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Pairing down signaling complexity

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Scanning pairs of signaling agonists predicts how cells react to arbitrary combinations of external stimuli

Cells process a multitude of external stimuli through receptor and adaptor proteins that converge on a core set of signal transduction pathways². How are such complex inputs converted into informative signaling outputs? In this issue, Chatterjee *et al.*¹ describe an experimentally tractable method for predicting signaling responses to the enormous number of stimulus ‘cocktails’ that a cell might encounter. Their test case is calcium signaling in platelets, but the approach holds promise for modeling signal integration in any cell.

If every signaling output were unique to an associated receptor, one could simply characterize signaling responses to individual stimuli and then infer the global response to any stimulus combination by linear superposition. Of course, crosstalk in signaling is more the rule than the exception, and so merely focusing on individual inputs would overlook key network properties. However, crosstalk does not require one to test all stimulus combinations to reveal important signal-transduction properties of a network. Previous studies have shown that simple pairwise stimulation of cells can provide unique information about the activation states of a signaling network^{3–5}. But it has remained unclear whether pairwise stimulation captures the major facets of signal integration or whether higher-order combinations of three or more stimuli are necessary.

Cells almost certainly encounter such complex stimulus combinations in their *in vivo* microenvironment. However, as the number of stimuli increases beyond two, the number of possible combinations quickly explodes, and the chance that a cell will see any particular higher-order combination becomes exceedingly small. Triple-stimulus combinations (A+B+C) would thus be too rare to evolve their own specific signal-integration mechanisms beyond those provided by the subsets of two stimuli (A+B, B+C, and A+C).

The work by Chatterjee *et al.*¹ and two other recent reports^{6,7} provide experimental support for this theoretical argument. Using different approaches, the three groups found that higher-order stimulus combinations could be accurately predicted from pairwise information. As emphasized in one study⁶, the practical implications of this finding are substantial, because it substantially reduces the number of stimulus cocktails that must be tested experimentally. With existing high-throughput approaches⁸ one should easily be able to quantify signaling responses to all pairwise combinations of 20–40 ligands that a cell type is most likely to encounter. Pairwise measurements could then enable data-driven predictions of any higher-order ligand combination as the need arises. These predictions could readily be incorporated into tissue-level models composed of multiple cell types and stimuli that vary in time and space. A comprehensive and predictive model of signal integration also would provide a means to search for cocktails that amplify desired signaling outputs or dampen undesirable ones.

The method of Chatterjee *et al.*¹, called pairwise agonist scanning (PAS), systematically exploits the power of pairwise measurements to make network-specific predictions and diagnoses. The authors focused on the calcium response of platelets to agonists of blood clotting, or thrombosis. Understanding the environmental factors and intrinsic network properties that control platelet activation is of considerable clinical interest because too much or too little clotting can be fatal. Furthermore, as a model for signal transduction, the platelet is ideal—no nucleus, a few thousand mRNA species and a collection of signaling proteins dedicated to one major function.

Platelet agonists mobilize calcium from intracellular stores to drive thrombosis. Calcium second-messenger dynamics are readily monitored by sensitive fluorescent dyes whose spectral properties change with the concentration of intracellular calcium. Chatterjee *et al.*¹ established a cell-based assay of intracellular calcium concentration with one such dye and used the assay to define the half-maximal effective concentration (EC_{50}) for six orthogonal platelet agonists. They then devised a high-throughput method for measuring the intracellular calcium concentration profile of a platelet suspension four minutes after stimulation with all possible combinations of six agonists at 0.1 $\times$, 1 $\times$, or 10 $\times$ their EC_{50} in a single 384-well plate (Fig. 1). Notably, the resulting 135 stimulus pairs fell right where the combinatorics start to become unwieldy—for instance, it would take four times as many measurements to assay all stimulus triples.

In total, PAS experiments yielded tens of thousands of intracellular calcium concentration measurements for each platelet sample. The authors then extracted pairwise information from these data by defining a synergy score for the time-integrated intracellular calcium concentration response to agonist combinations. This score captures synergy, antagonism and additivity in a single metric and compresses each measurement into a 135-element synergy score vector that describes the synergy ‘signature’ for a platelet population.

Remarkably, when the authors applied PAS to platelets from 10 human donors, they found that synergy signatures could reproducibly organize donors into subpopulations with greater fidelity than previous studies had reported. Such patient grouping based on platelet phenotype has long been desired for predicting adverse events to coagulant, anticoagulant, and thrombolytic drugs. To translate the applications of this work further toward patient prognosis, it will be important to connect PAS-based discrimination with key clinical variables or platelet aggregopathies.

PAS signatures may be reproducible and donor-specific, but do they truly capture the higher-order signal transduction properties of cells? To compile the PAS data in a format that enables predictions for new cocktails of agonists, Chatterjee *et al.*¹ used a history-dependent form of neural-network modeling⁹ (Fig. 1). With reasonably few fitted parameters, they built a model to predict the platelet intracellular calcium concentration time course given an arbitrary combination and timing of the six selected agonists. When challenged with new cocktails added together or sequentially, the model performed surprisingly well. Overall, the authors observed a strong correlation between predicted and measured synergy scores, although predicted intracellular calcium concentration responses occasionally did not match experimental measurements when several agonists were added together at 10 $\times$ EC_{50} . Importantly, the predictions were achieved using less than 4% of the data that would be required to measure the six-factor agonist space exhaustively. PAS efficiency would become even more dramatic if more agonists were included in the stimulus panel.

The utility of PAS should extend well beyond the particular network studied in this paper. For example, with proper signaling readouts, PAS could be used in pharmaceutical

development to investigate the primary hits from a small-molecule screen. PAS in the presence and absence of primary compound would quickly reveal contexts in which the drug has perturbed the normal signal-transduction machinery. Overall, PAS will be most successful in situations where the convergent intracellular pathways are established and where time-dependent signaling readouts are easily obtained for hundreds to thousands of samples.

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Figure 1.

Combining pairwise agonist scanning (PAS) with neural-network modeling to capture a complex stimulus landscape. Chatterjee *et al.*¹ used PAS to measure the dynamic intracellular calcium response of platelets to all possible pairs of six agonists added at different dosings (agonists A–F, where smaller labels indicate lower doses). PAS data were then used to train a neural-network model of intracellular calcium responses. The model predicted sequential and higher-order agonist combinations (red), suggesting that most of the overall stimulus landscape had been captured with pairwise data from PAS.