicts drug sensitivity under flow for said individual.

15 23. The method of claim 18, wherein said signaling response identifies a defective cellular function or pathway related to a specific gene mutation in said individual.

24. The method of claim 23, wherein said mutation occurs in a TP receptor.

20 25. The method of claim 23, wherein identification of said specific gene mutation indicates that said individual is unresponsive to a therapeutic drug or therapy.

26. The method of claim 25, wherein said therapeutic drug or therapy comprises indomethacin or a salt thereof.

27. The method of claim 11, where said neural network is trained from said at least one cell obtained from said individual, and wherein said network is

embedded in a larger calculational model that predicts cellular response as a function of space or time.

28. The method of claim 27, wherein said neural network is used to predict disease progression rates in said individual or used to predict responses to a
- 5 therapeutic drug or treatment in said individual.

Fig. 1a

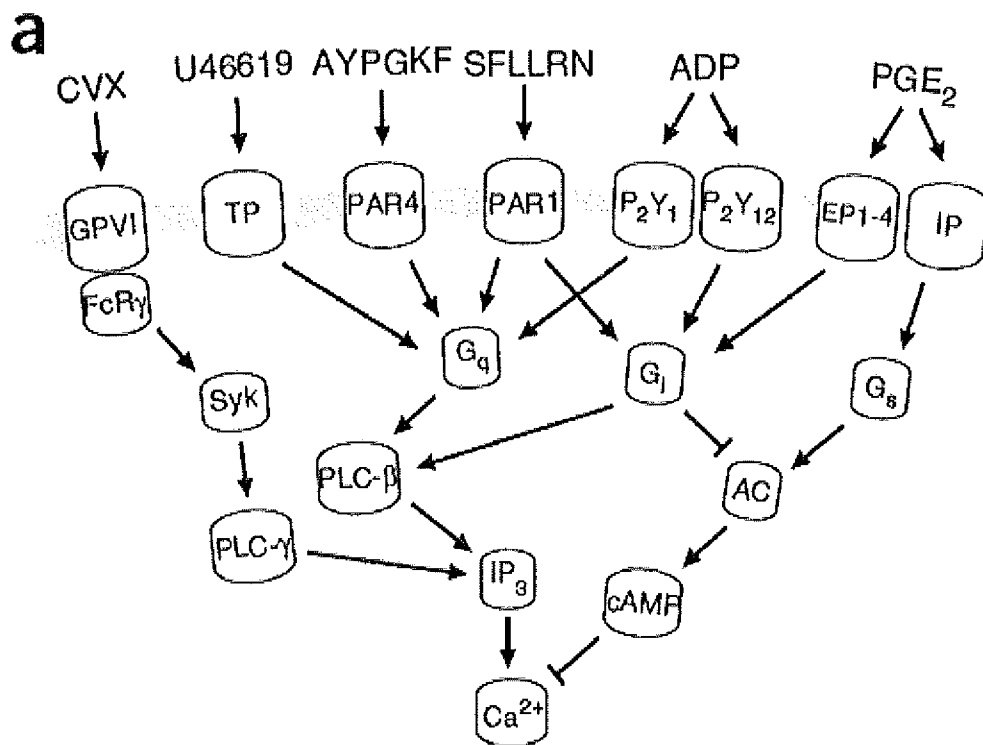


Fig. 1b

b

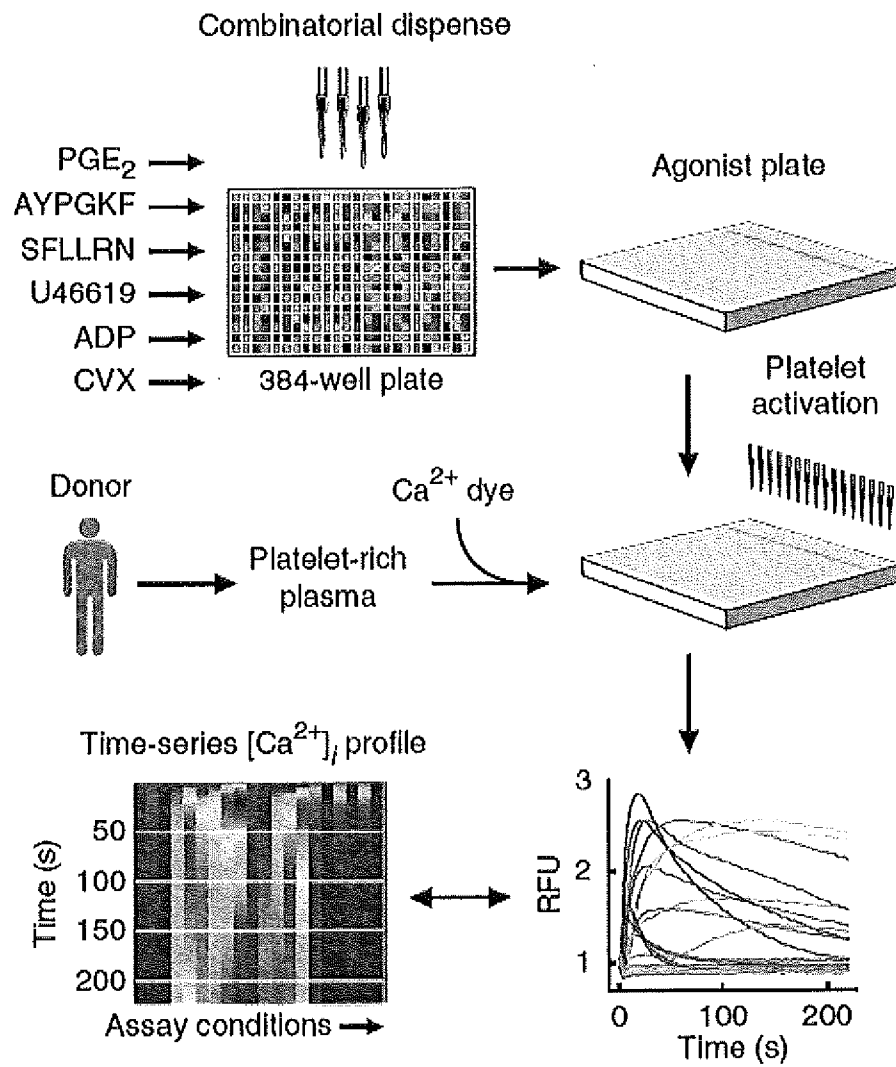


Fig. 1c

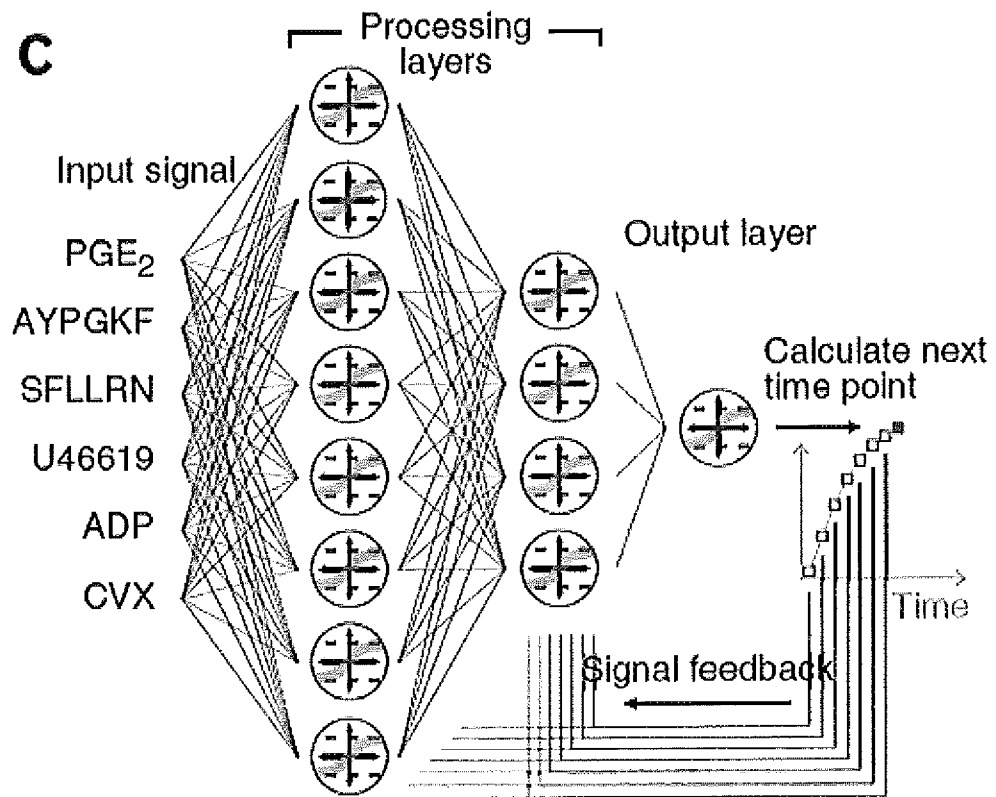


Fig. 2a

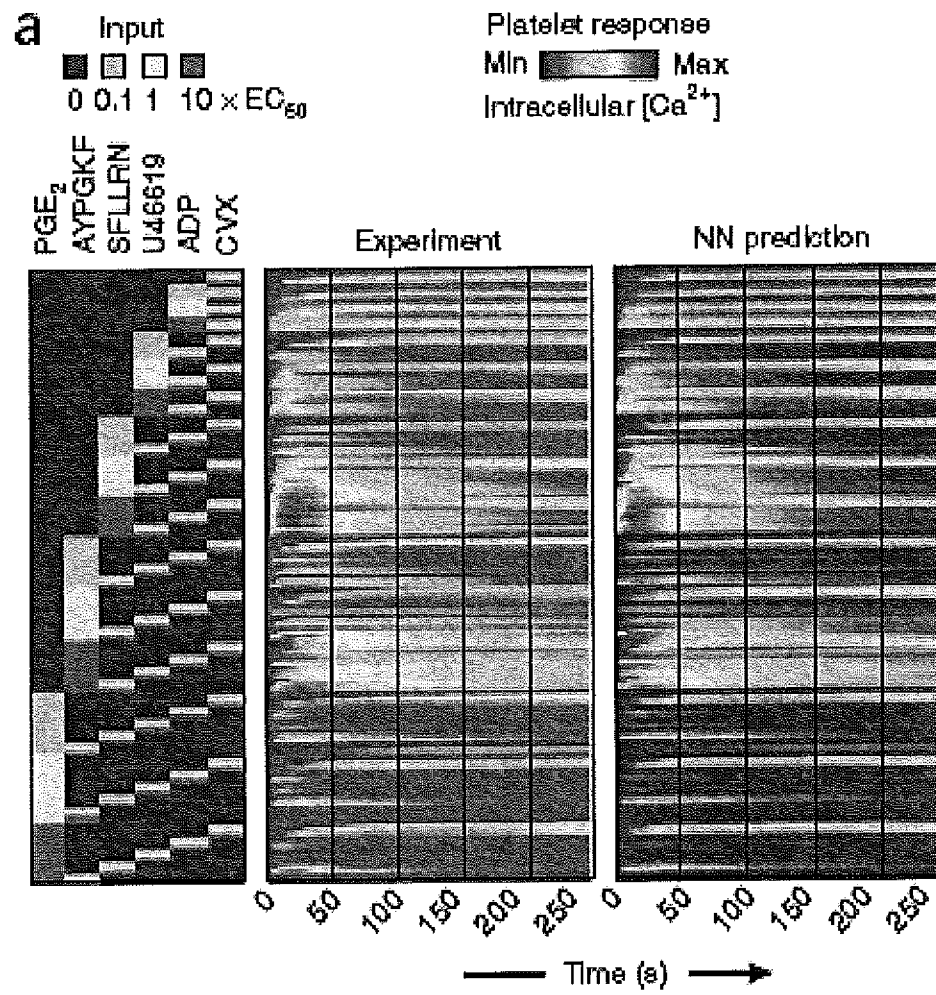


Fig. 2b

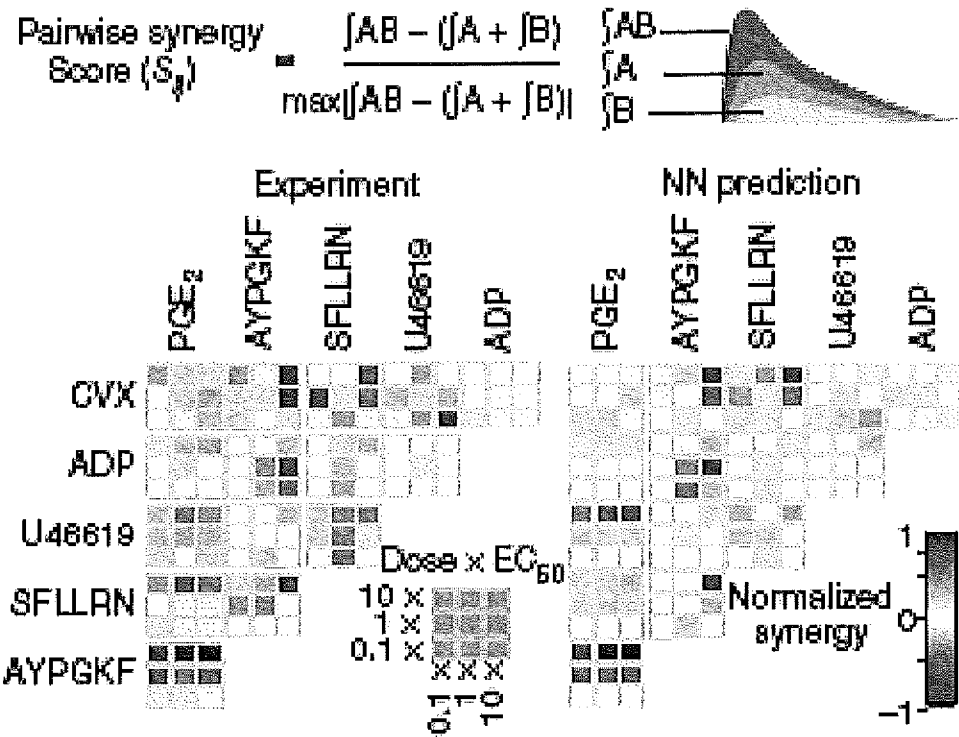


Fig. 3a

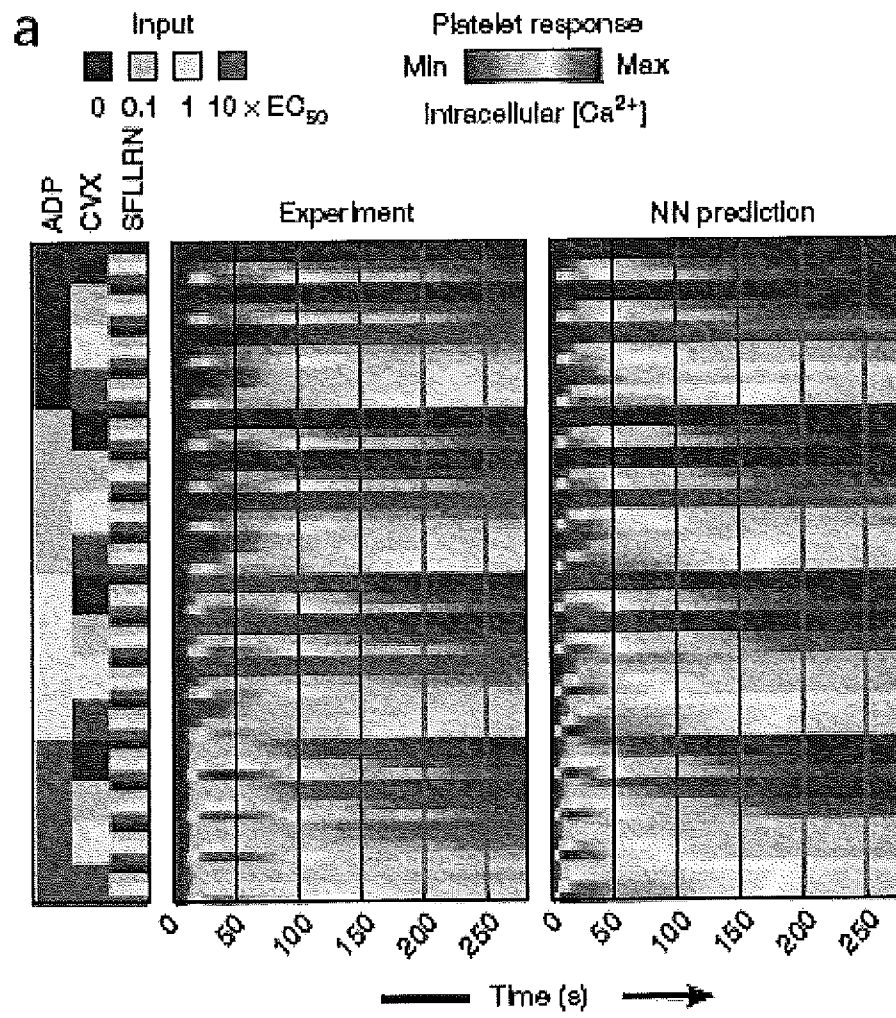


Fig. 3b

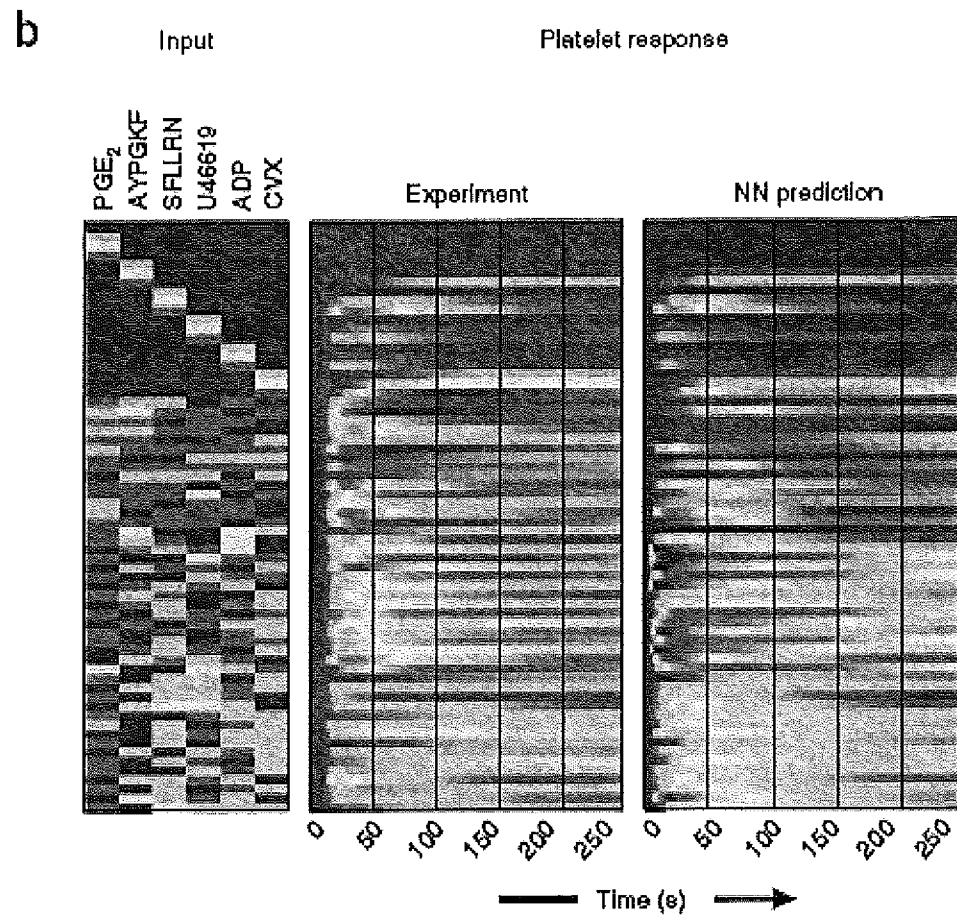


Fig. 3c

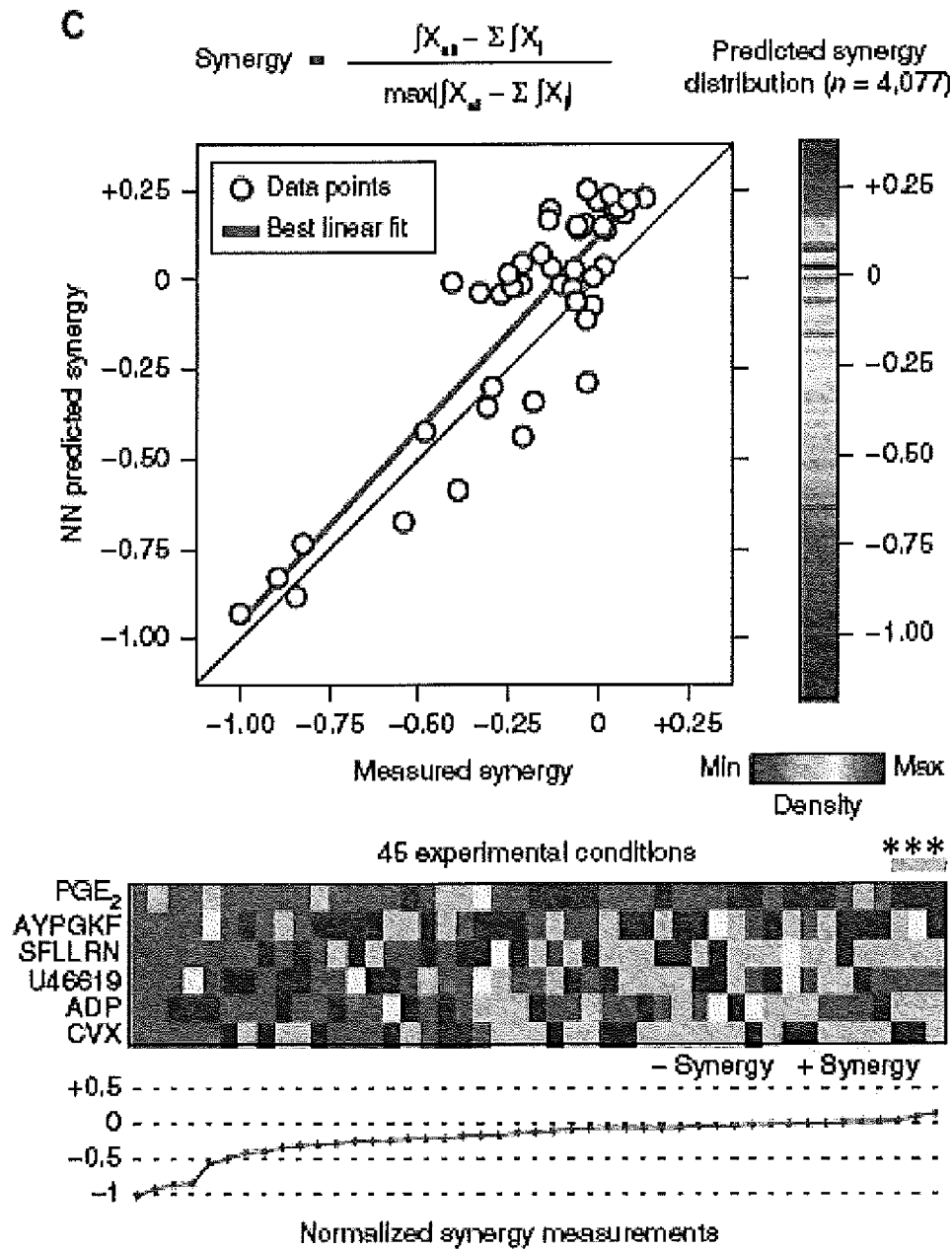


Fig. 3d

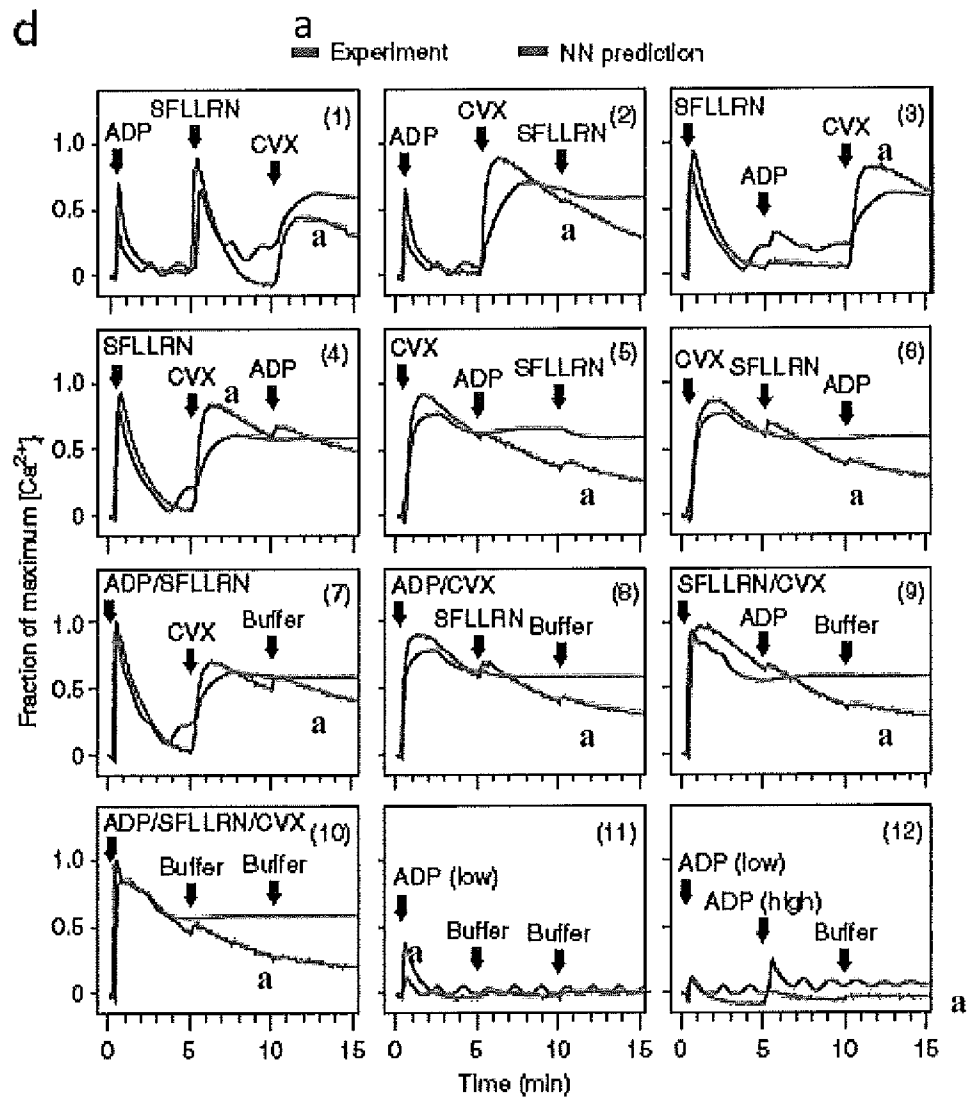


Fig. 4

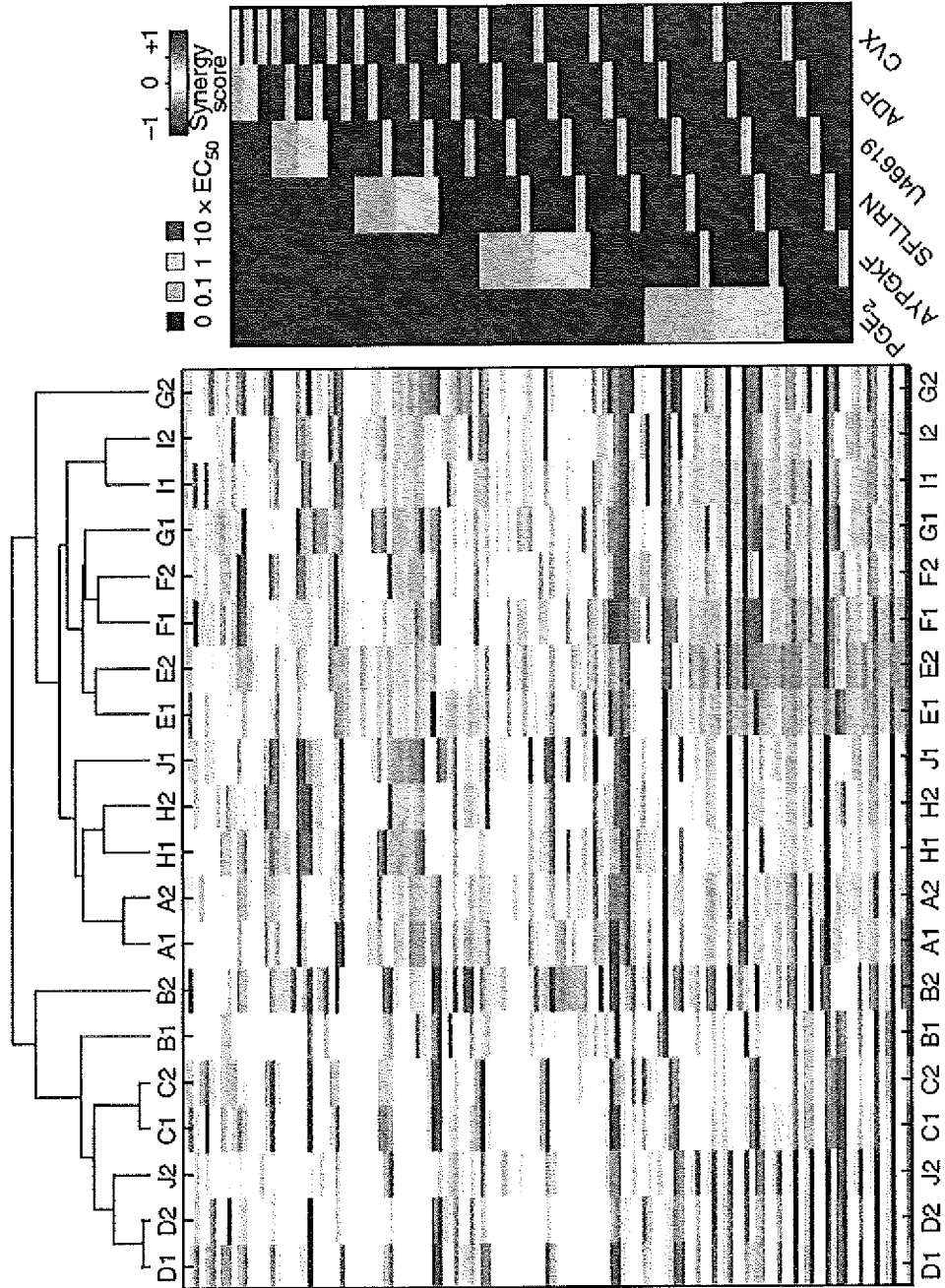


Fig. 5a

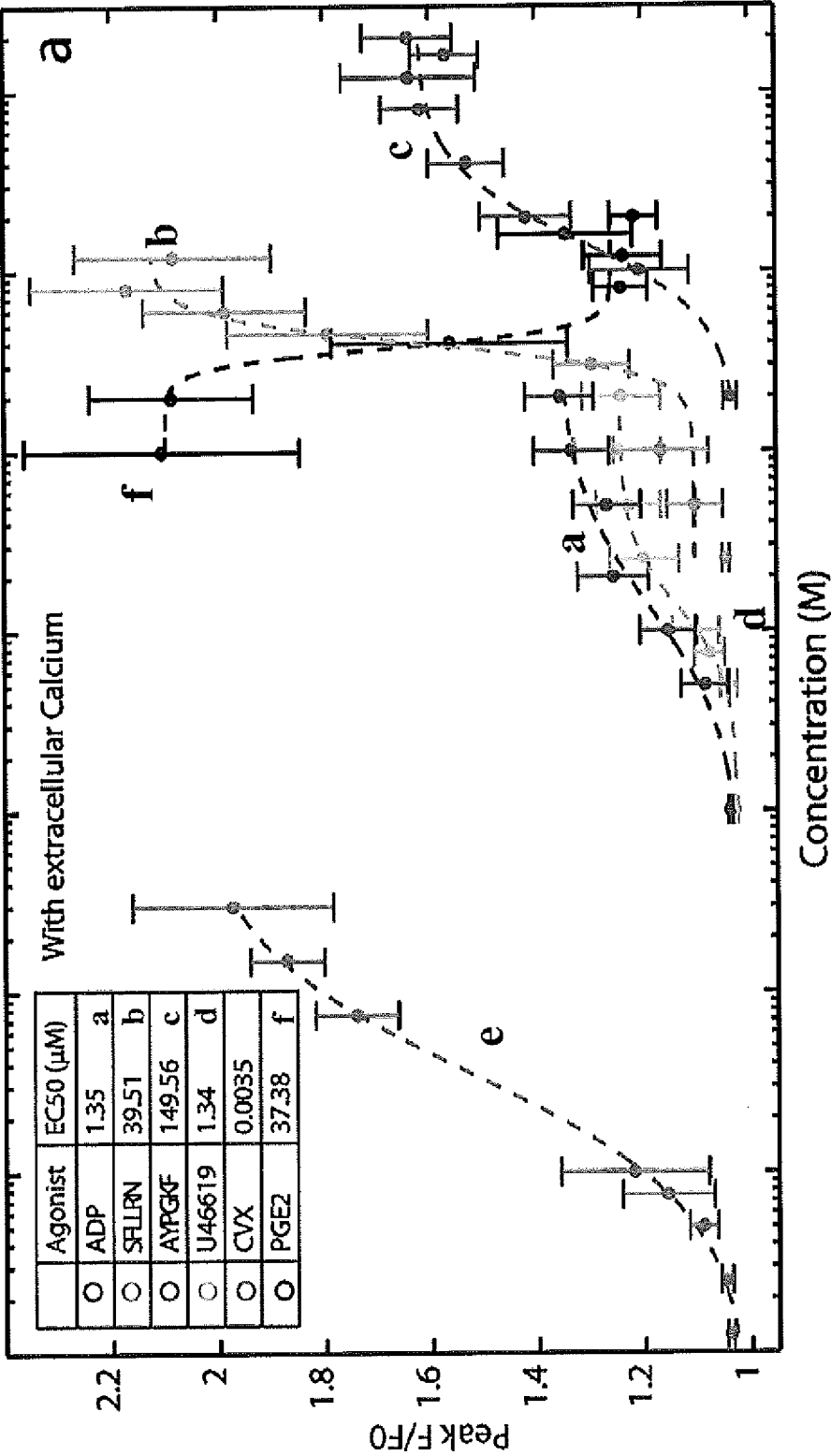


Fig. 5b

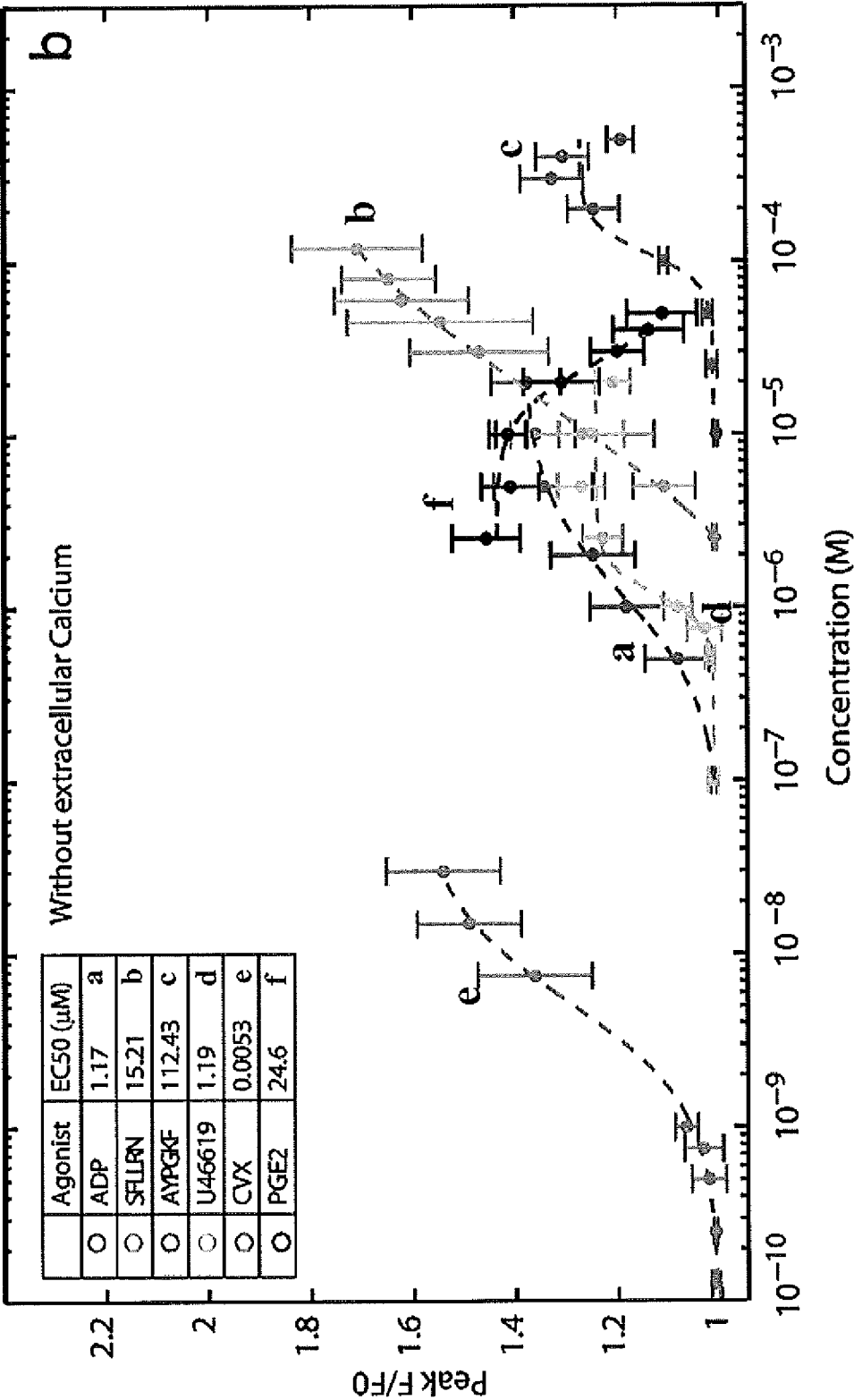


Fig. 6a

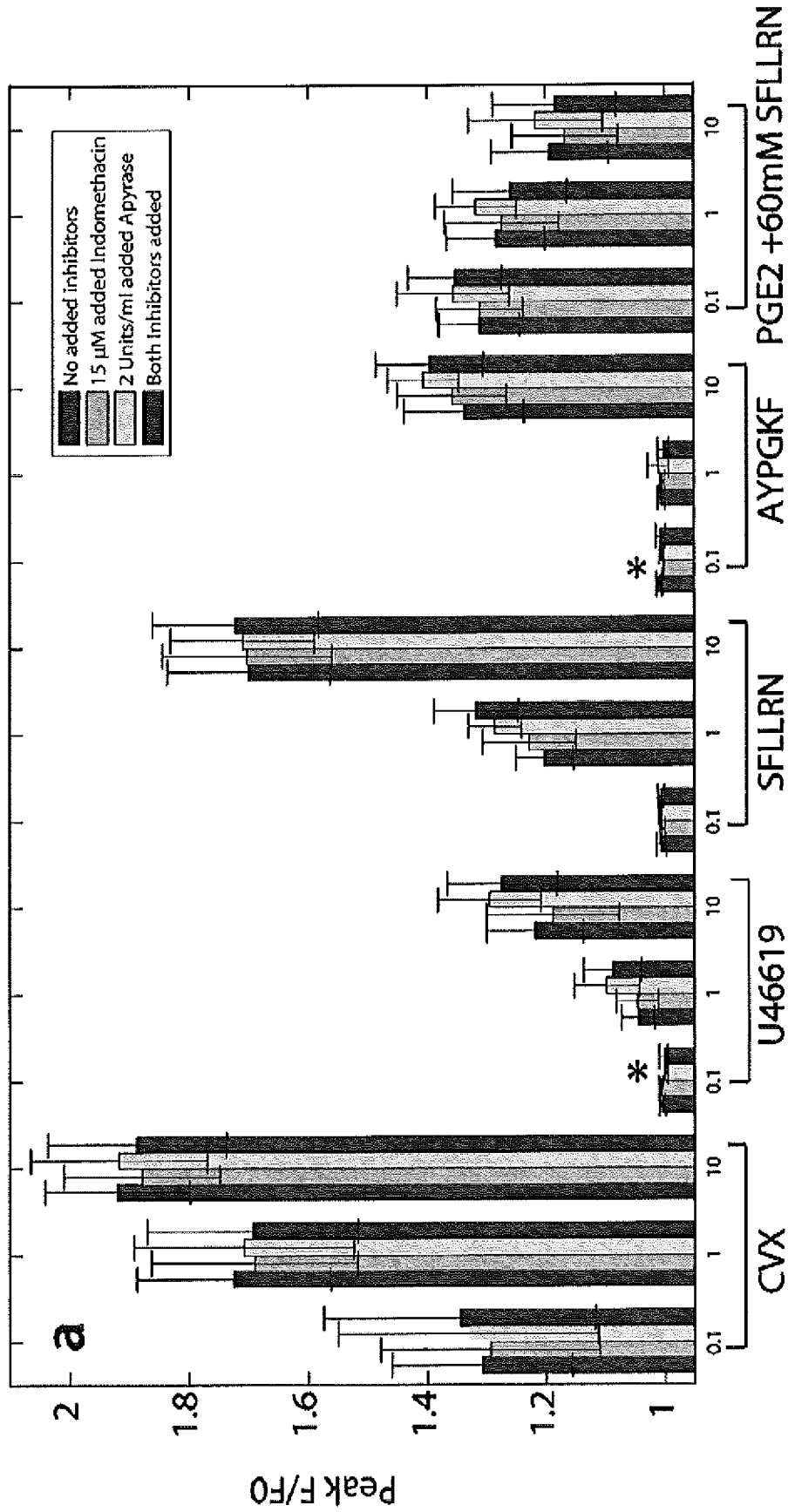


Fig. 6b

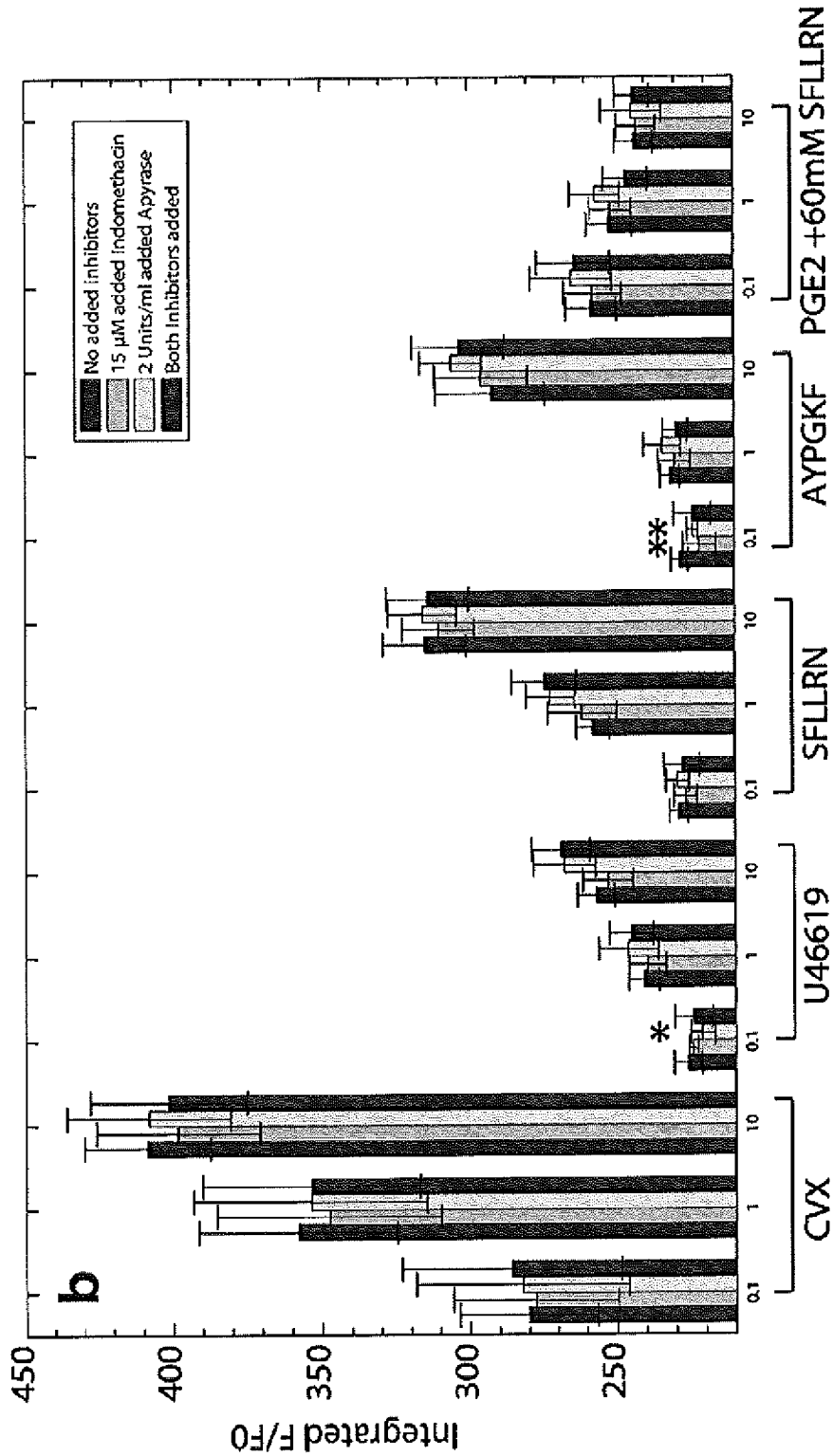


Fig. 7

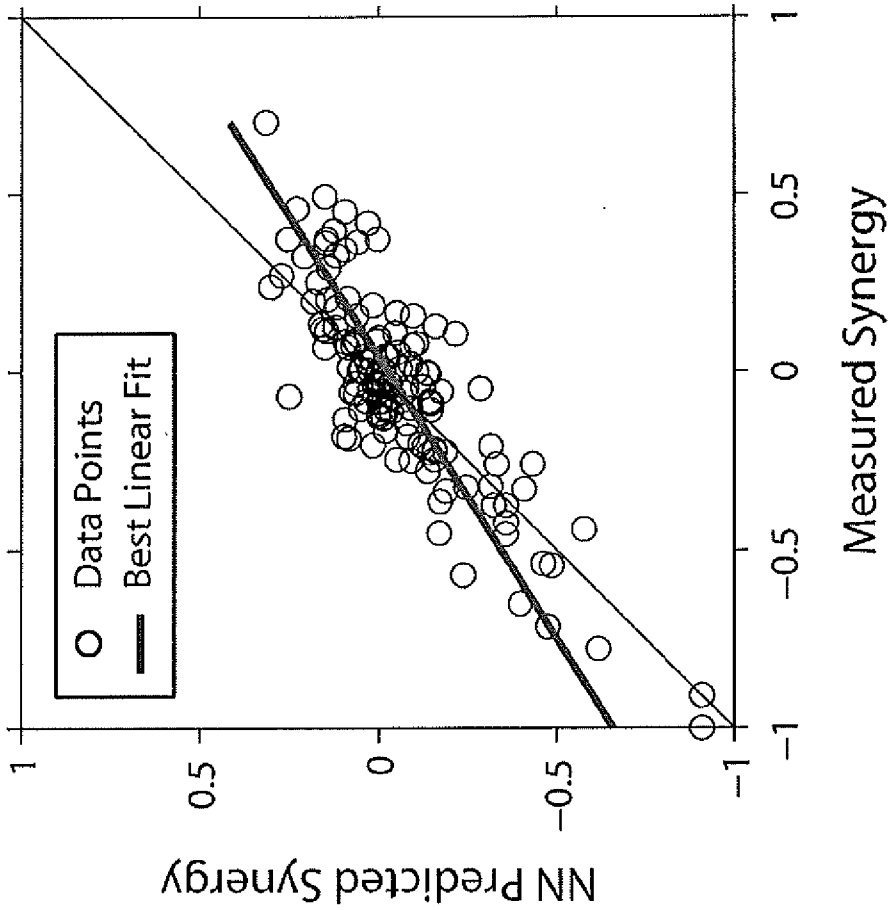


Fig. 8

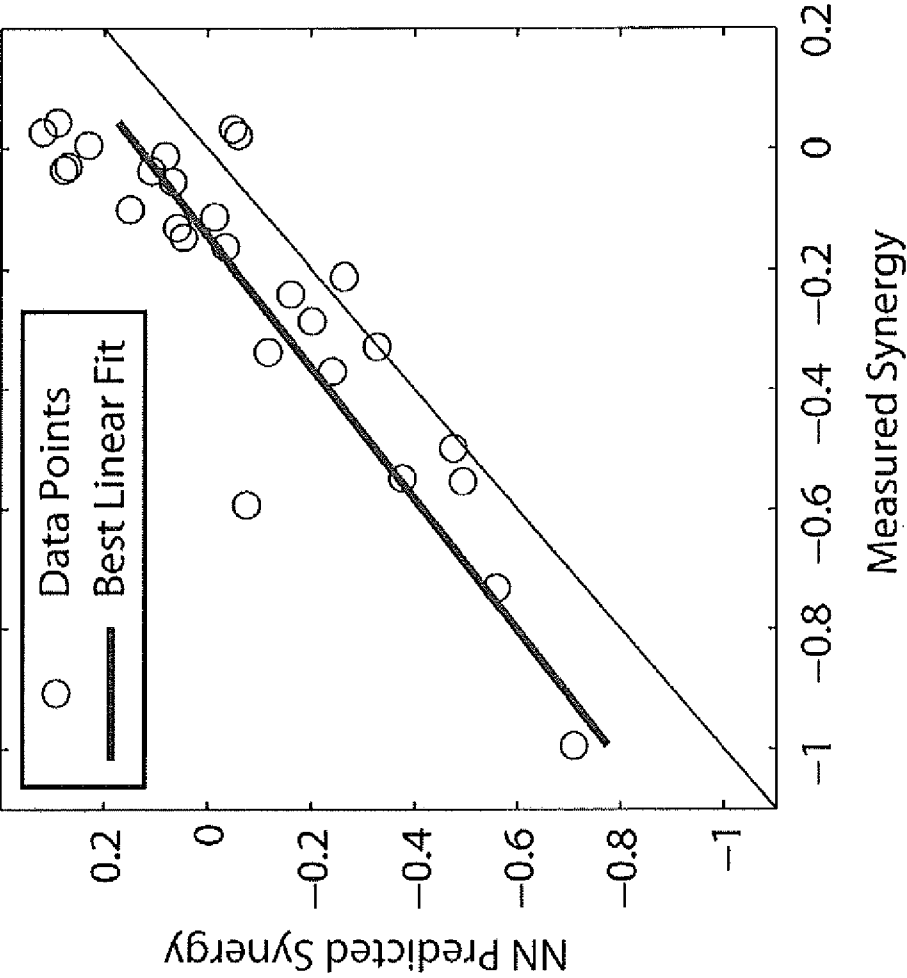


Fig. 9

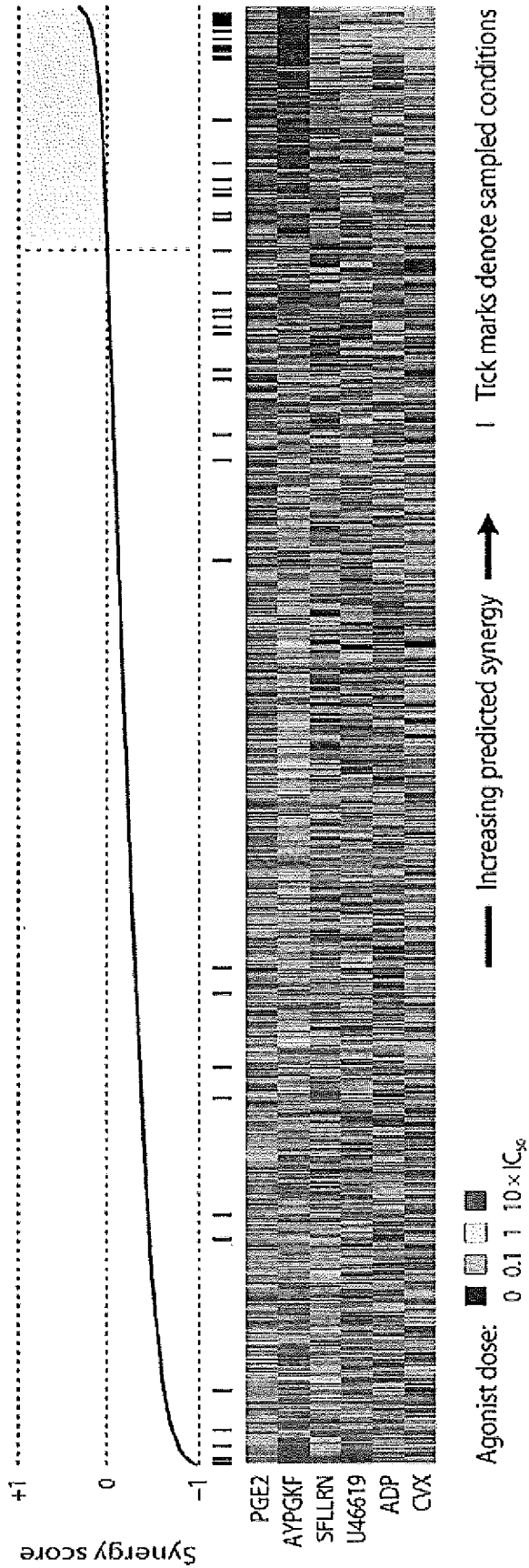


Fig. 10a

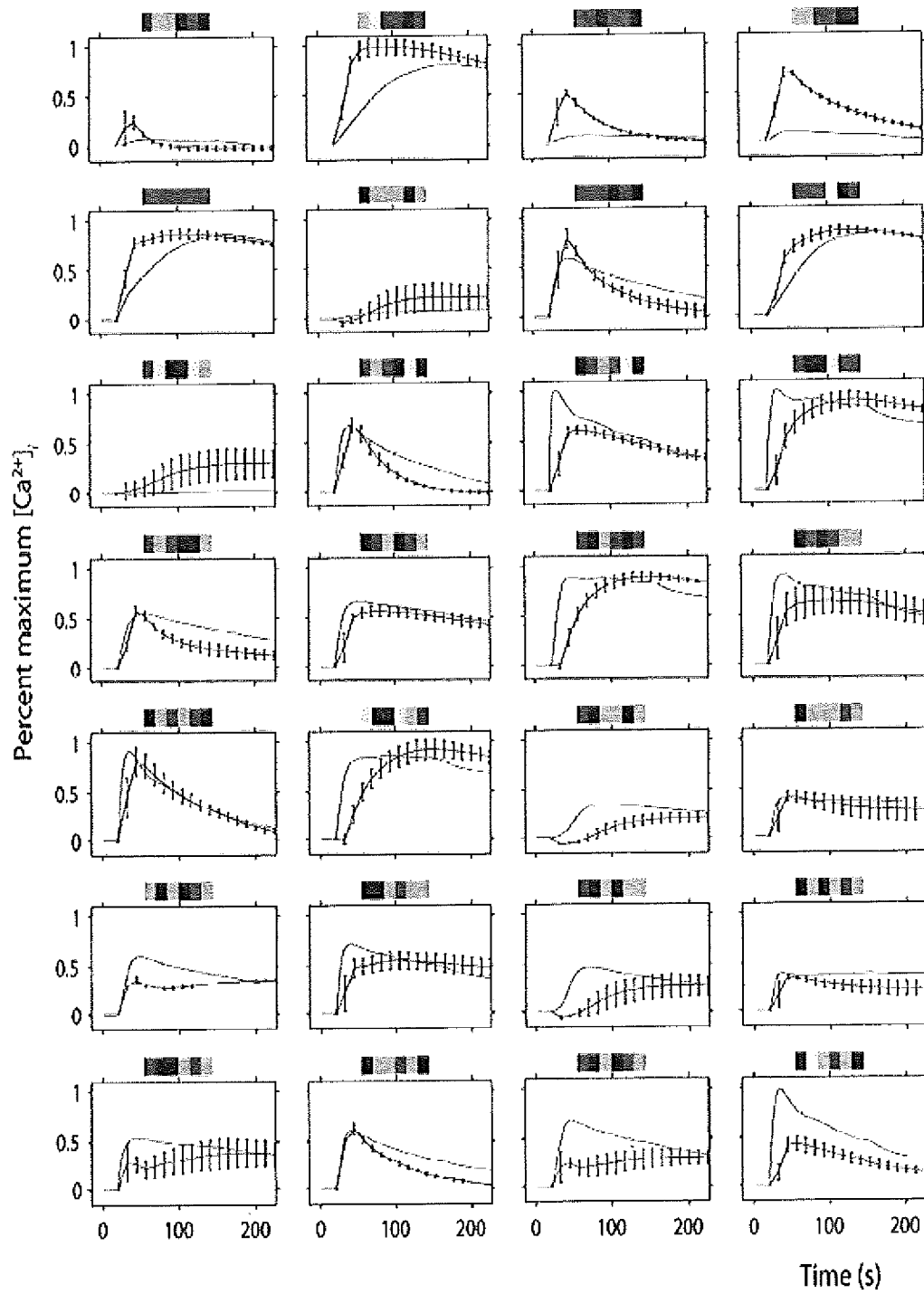


Fig. 10b

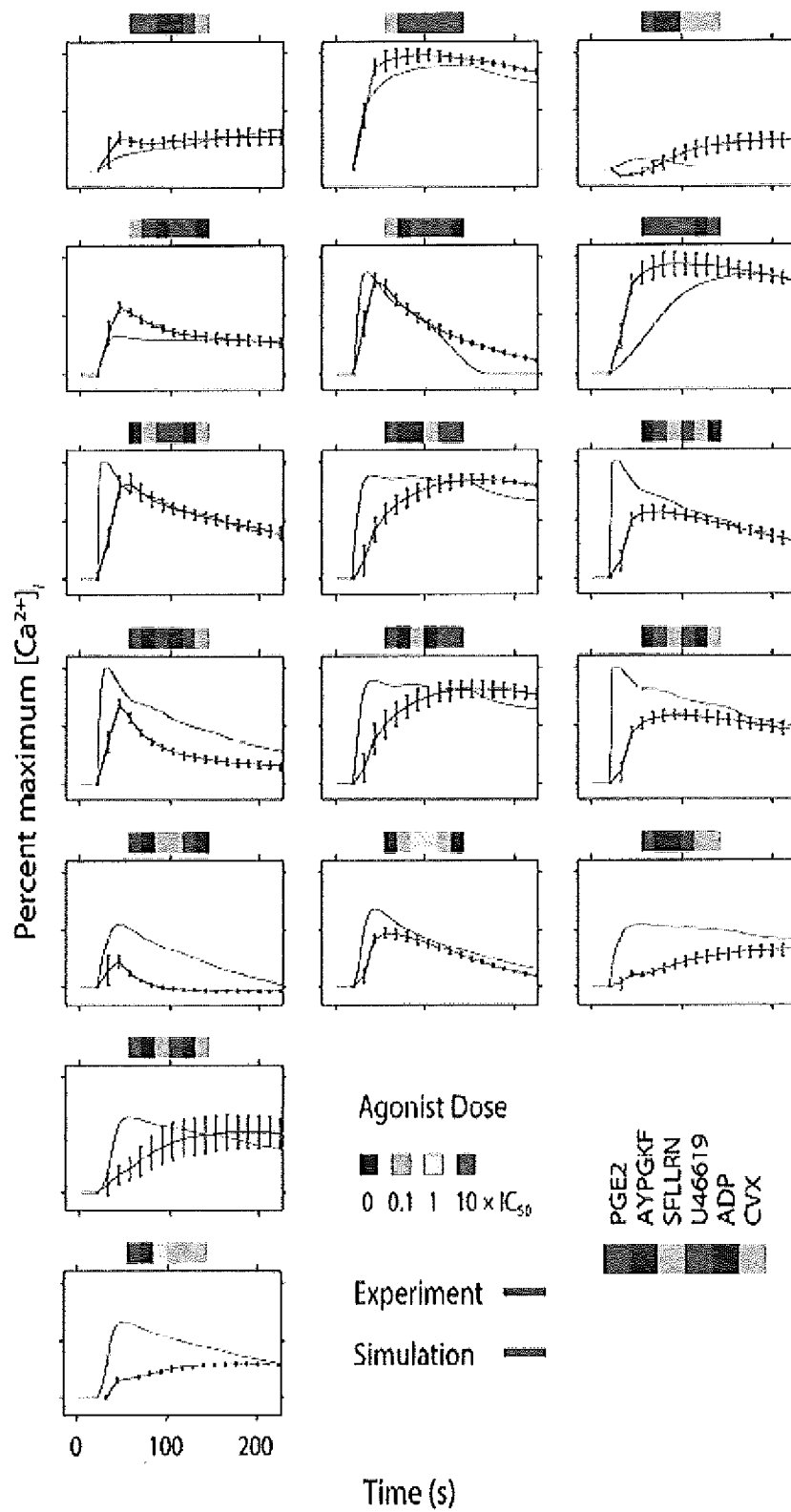
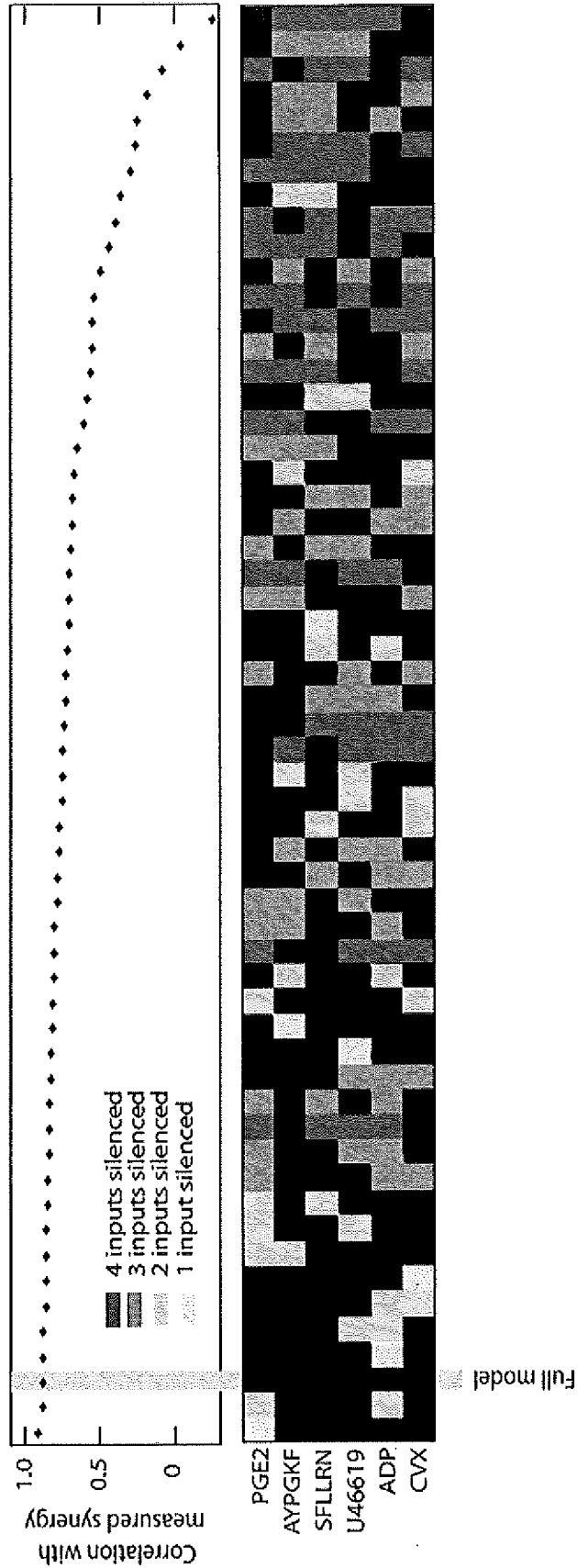


Fig. 11



$\triangle$

Fig. 12A

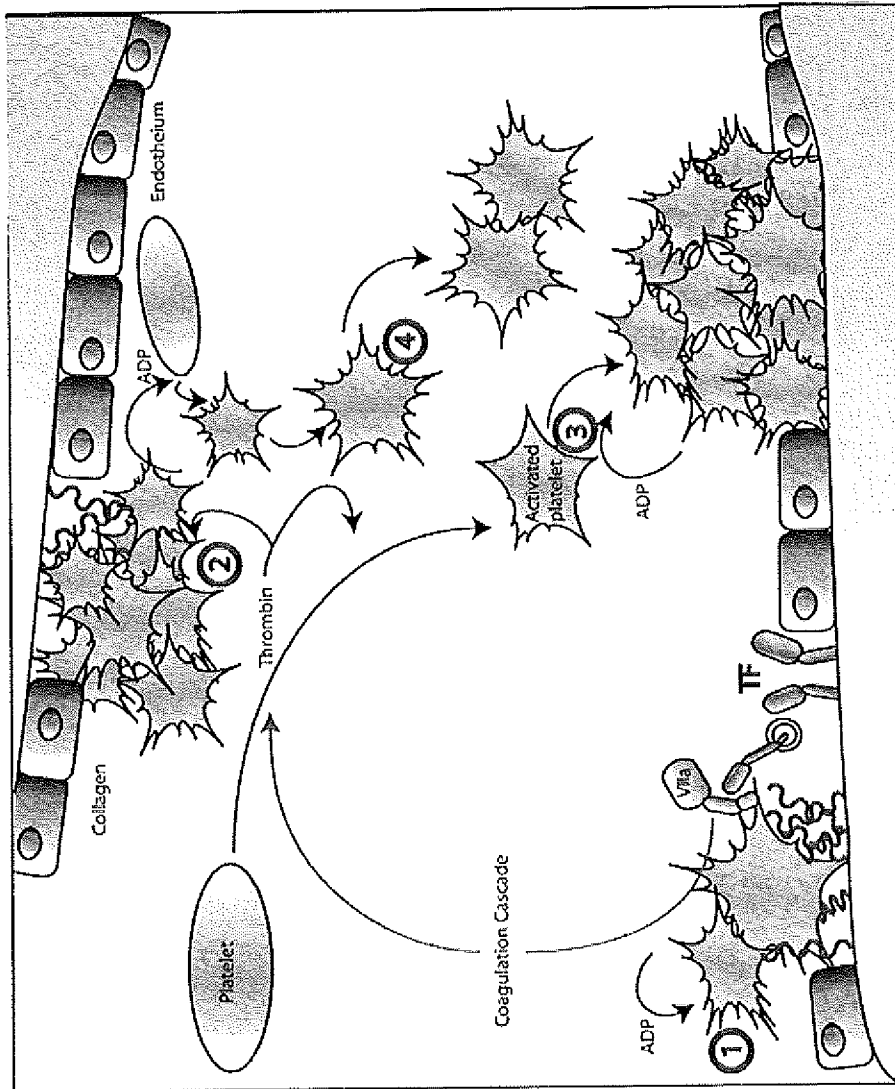


Fig. 12b

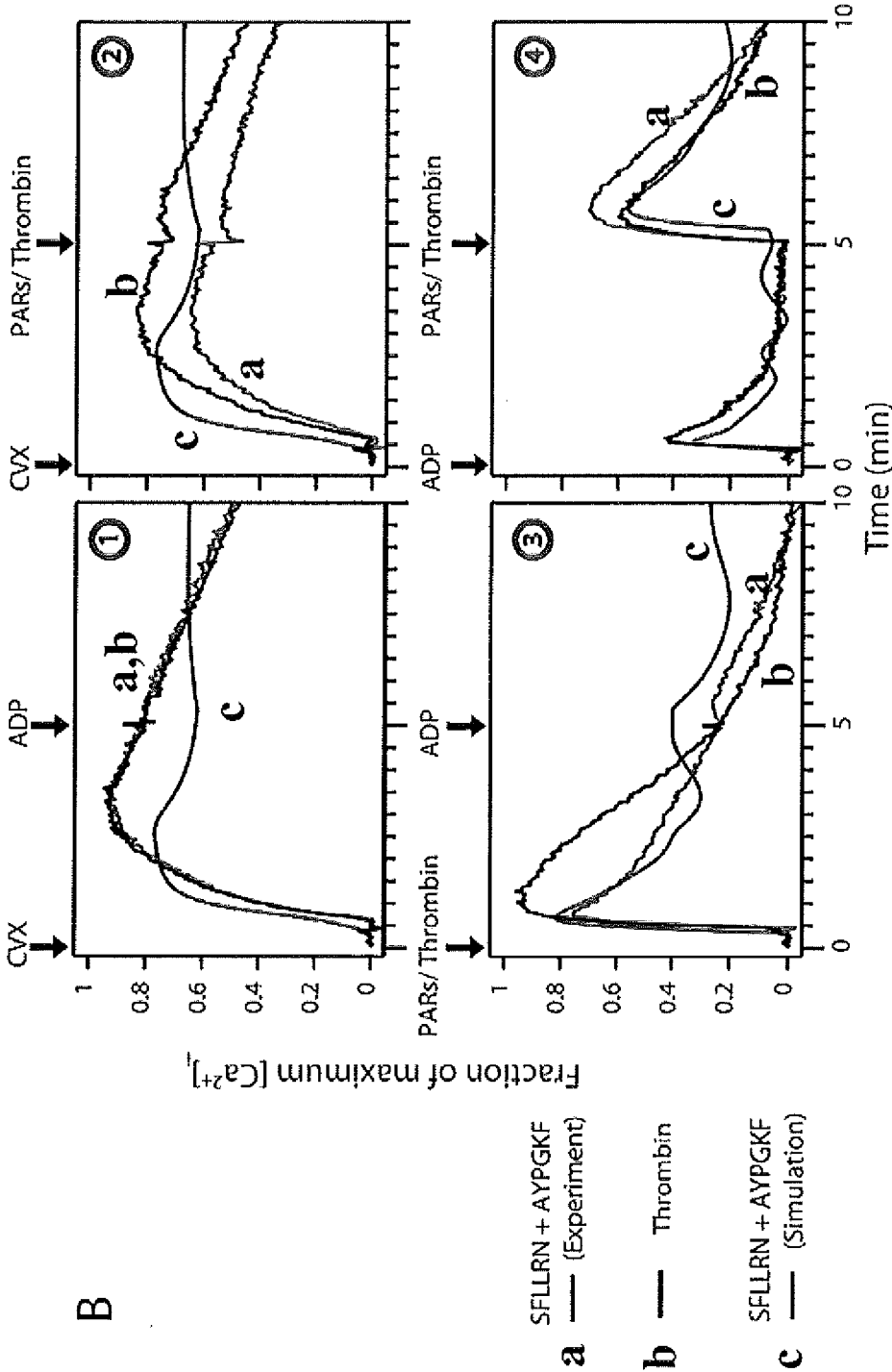
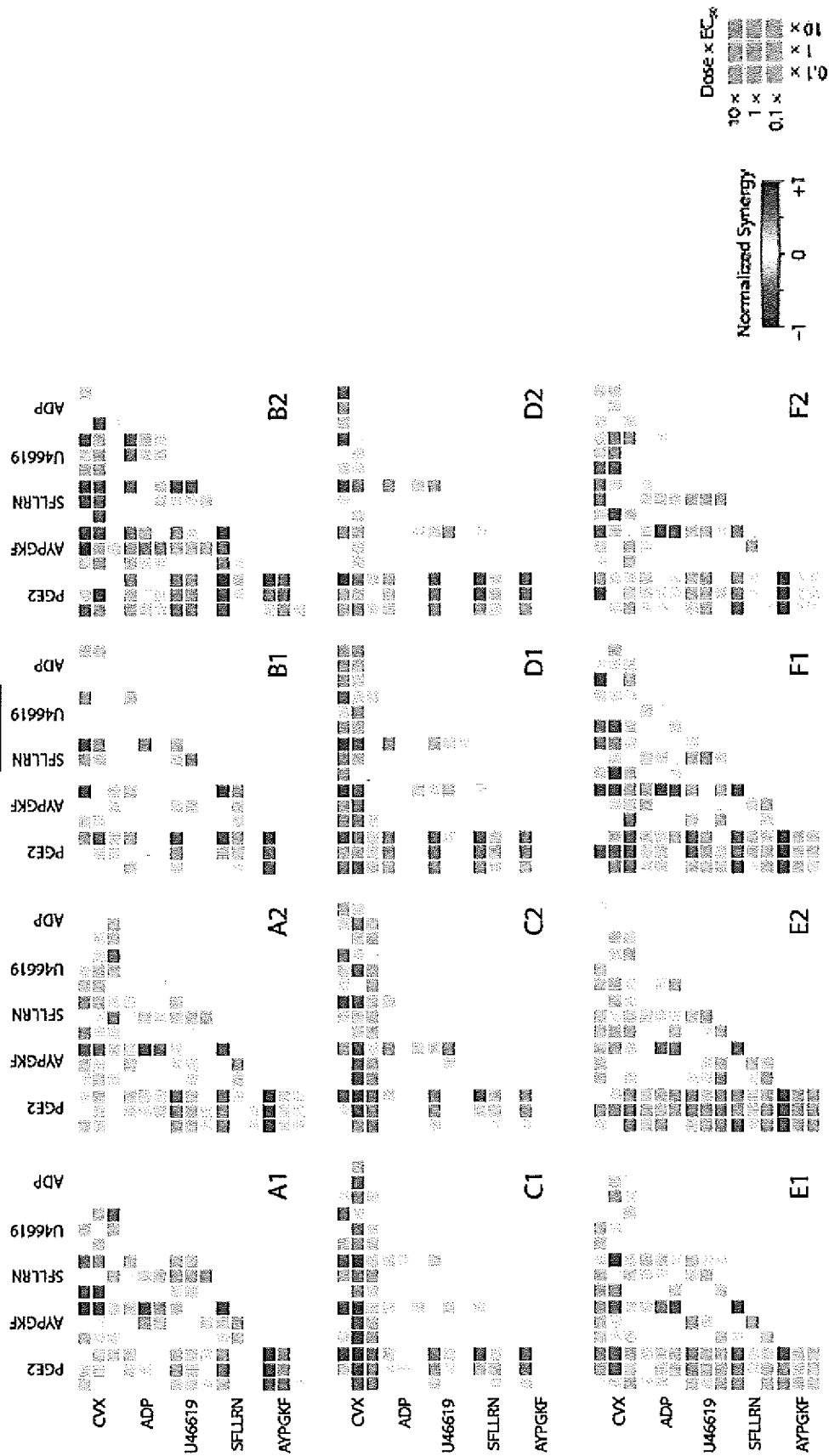


Fig. 13a



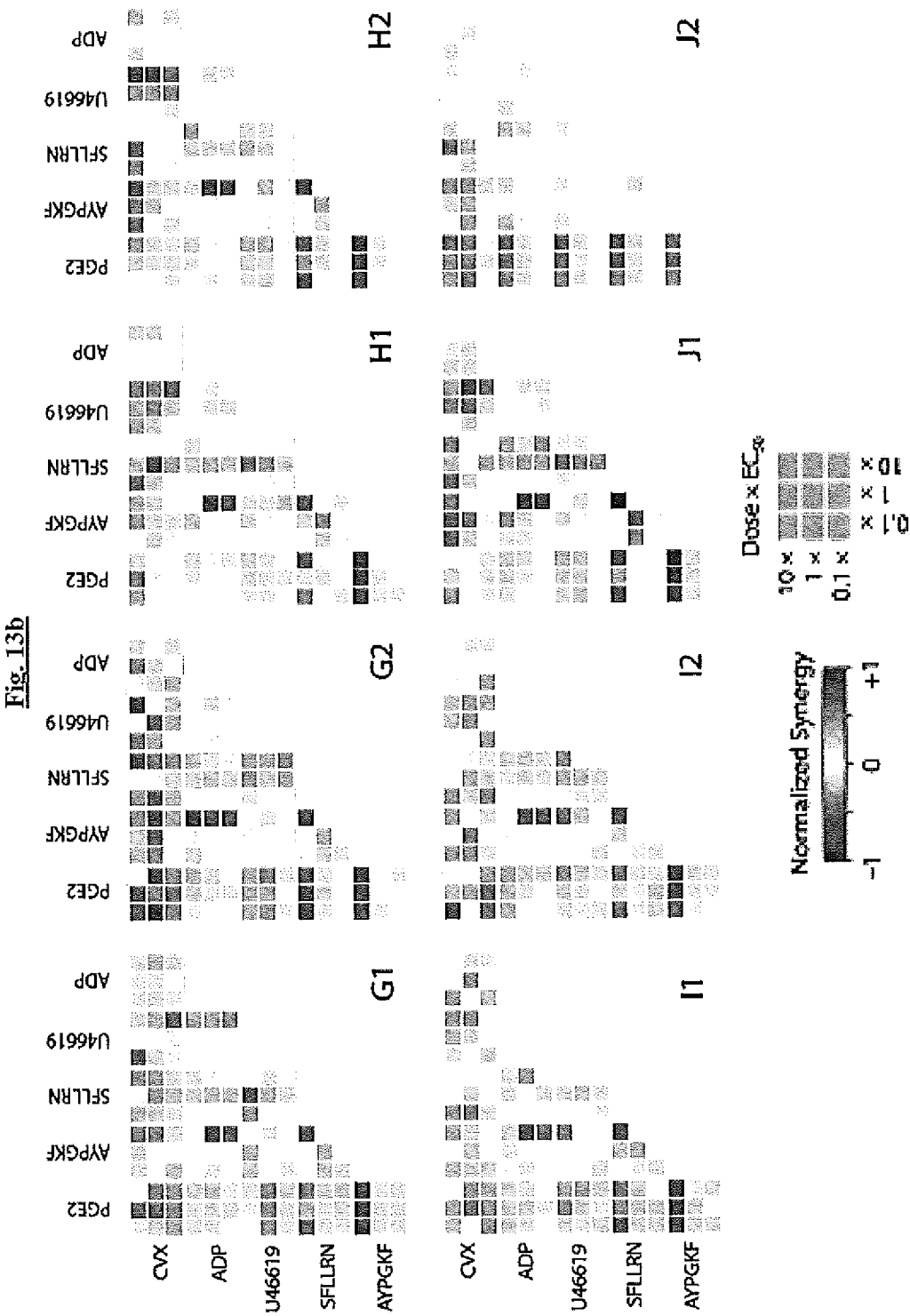


Fig. 14

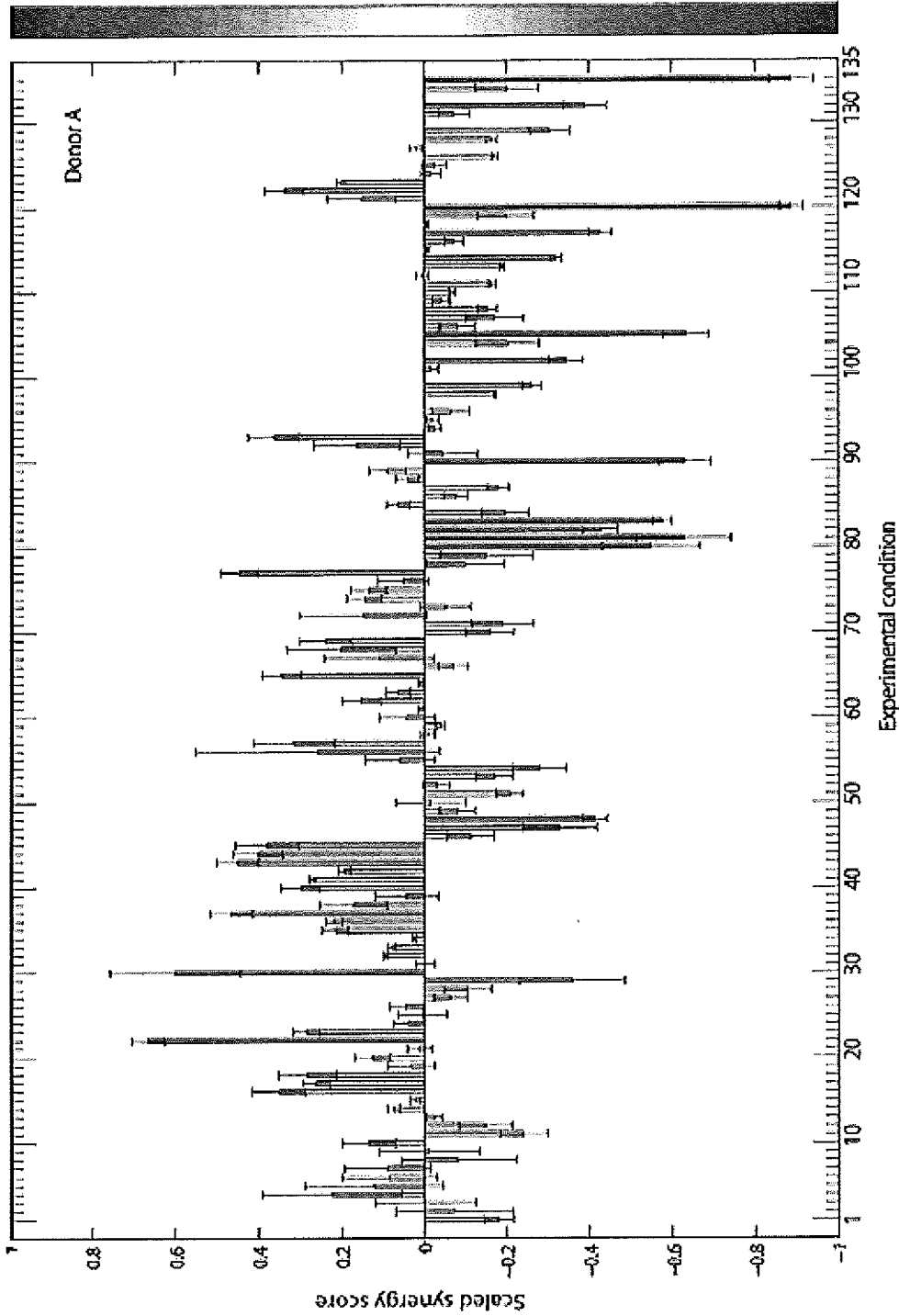


Fig. 15

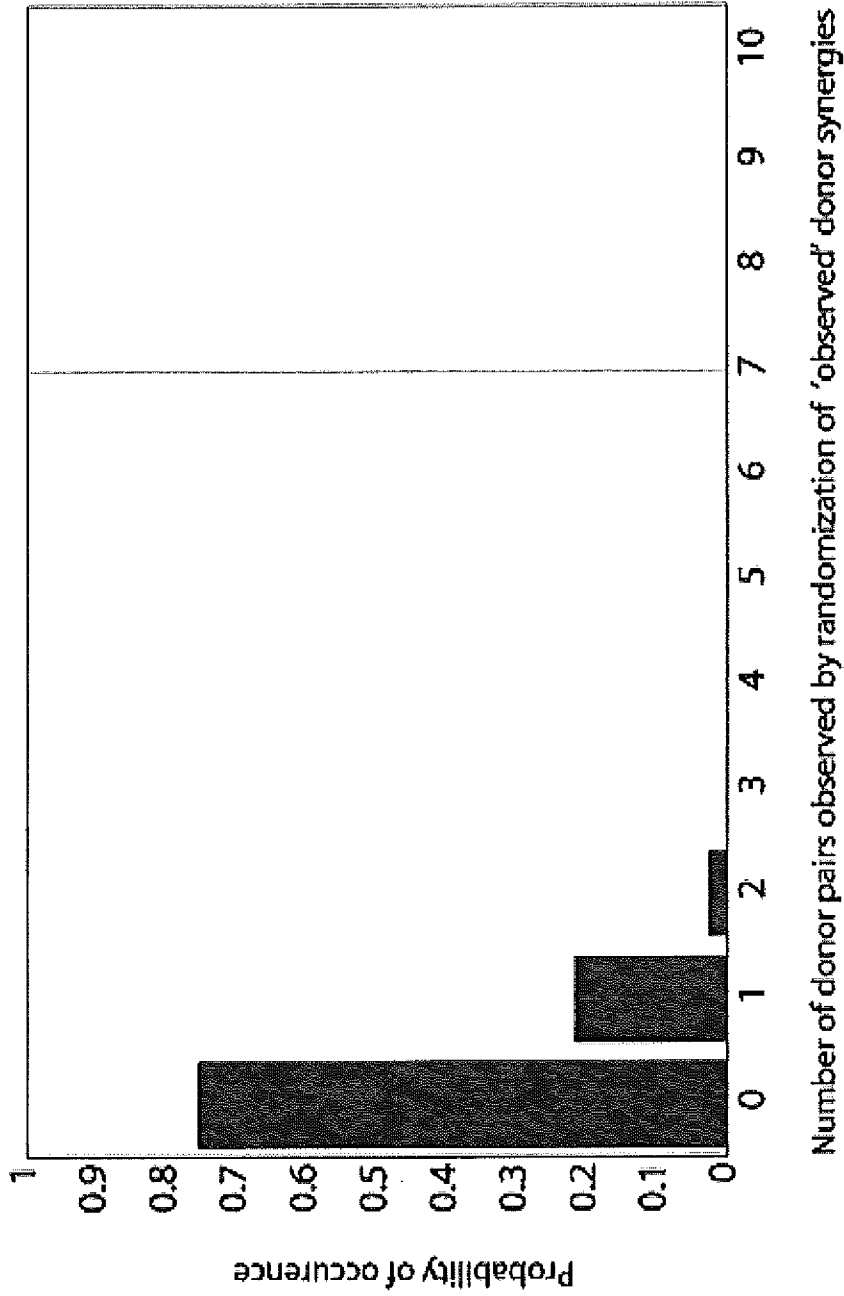


Fig. 16

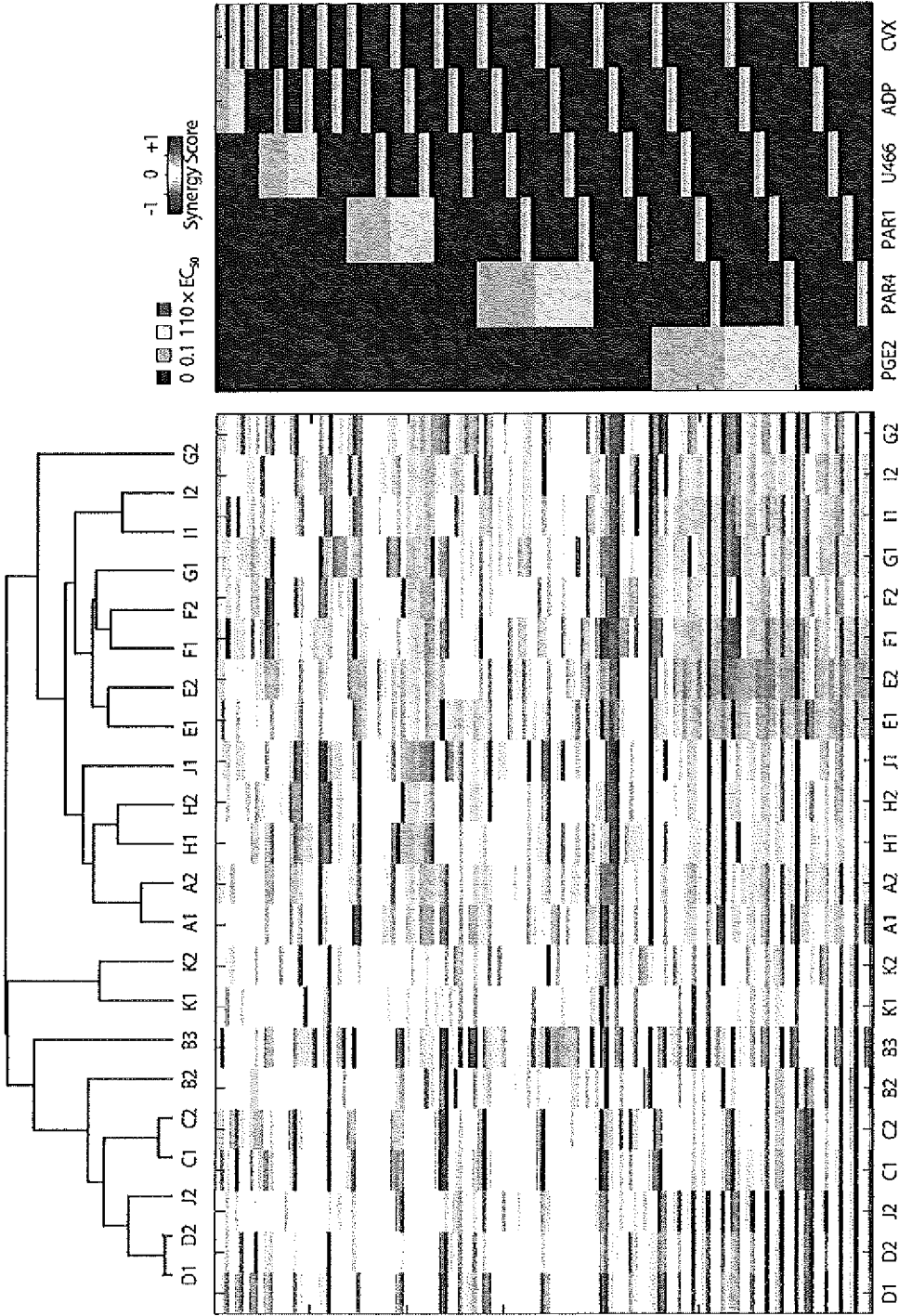


Fig. 17

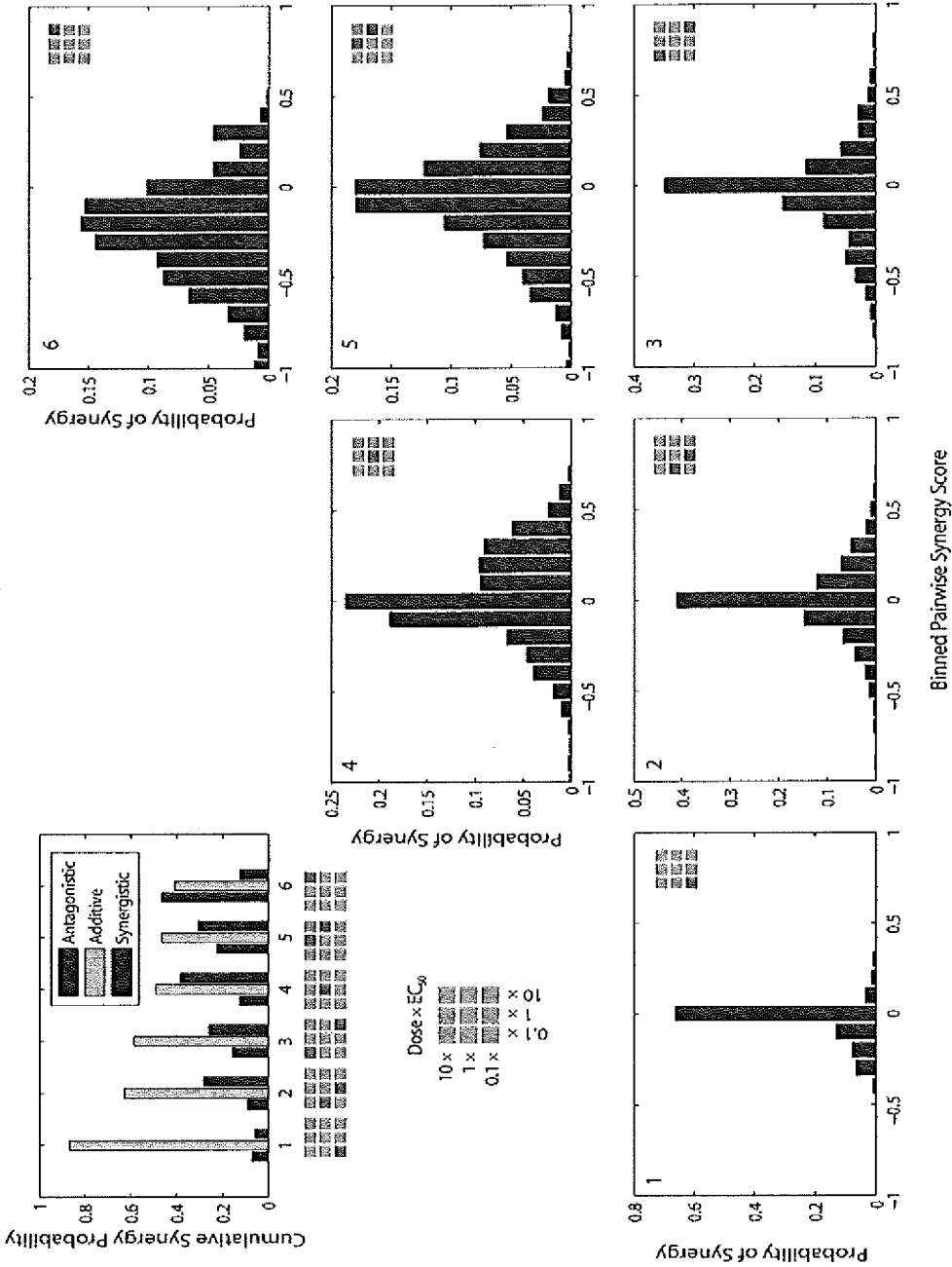


Fig. 18

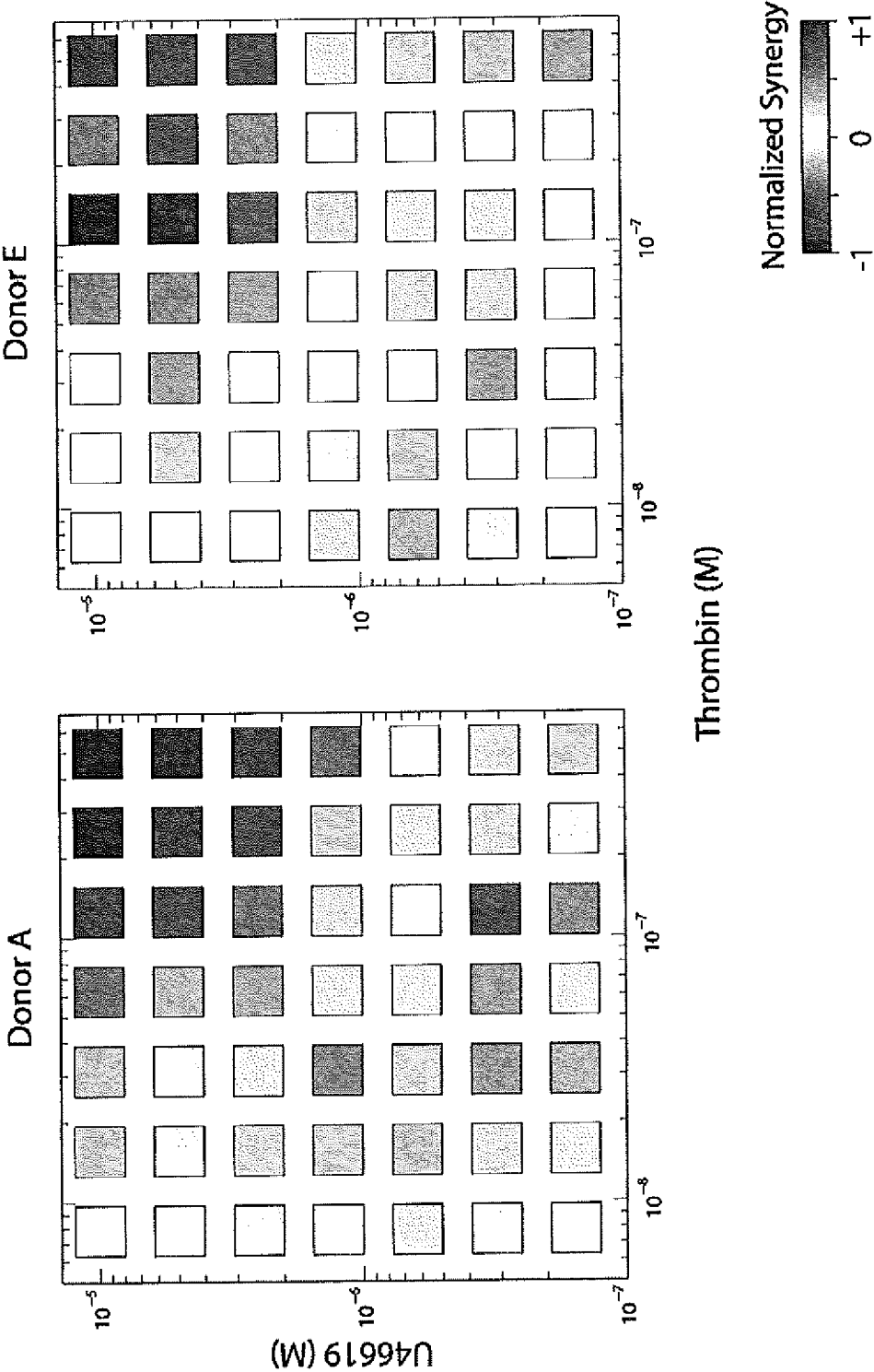


Fig. 19a

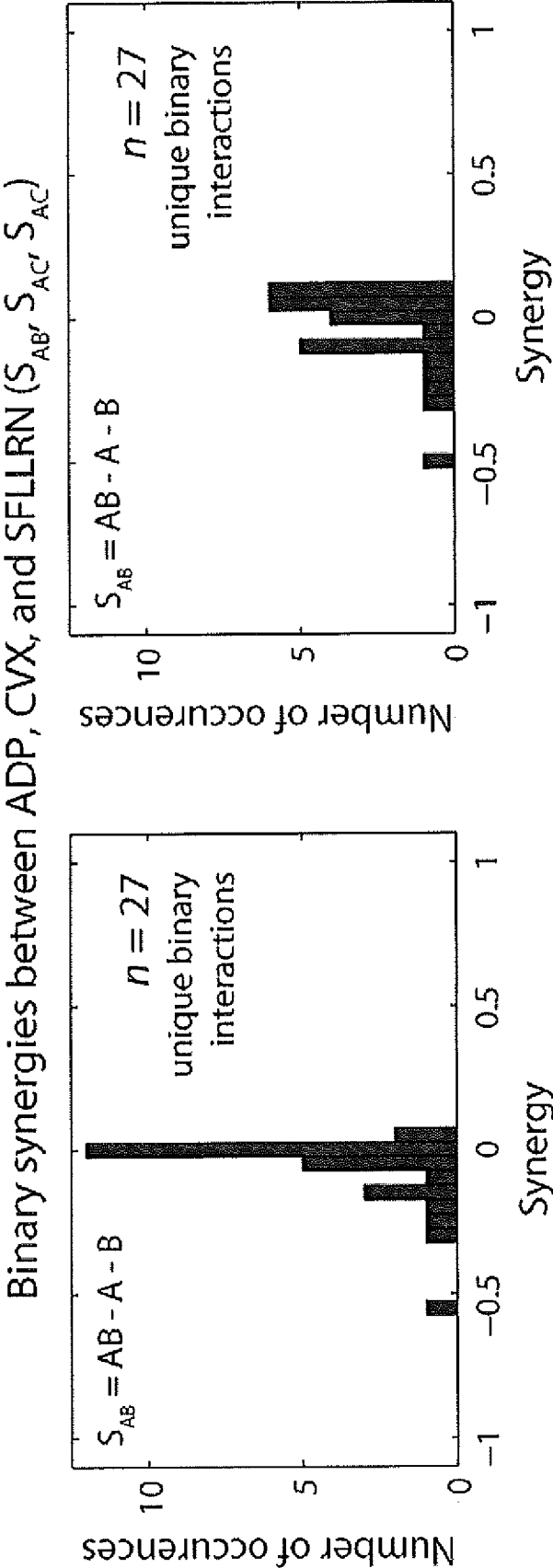


Fig. 19b

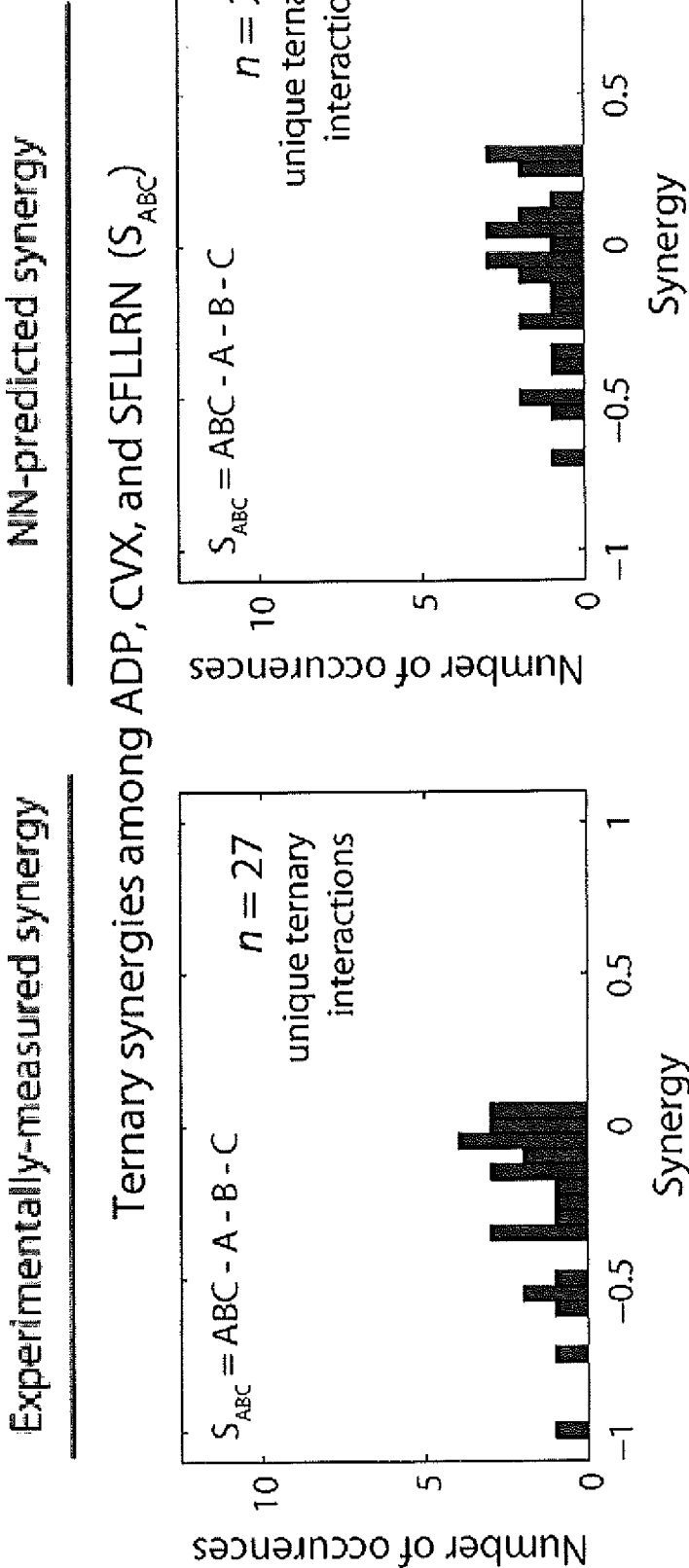


Fig. 19c

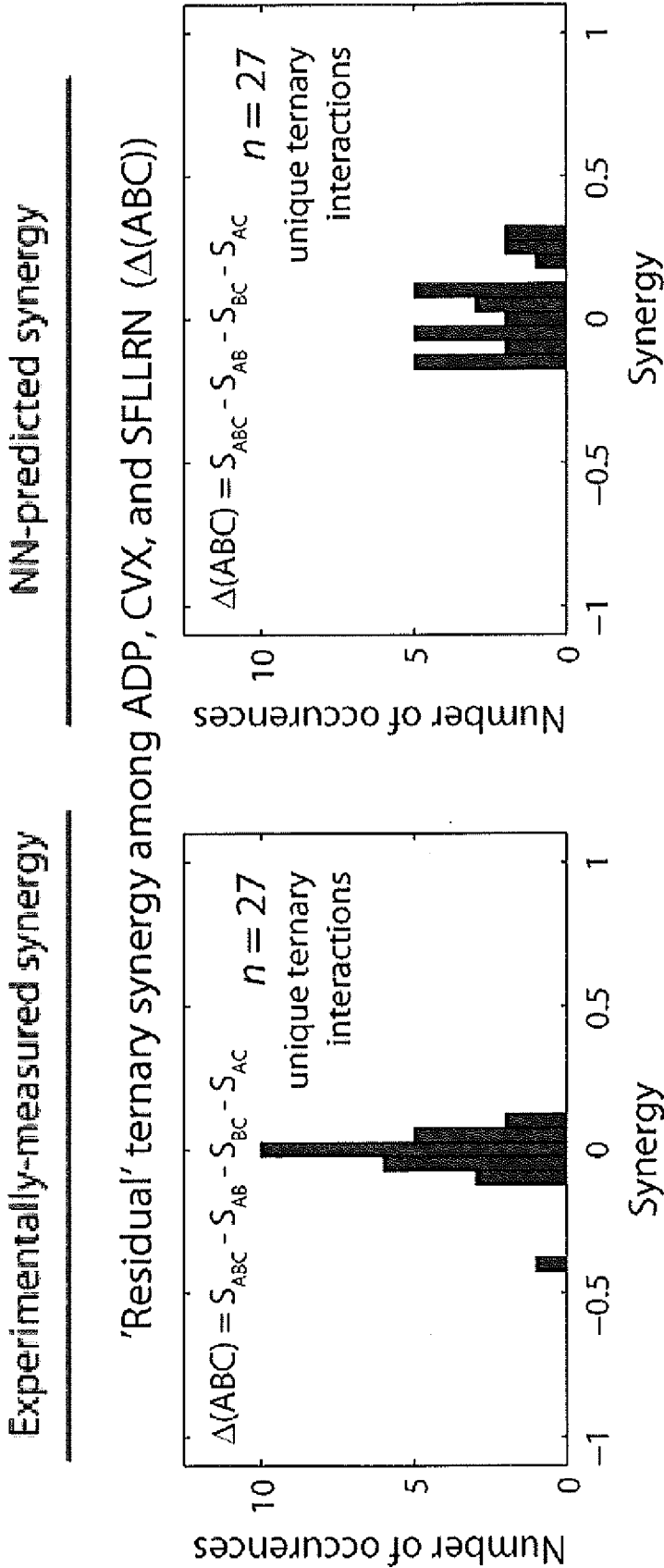


Fig. 20a

Time course traces for pairwise agonists

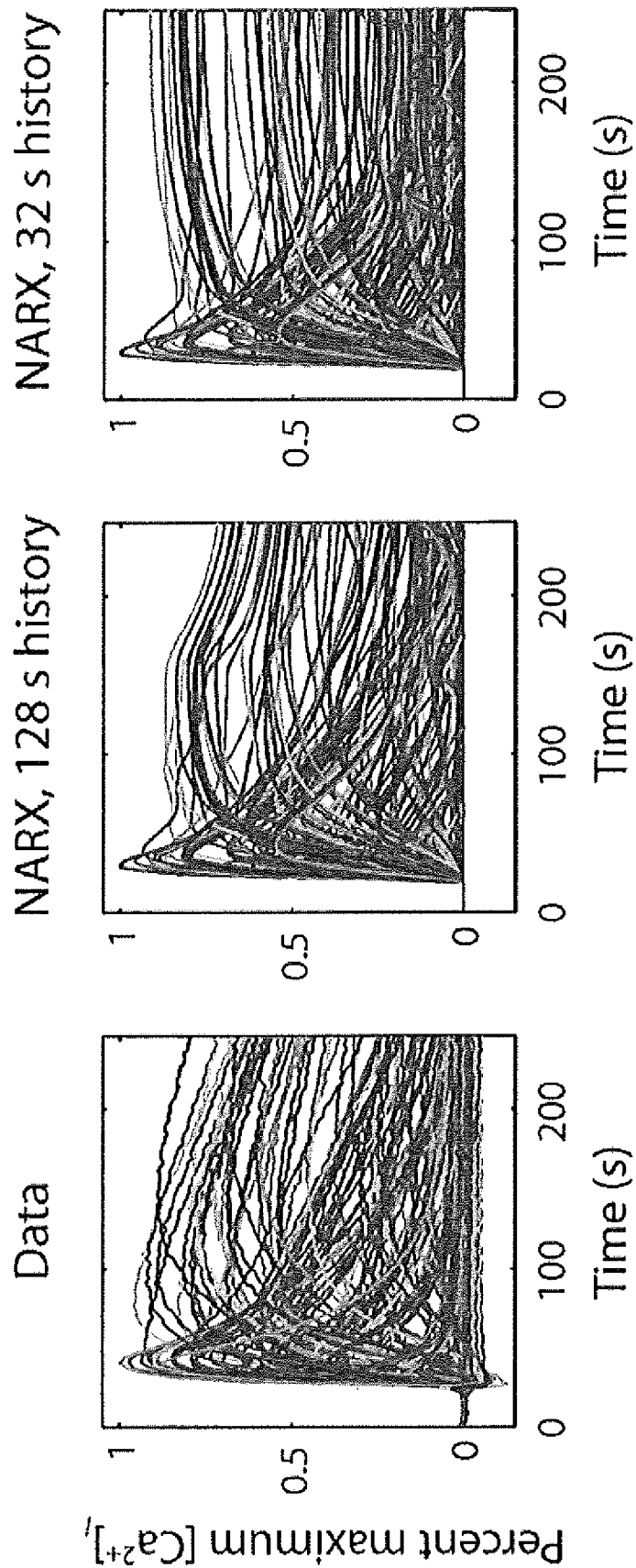
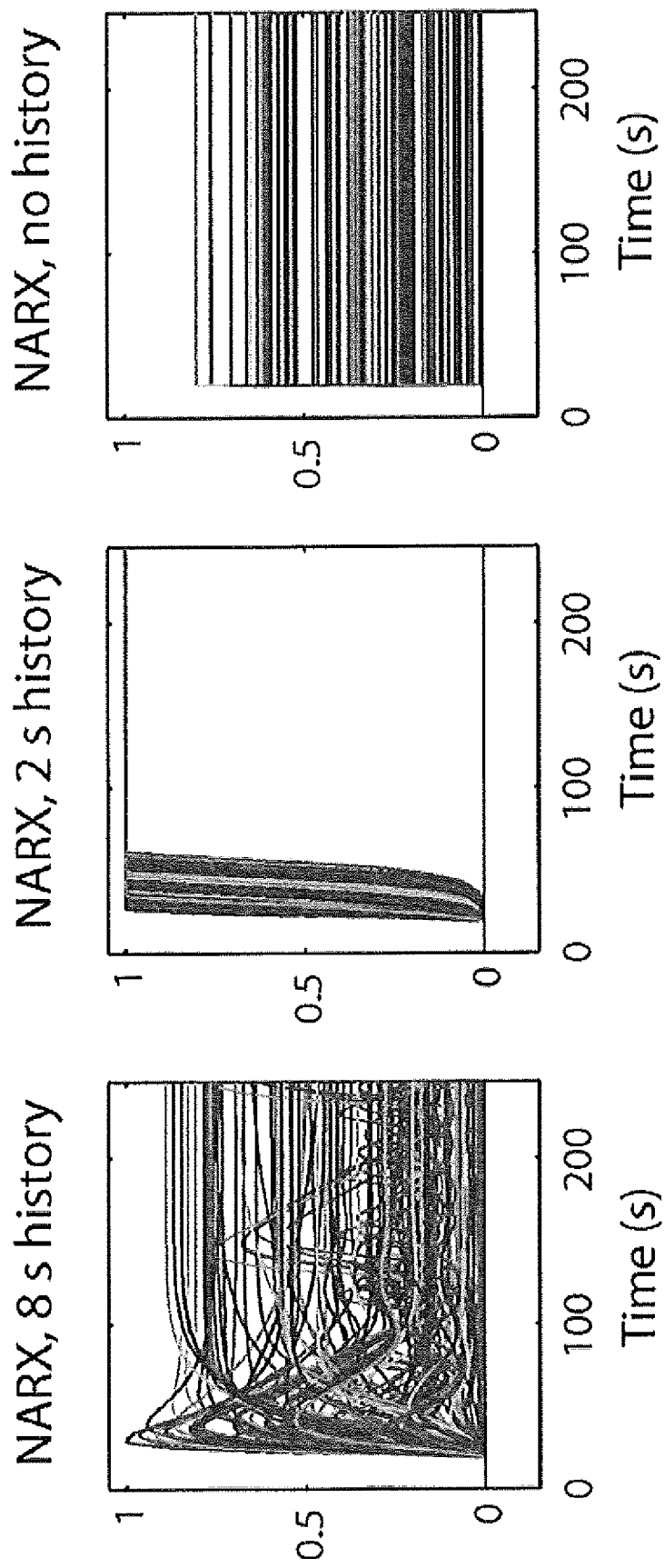


Fig. 20b

Time course traces for pairwise agonists



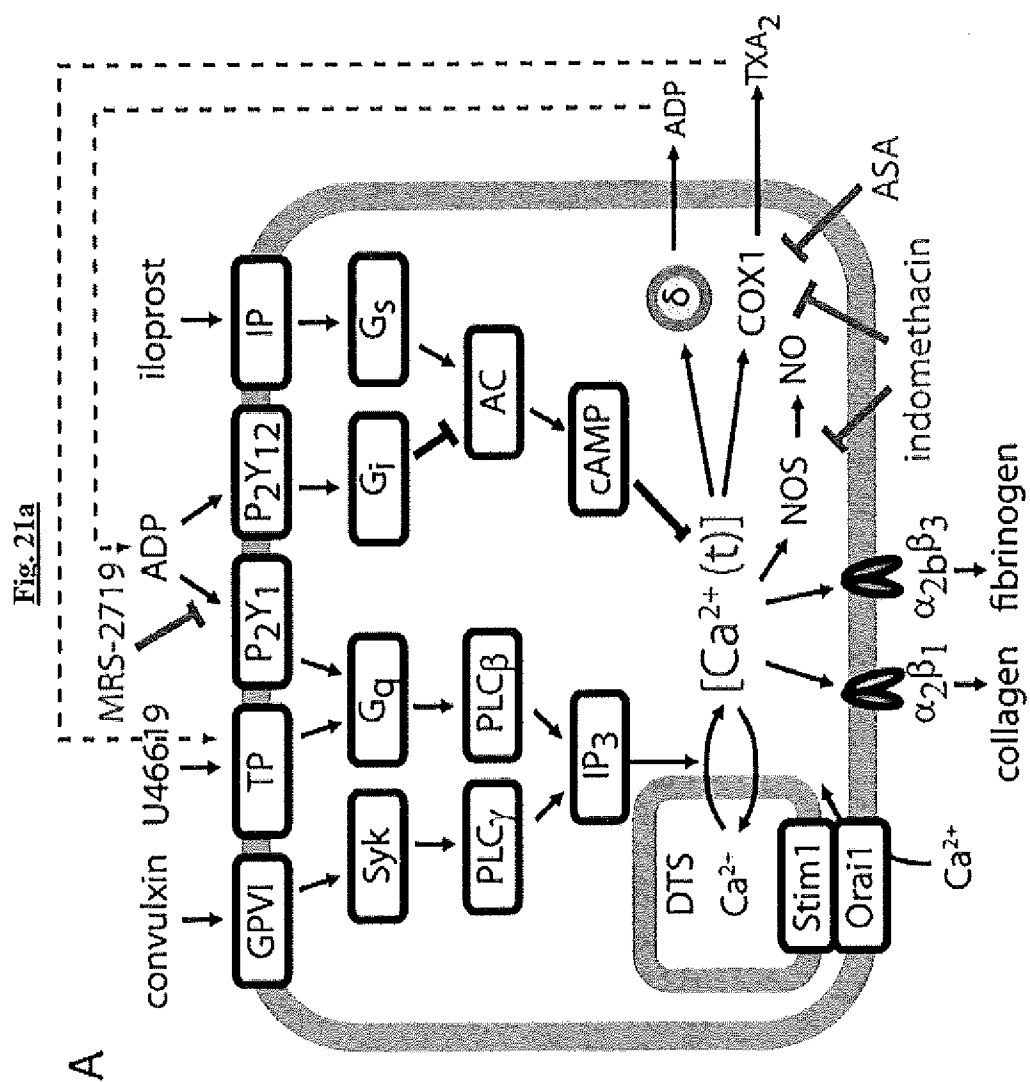
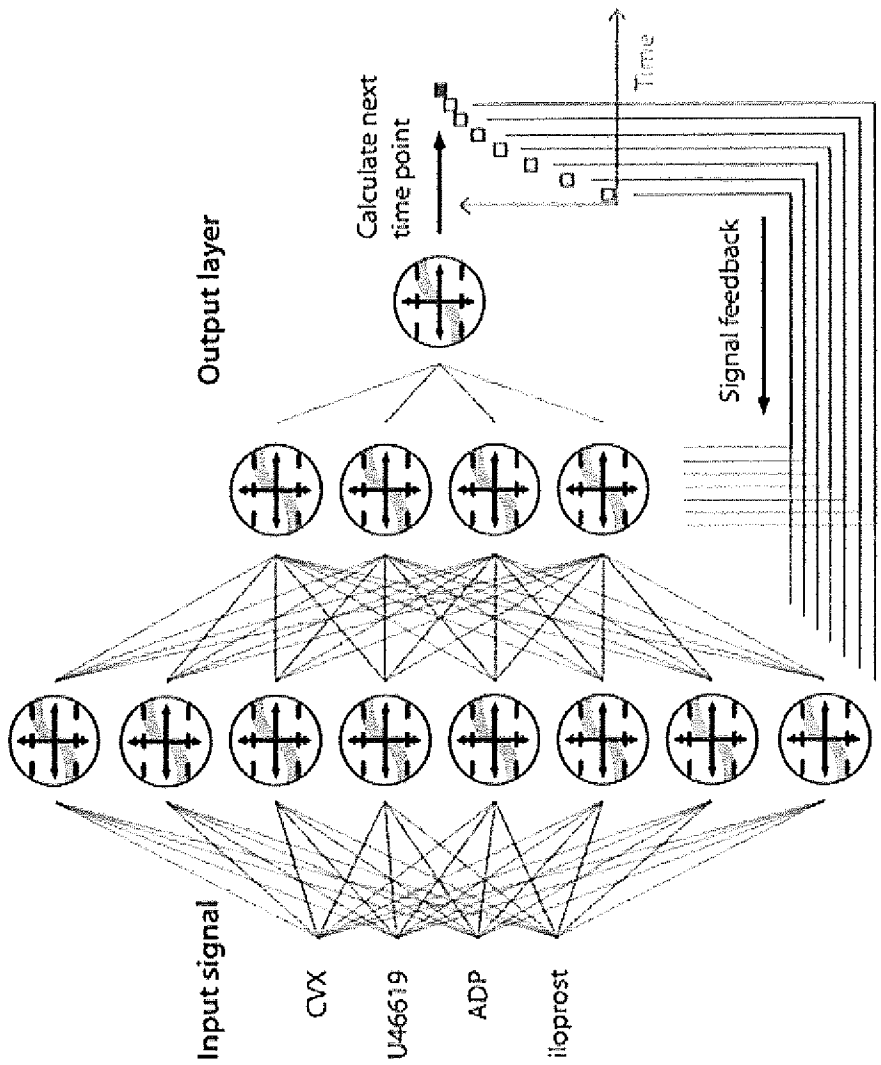


Fig. 21b

Processing layers



B

Fig. 21c

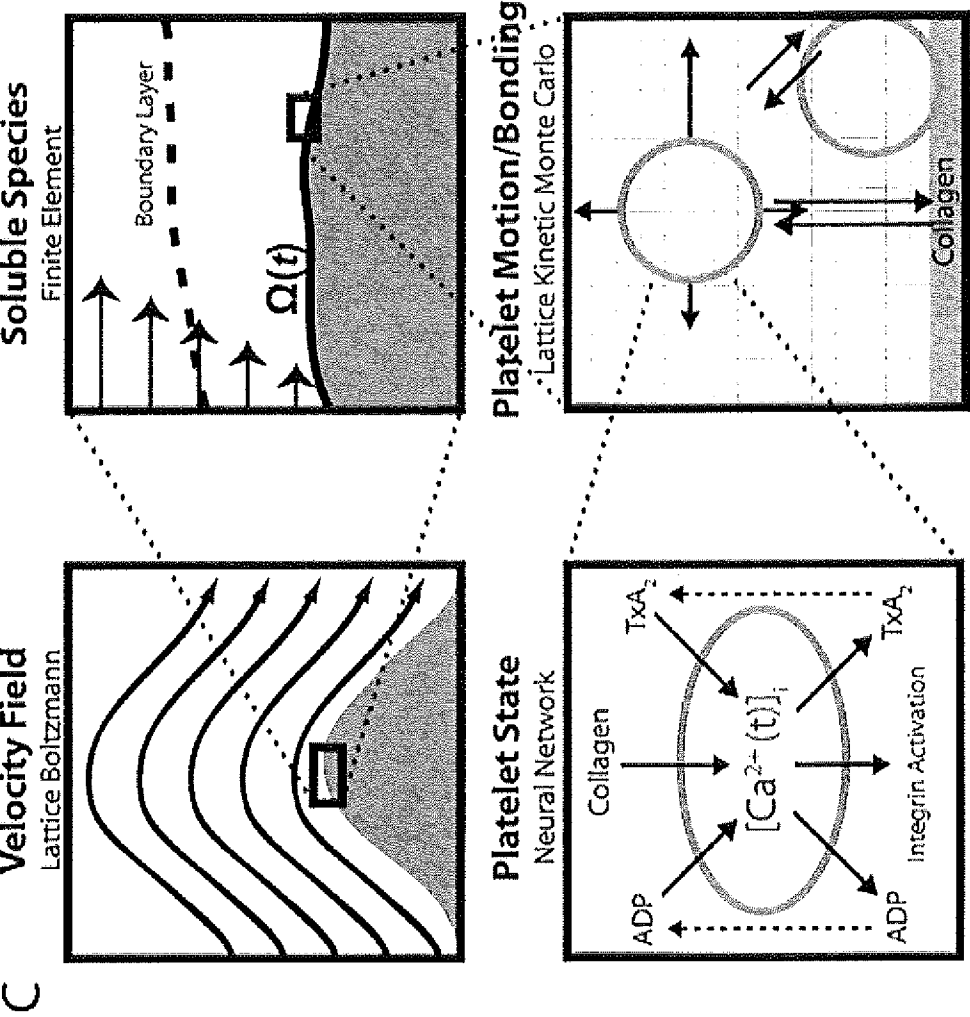


Fig. 21d

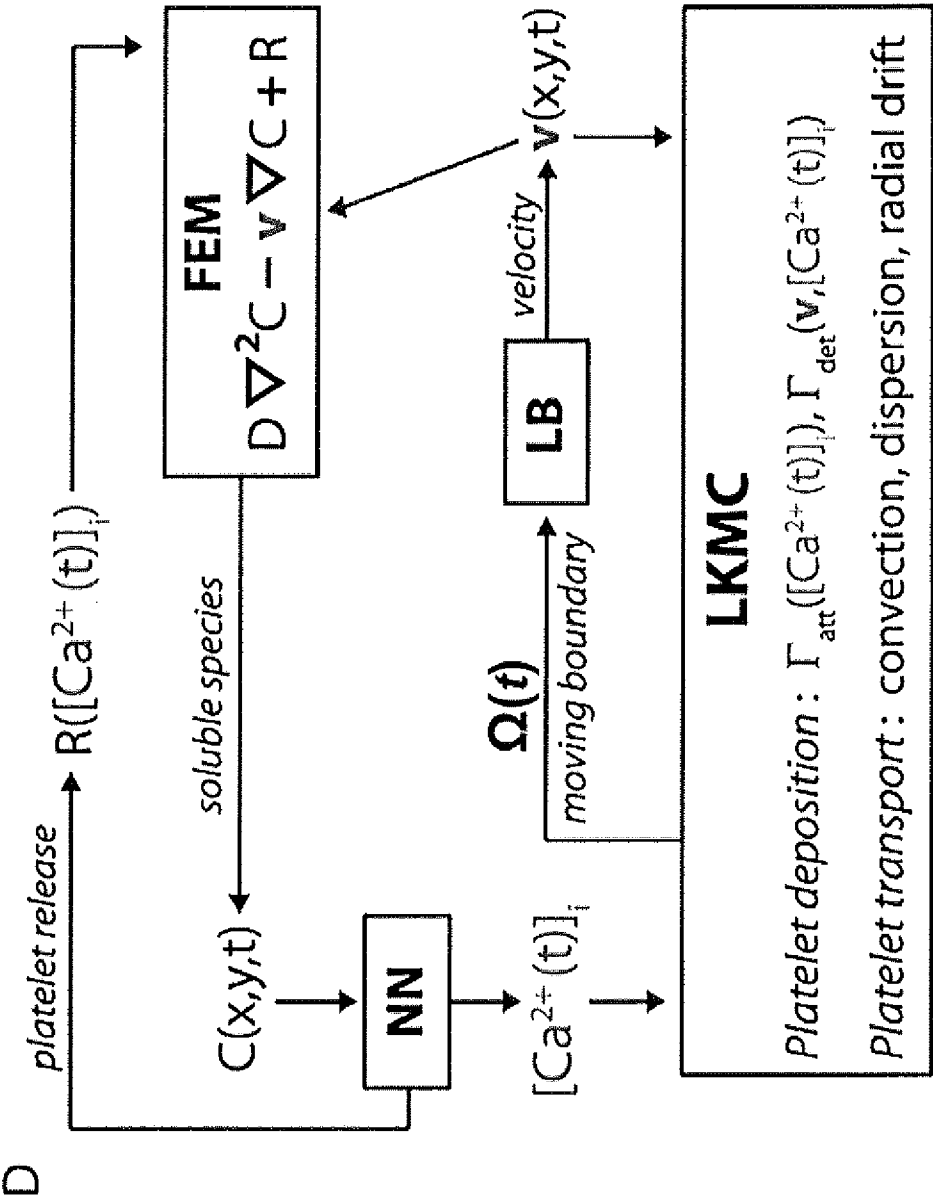


Fig. 22a

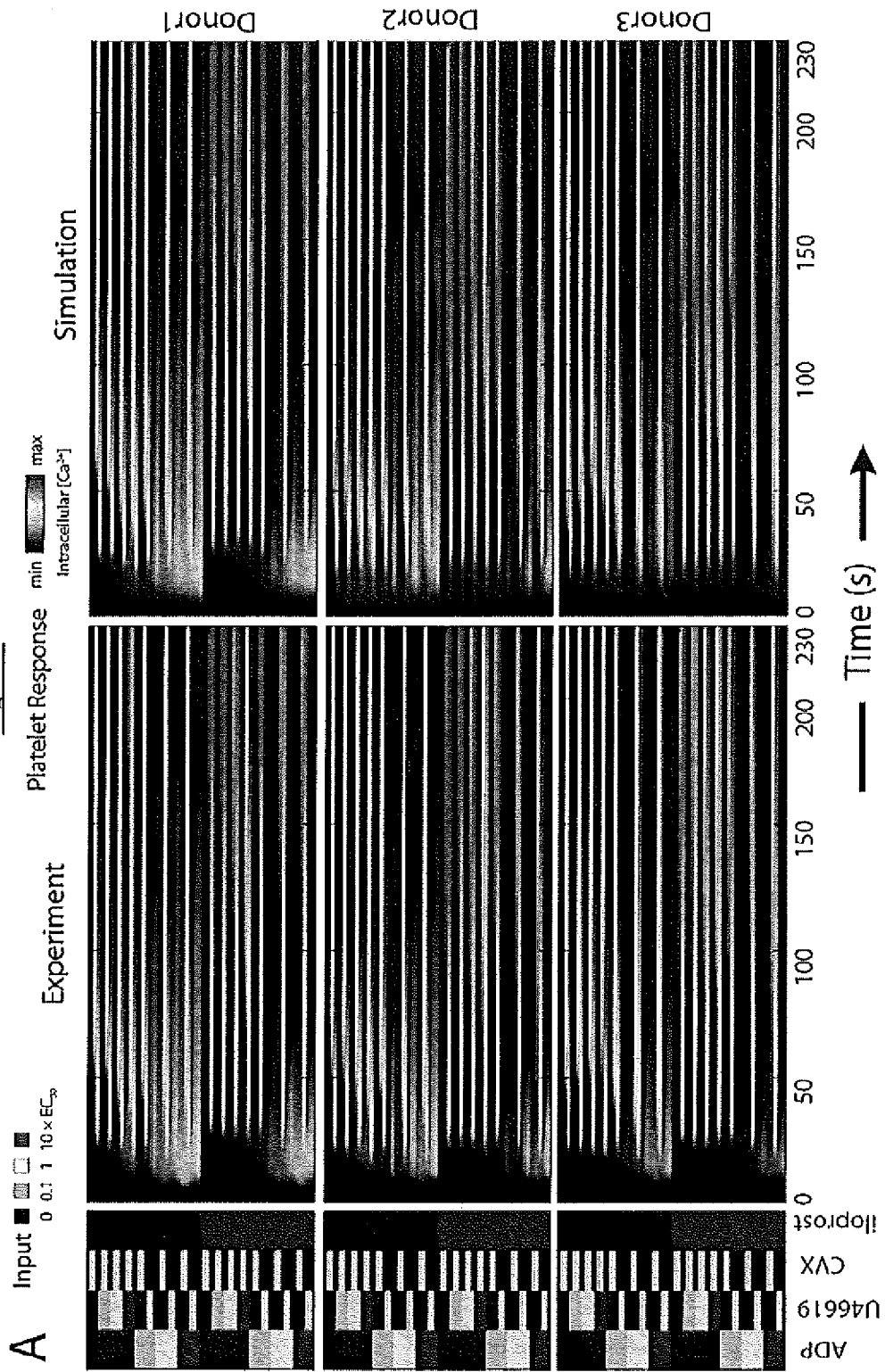


Fig. 22b

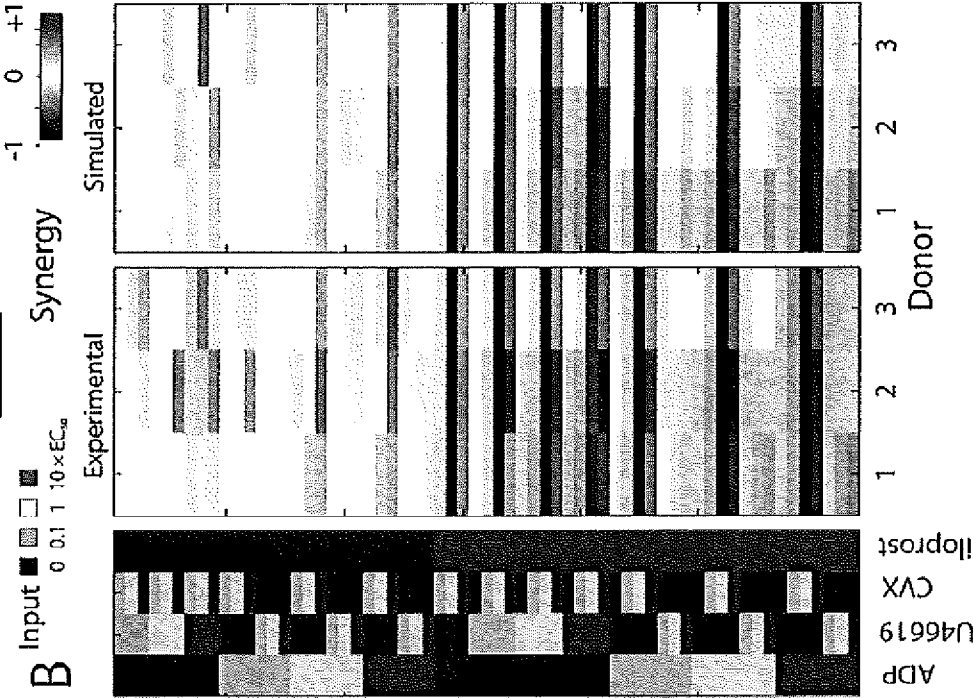


Fig. 22c

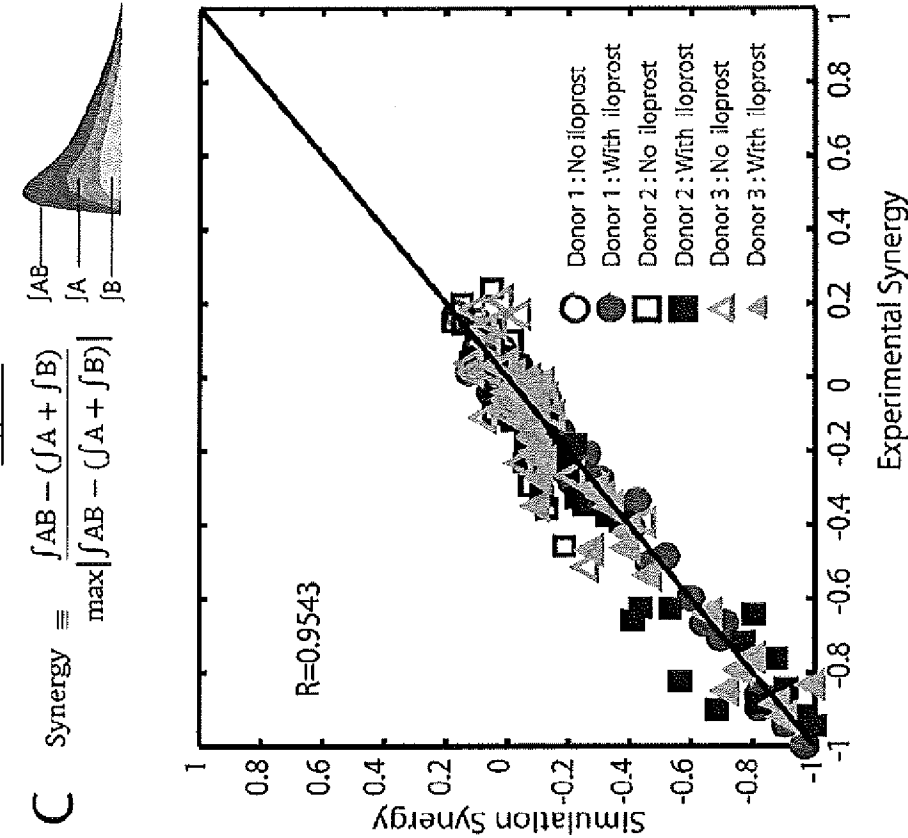


Fig. 23a

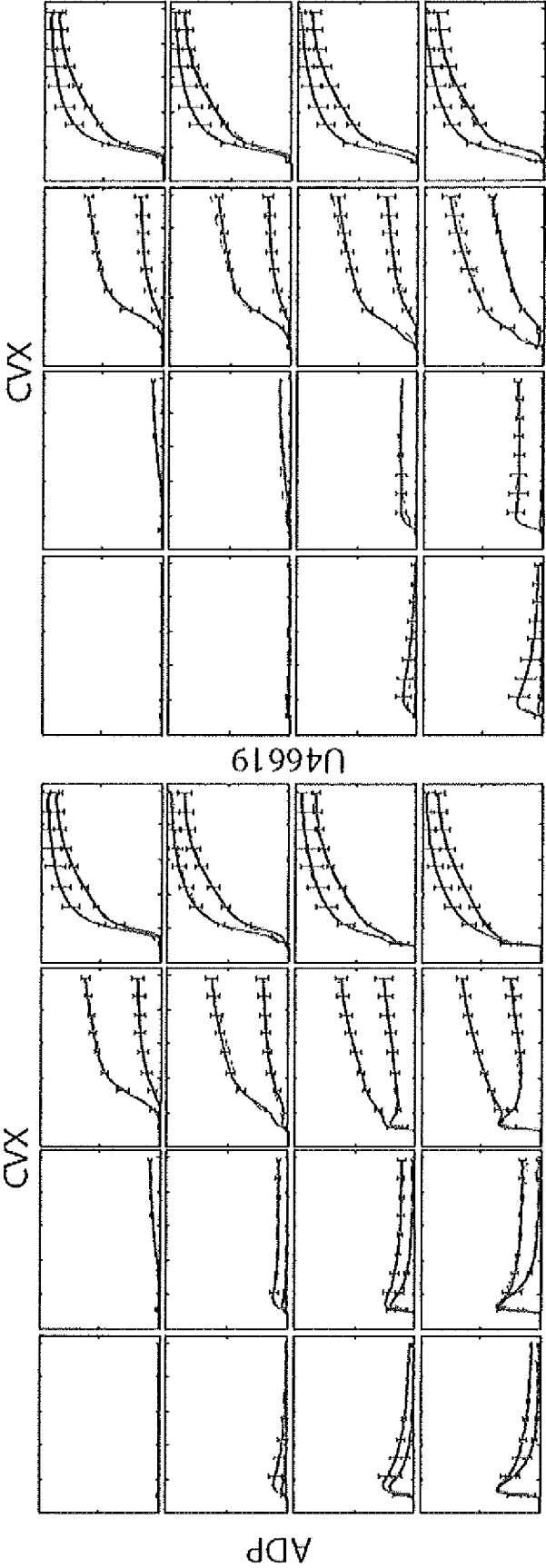


Fig. 23b

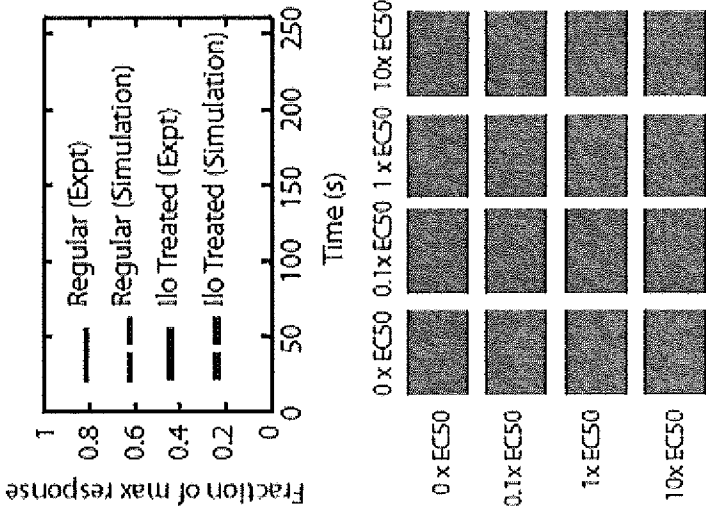
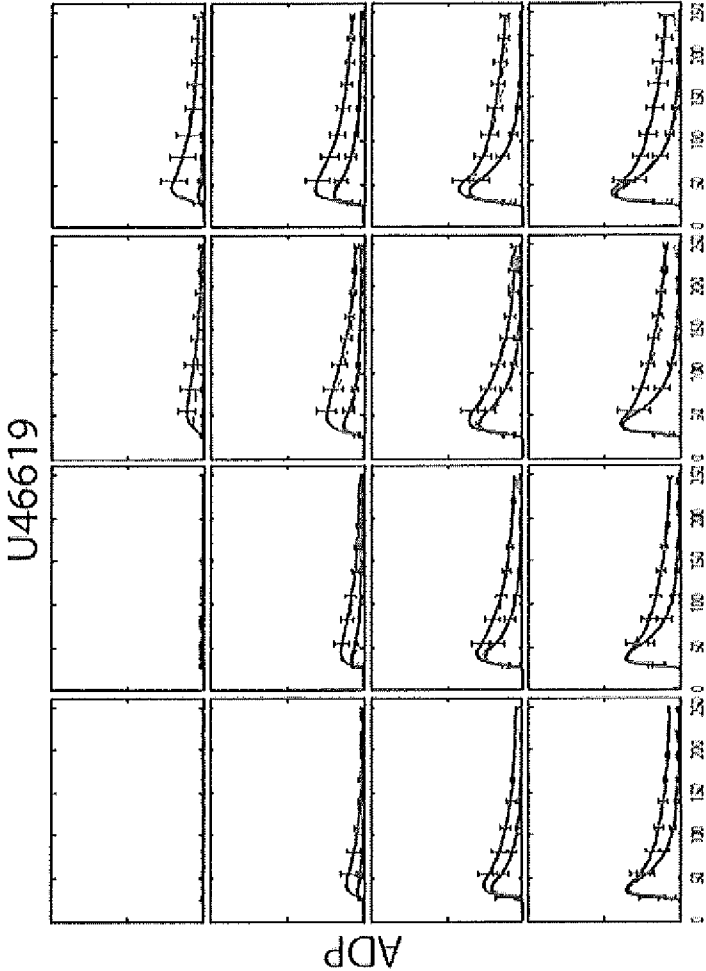


Fig. 24

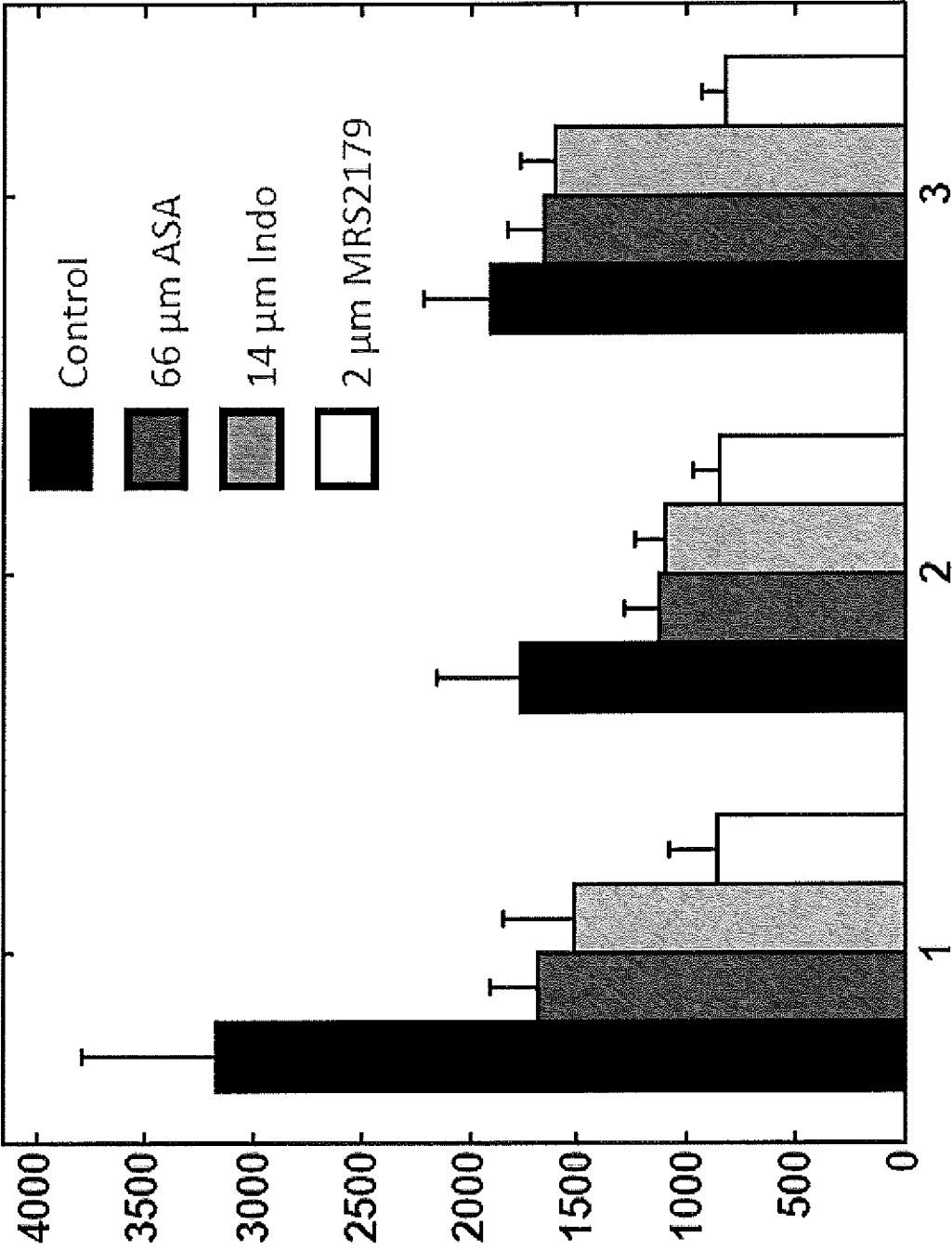


Fig. 25

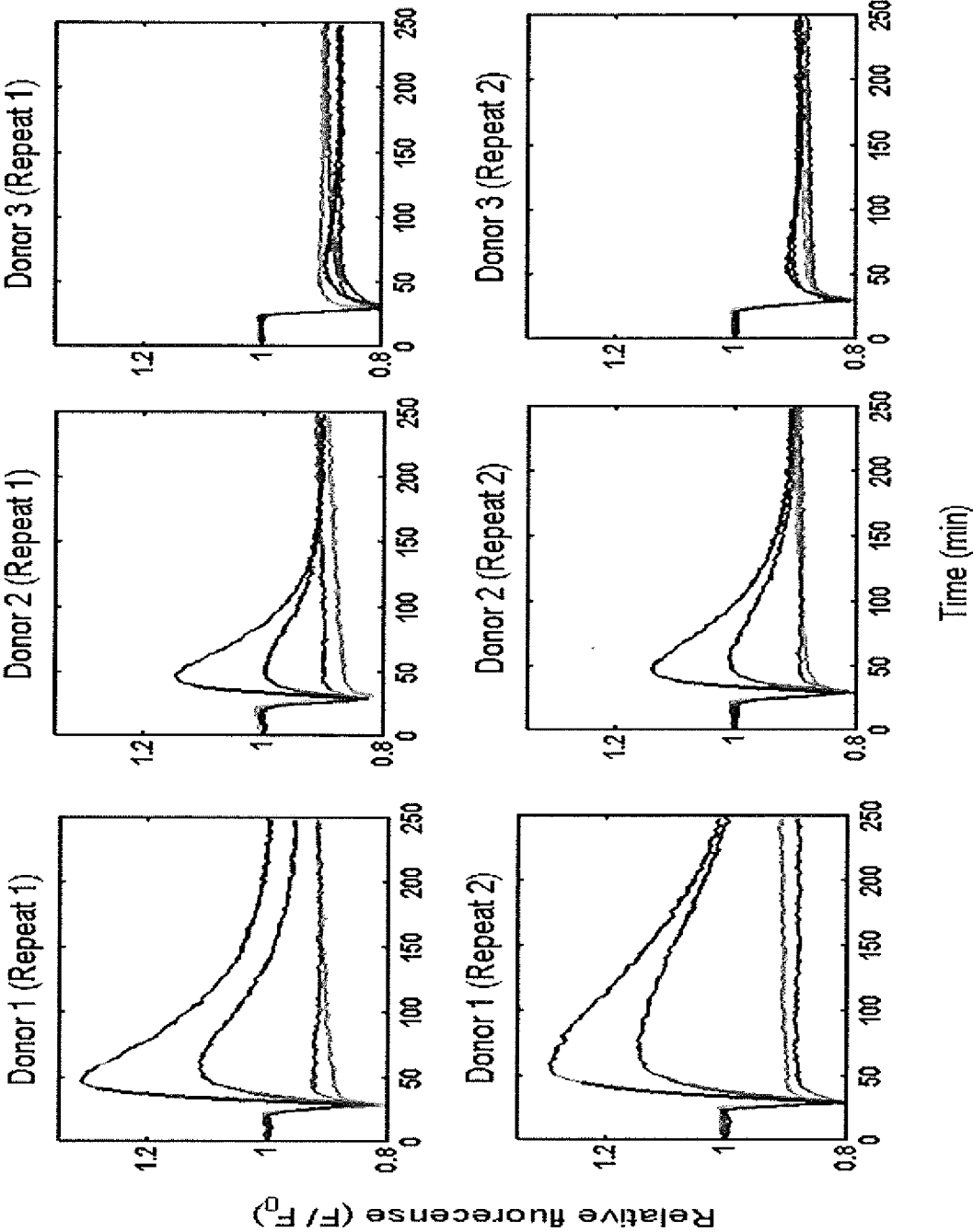


Fig. 26

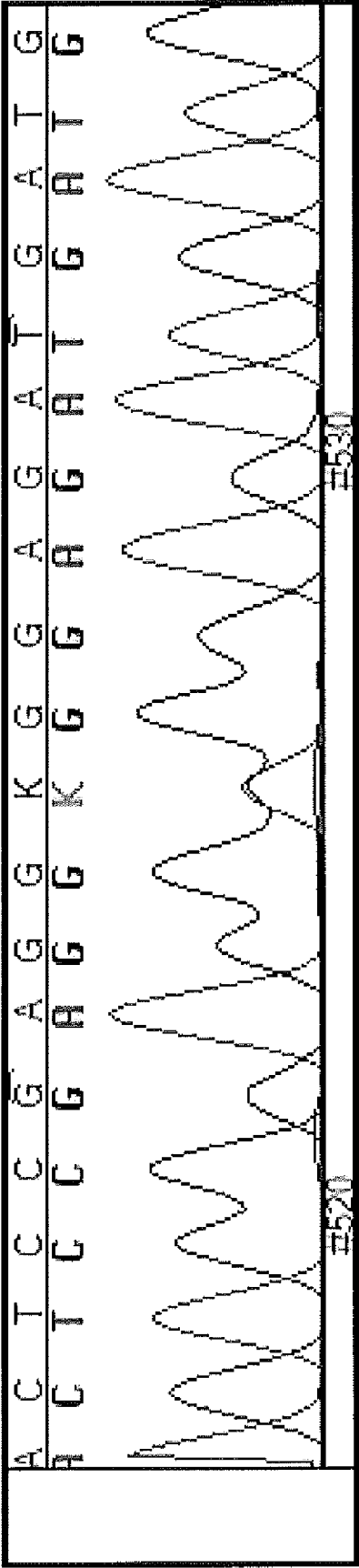


Fig. 27

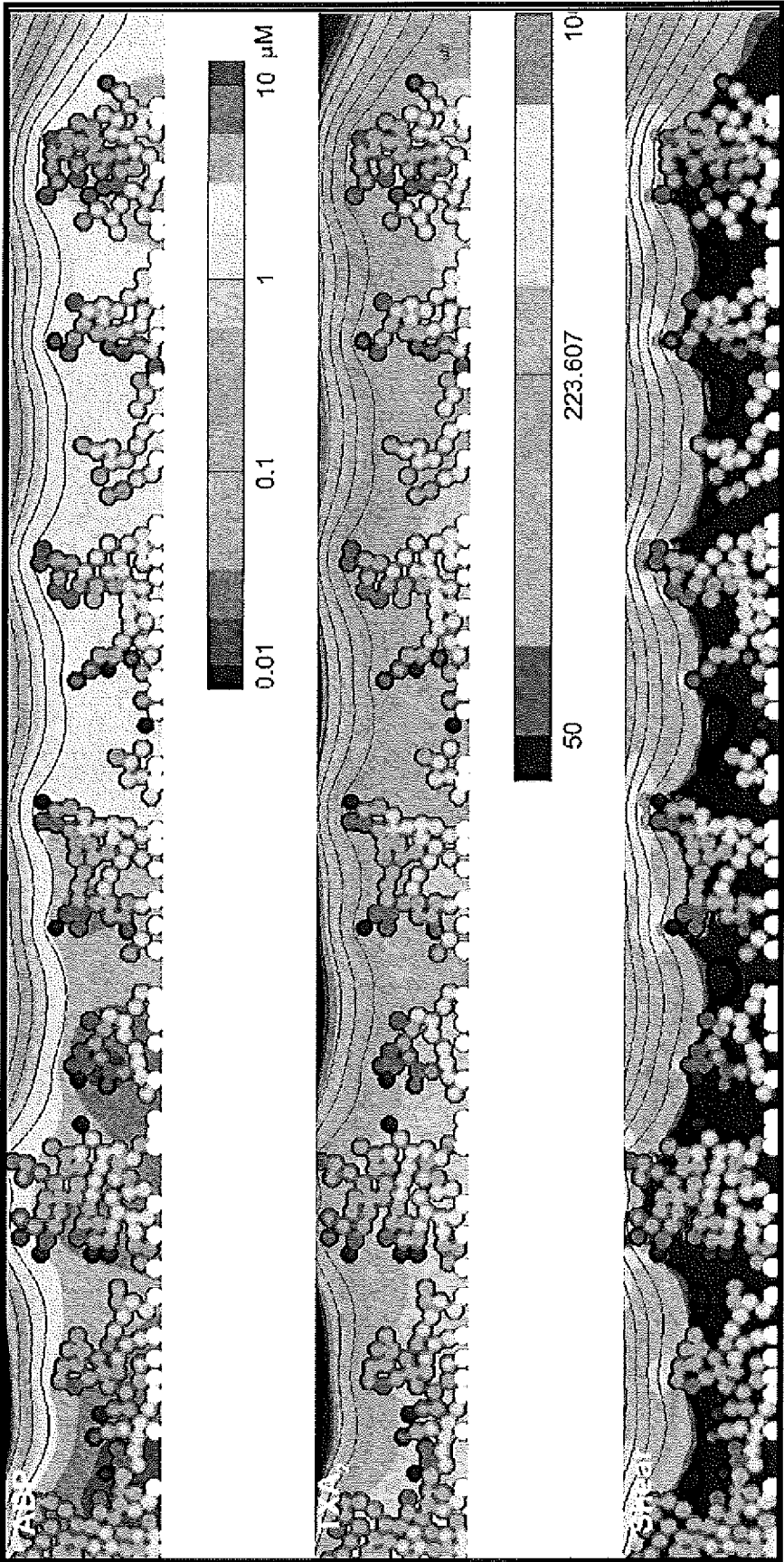


Fig. 28a

MEASUREMENT OF CLOTTING UNDER FLOW

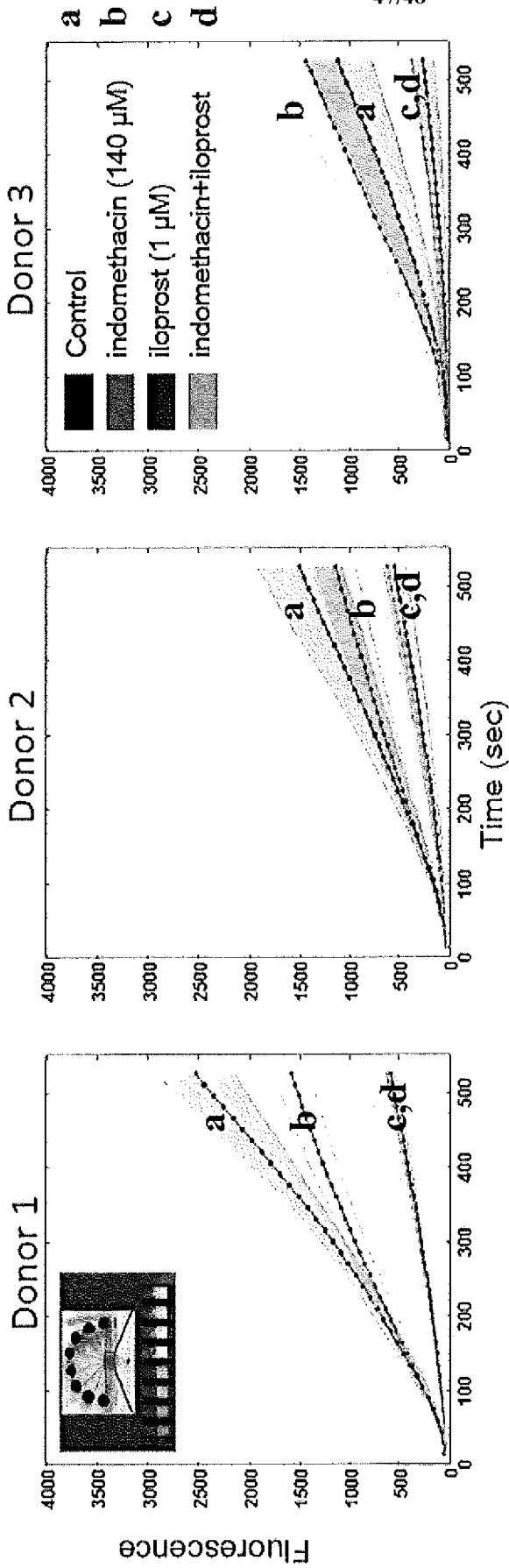
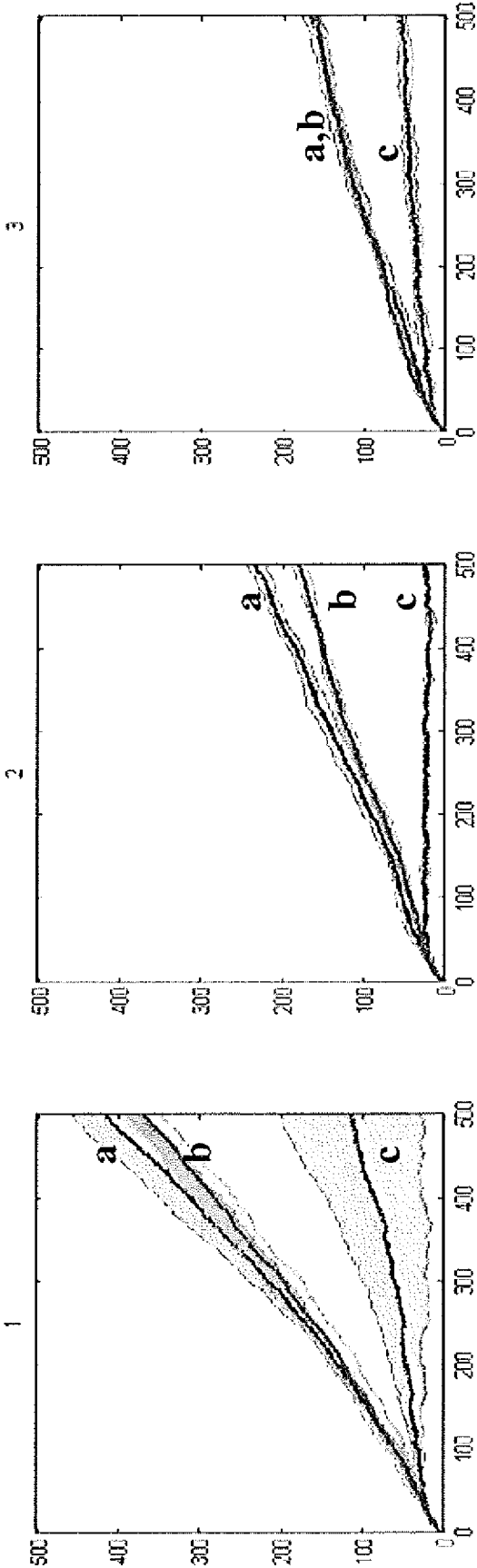


Fig. 28b

COMPUTER SIMULATION



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 11/40712

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C40B 30/06; G06G 7/58 (2011.01)

USPC - 506/10; 703/11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8)- C40B 30/06; G06G 7/58 (2011.01);

USPC- 506/10; 703/11

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PubWest (US Pat, PgPub, EPO, JPO), Google Scholar (PL, NPL), FreePatentsOnline (US Pat, PgPub, EPO, JPO, WIPO, NPL): predict, signal, response, cell, disease, disorder, stimulus, model, neural, learn, supervise, network, set, platelet, iloprost, indomethacin

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2006/0134605 A1 (FOLKMAN et al.) 22 June 2006 (22.06.2006) para [0011], [0016], [0020], [0024], [0065], [0073], [0085], [0088], [0091], [0096]-[0111], [0165], [0170]-[0187], [0203]-[0208], [0212], [0228], [0237], [0239], [0256], [0263], [0269], [0272], [0276]	1-13, 15-17, 27-28 ----- 14, 18-26
Y	US 5,728,808 A (ABRAMOVITZ et al.) 17 March 1998 (17.03.1998) col 1, ln 32-55; col 2, ln 18 to col 3, ln 4; col 7, ln 39 to col 9, ln 55; col 11, ln 63 to col 12, ln 50; col 13, ln 14-33	14, 18-26
A	WO 2010/019952 A2 (SCHOEBERL et al.) 18 February 2010 (18.02.2010) pg 3-62	1-28
A	US 2008/0288227 A1 (HIGGINS et al.) 20 November 2008 (20.11.2008) para [0045]-[0100]	1-28
A	US 2007/0093969 A1 (MENDRICK et al.) 26 April 2007 (26.04.2007) para [0008]-[0212]	1-28

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search

30 September 2011 (30.09.2011)

Date of mailing of the international search report

25 OCT 2011

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

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