Elucidation of Drug Responses by Profiling Signal Transduction Pathways Using Cytokine and Biomarker Panels

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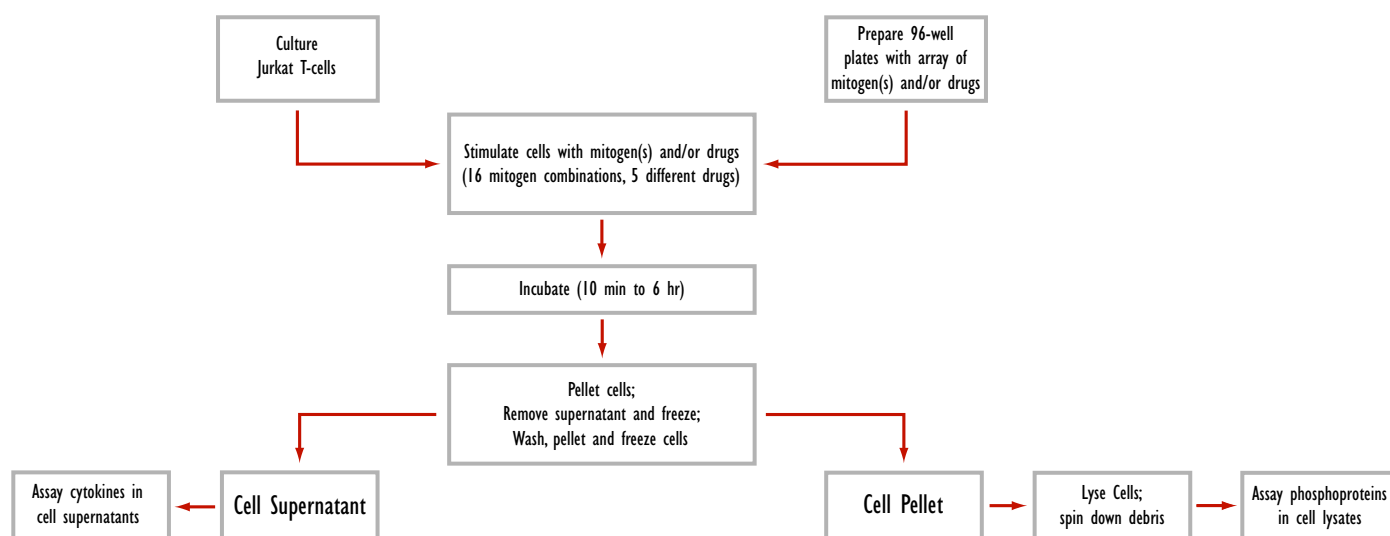
❶ Abstract

Human T-cells can be perturbed in a controlled manner using well-defined modulators and are an effective model system for studying stimulus-evoked transcriptional control. Recent studies utilizing high throughput cell-based transcriptional assays in which the transcriptional output of T-cells was activated by a combinatorial array of T-cell mitogens and gated by multiple immunomodulatory agents provide high dimension data and insights into the mechanisms through which molecular signaling events are integrated in the nucleus to influence gene expression. We extended this approach by measuring correlatory changes in the proteome associated with these signal transduction events including gene regulatory endpoints (cytokine gene products) and upstream molecular signaling intermediates (phosphoprotein biomarkers). Thalidomides are a class of drugs (now in several clinical trials) that have immunomodulatory properties and significant potential as anti-cancer agents. We used the combined transcriptional and proteomics approach to measure the therapeutic targeting of thalidomide and three of its clinically active analogues: CPS11, CPS45, and CPS49. We analyzed 10 human cytokines and 4 phosphoproteins in Jurkat cells treated with combinatorial mitogen stimulation and obtained dose and time course information for each drug. The measurements were enabled using new multiplexing tools that increased the data set completeness and sped its acquisition. These data show patterns and other interesting differences in cellular responsiveness to drugs and various stimuli. In combination with transcriptional target activation, these experiments suggest critical determinants that may help reveal the drug action/response in this system.

❷ Experimental Methods and Sample Preparation

```
┌──────────────┐                          ┌─────────────────────┐
│   Culture    │                          │  Prepare 96-well    │
│ Jurkat T-cells│                         │  plates with array of│
└──────┬───────┘                          │ mitogen(s) and/or drugs│
       │                                  └──────────┬──────────┘
       │        ┌──────────────────────────────────┐ │
       └───────▶│ Stimulate cells with mitogen(s) and/or drugs │◀──┘
                │ (16 mitogen combinations, 5 different drugs)  │
                └──────────────────┬───────────────┘
                                   │
                      ┌────────────▼─────────────┐
                      │  Incubate (10 min to 6 hr) │
                      └────────────┬─────────────┘
                                   │
                      ┌────────────▼─────────────┐
                      │       Pellet cells;       │
                      │ Remove supernatant and freeze; │
                      │  Wash, pellet and freeze cells │
                      └──┬─────────────────────┬──┘
                         │                     │
```

Assay cytokines in cell supernatants ◀── **Cell Supernatant**

Cell Pellet ──▶ Lyse Cells; spin down debris ──▶ Assay phosphoproteins in cell lysates

❸ Electrochemiluminescence

LIGHT — Measured signal is light

*Ru(bpy)$_3$$^{2+}$

TPA$^.$

-H$^+$

Luminescence
Emitting Light

Ru(bpy)$_3$$^{2+}$ — Ru(bpy)$_3$$^{3+}$ — TPA — TPA$^{.+}$

Chemi-
Chemical Energy

e- — Electrode — e-

Electro-
Electrochemically Initiated

- Extremely sensitive (attomole) detection
- Wide dynamic range (>5 logs)
- Selective, homogeneous
- Robust, stable
- Only label bound near electrode is detected
- Emission in red region of visible spectrum, reduces color quenching

Spectrum of Ru-bpy TAG ECL

Intensity (normalized) vs Wavelength (nm)

Ruthenium (II) tris-bipyridine-(4-methylsulfone) NHS ester (MSD SULFO-TAG™ label)

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❹ MSD MULTI-ARRAY™ Technology

96-well Single   4-Spot

7-Spot   10-Spot
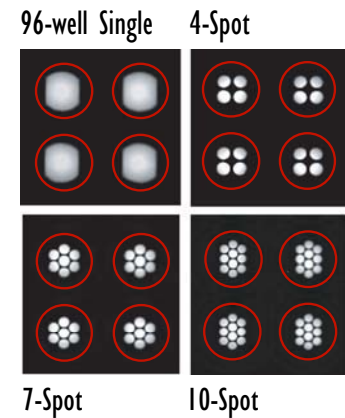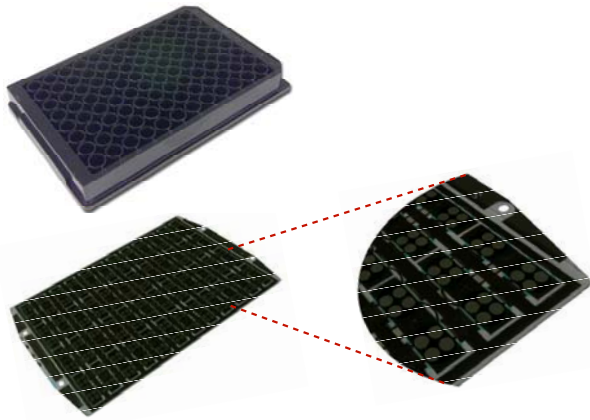
Patterned Surface Arrays

- Patterned electrodes
    - 24-well, 96-well, 384-well
- Multiplexing
- High throughput
- Sample volumes 10-20 μL for 96-well and 384-well plates
- Reduced noise
- Multiple plate coatings
    - Streptavidin, avidin
    - Antibody (primary or secondary)
    - Gluthathione
    - Nickel
    - Polyethyleneimine

Multiple Instruments

- Two imaging instruments for HTS
- Two personal readers for assay development
- Very fast read time

Applications

- Cytokines
- Biomarkers
- Phosphoproteins
- GPCRs
- Kinase profiling
- Signaling Pathways
- Clinical studies
- Compound profiling
- High throughput screening

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❺ Cytokine 10-Plex Assay

Cell Supernatant Protocol

1. Add 20 μL of sample and calibrators, incubate 1hr at RT
2. Add 20 μL of detection antibodies, incubate 1hr at RT
3. Wash 3X with PBS
4. Add 150 μL/well read buffer and read

12 plates (11,520 data points) were easily processed in 4 hrs

10-Plex Human Panel MSD MULTI-SPOT® 96-Well Plate

IL-2
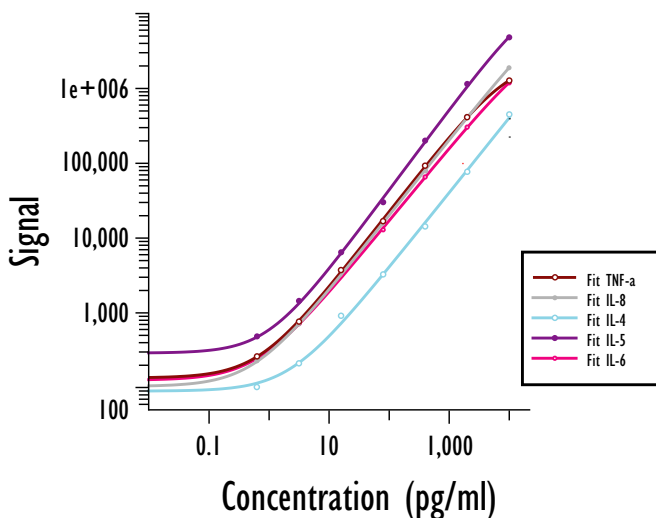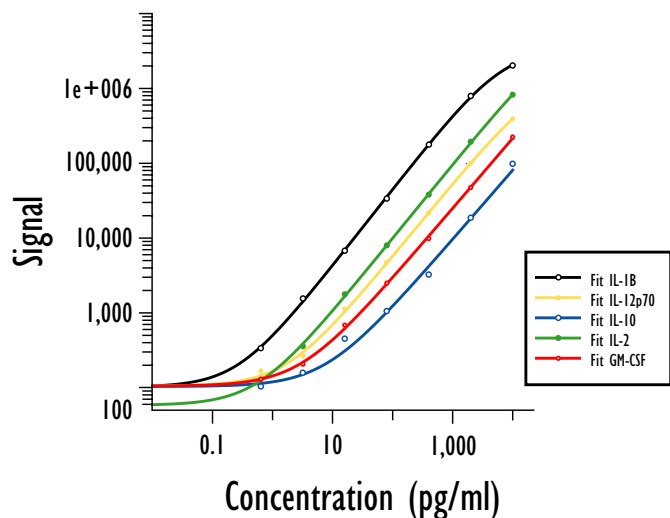IL-1b
GM-CSF
IL-12p70
IL-10

IL-4
IL-5
TNF-a
IL-6
IL-8

Labeled-Ab
TNF-α
Capture Ab
Working electrode

10 - 10,000 counts
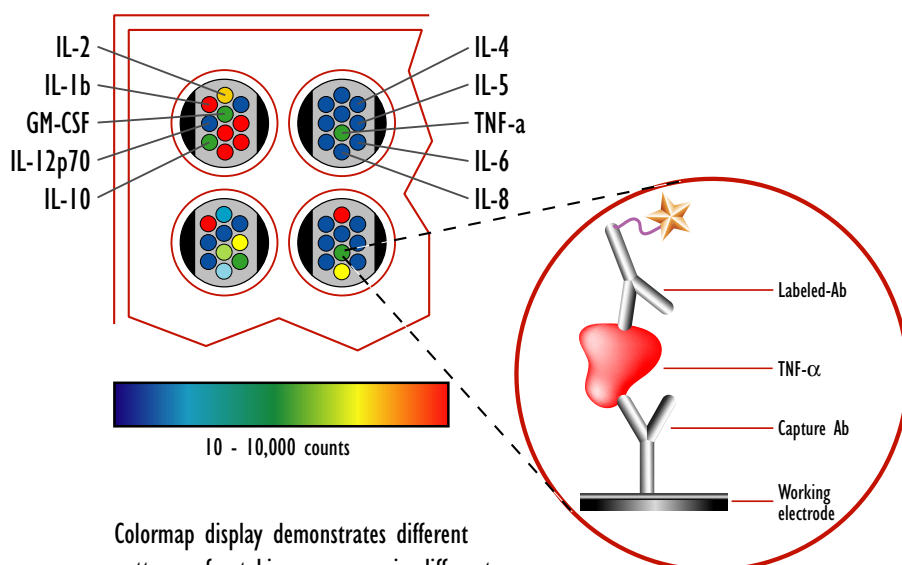
Colormap display demonstrates different patterns of cytokine responses in different wells of a 96-well 10-spot assay plate.

❻ Representative Cytokine Data and Detection Limits

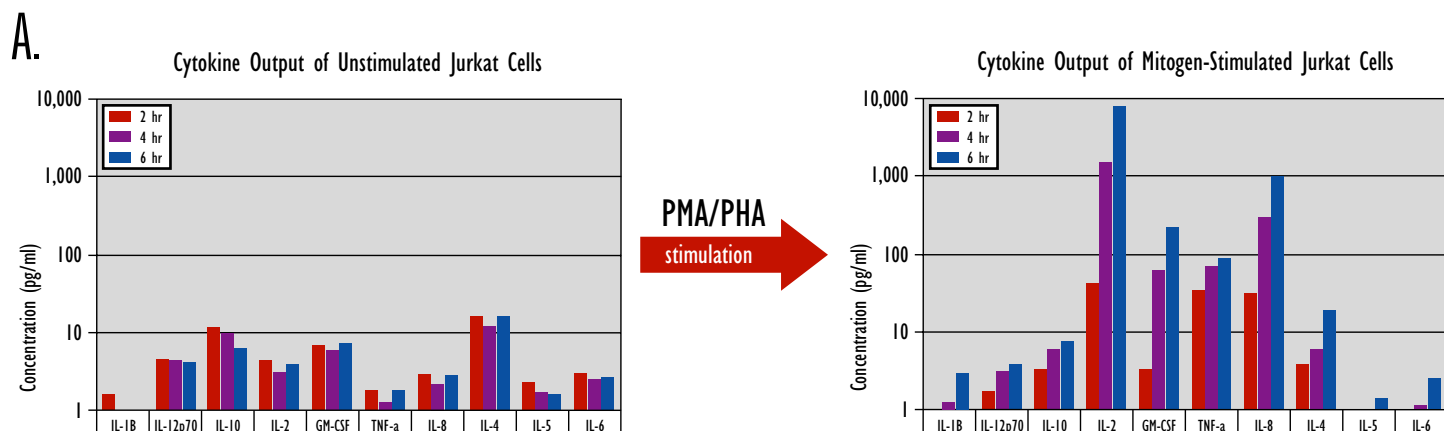| Cytokine	IL-1B	IL-12p70	IL-10	IL-2	GM-CSF	TNF-a	IL-8	IL-4	IL-5	IL-6
Detection Limit (pg/ml)	0.53	1.24	8.01	0.88	2.59	0.41	0.51	2.39	0.32	0.49

The figures show typical standard curves for the MSD ten-spot cytokine panel used in these studies and illustrate the sensitivity and dynamic range of the analysis platform. Calculated detection limits (2.5 sigma) for each cytokine in cell culture supernatant appear in the table.
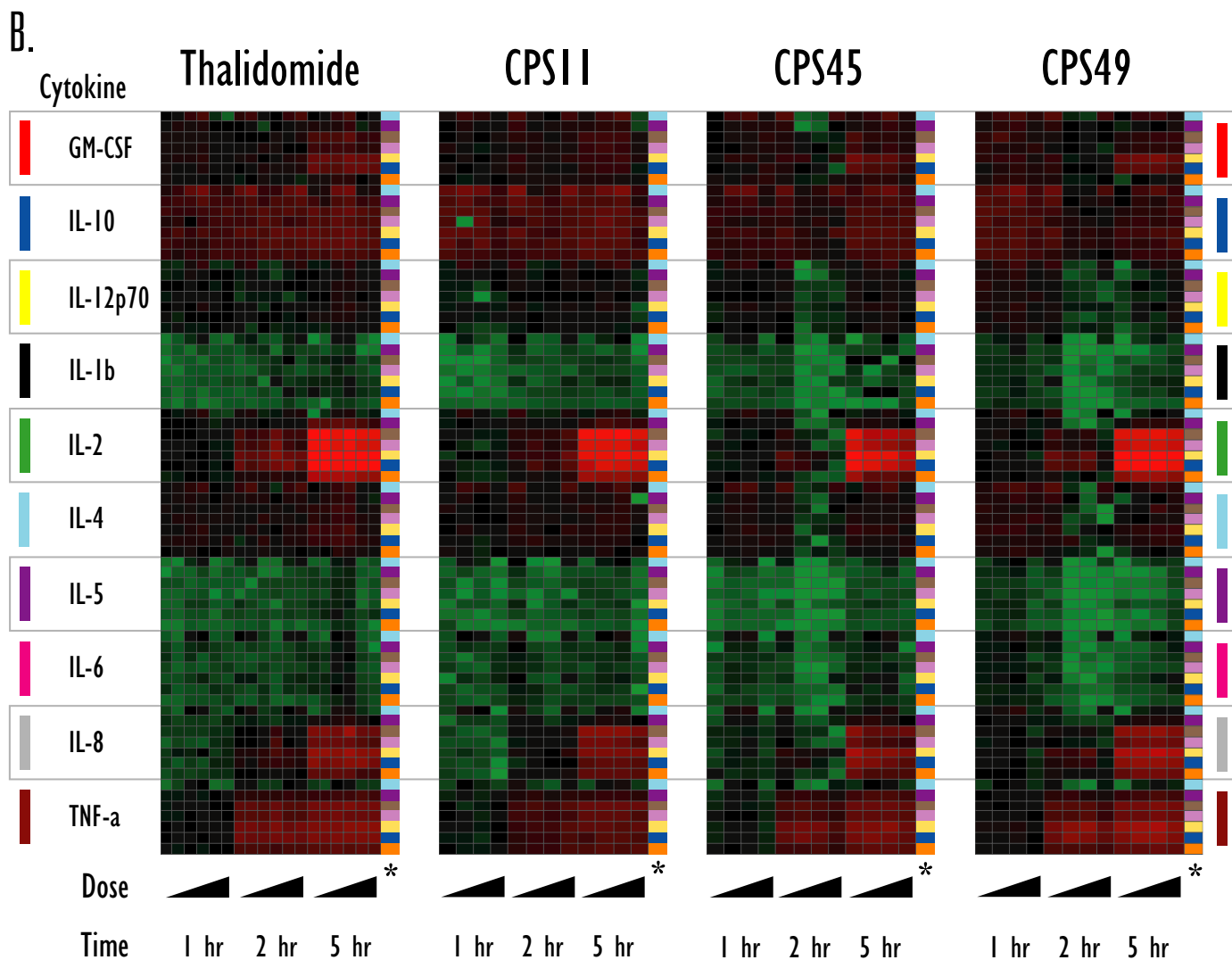
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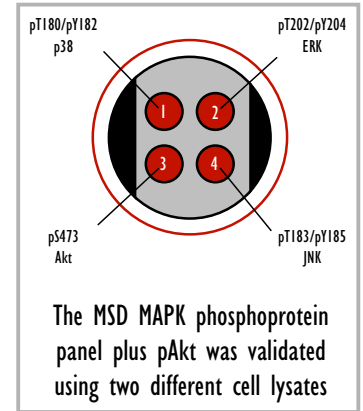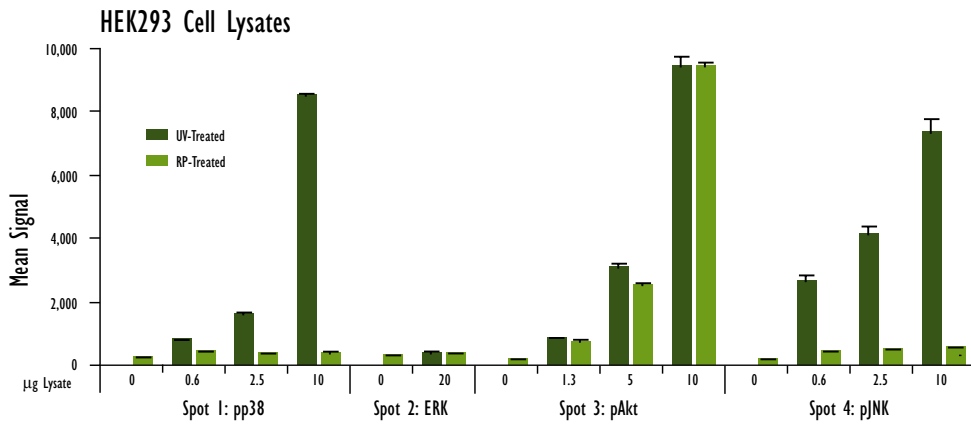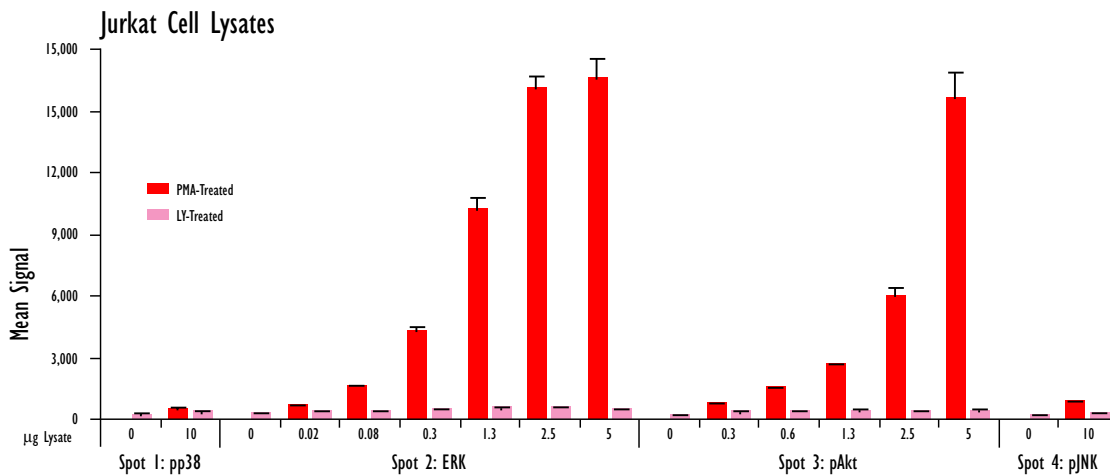# Elucidation of Drug Responses by Profiling Signal Transduction Pathways Using Cytokine and Biomarker Panels

❼ Cytokine Data

A.

Cytokine Output of Unstimulated Jurkat Cells

PMA/PHA
stimulation

Cytokine Output of Mitogen-Stimulated Jurkat Cells

Combinatorial Stimulation Conditions

Mitogens	Drug Doses	Drugs	Time Points
Unstimulated	0 nM	Thalidomide	10 min - 6 hr
PMA	1 nM	No Drug	
PMA/Ionomycin	10 nM	CPS11	
PMA/PHA	100 nM	CPS45	
PMA/Ionomycin/anti-CD28 Ab	1 μM	CPS49	
PMA/PHA/anti-CD28 Ab	5 μM		
PMA/anti-CD28 Ab/anti-CD3 Ab	10 μM		
	100 μM (thalidomide)		
	200 μM (thalidomide)		

Individual mitogens or mitogen combinations can be utilized to stimulate particular transcriptional pathways and induce downstream signaling and cytokine responses. The example (A) shows the excreted cytokine response of Jurkat cells to PHA/PMA stimulation over several hours. The output clearly depends on time and type of cytokine providing a pattern that suggests internal communication and signaling across multiple levels within the cell. (B) Multiplex cytokine data obtained from the supernatants of Jurkat cells treated with thalidomide or one of its analogues. The mitogen and drug conditions described in the table were evaluated to create a profile of response as a function of drug dose and time course. Data are presented in log scale to show the wide dynamic range (~4 logs) of cytokine responses measured.

❼ Cytokine Data (continued)

B.

Cytokine	Thalidomide	CPS11	CPS45	CPS49
GM-CSF				
IL-10				
IL-12p70				
IL-1b				
IL-2				
IL-4				
IL-5				
IL-6				
IL-8				
TNF-a				

Dose

Time: 1 hr, 2 hr, 5 hr (for each: Thalidomide, CPS11, CPS45, CPS49)

0.01 — 1 — 1000 pg/ml

* Mitogens
- Unstimulated
- PMA
- PHA/PMA/CD28
- PHA/PMA
- Ion/PMA/CD28
- Ion/PMA
- CD3/PMA/CD28

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❽ Phosphoprotein 4-Plex Assay Performance

HEK293 Cell Lysates

UV-Treated
RP-Treated

The MSD MAPK phosphoprotein panel plus pAkt was validated using two different cell lysates

Logarithmically growing HEK293 cells were treated with UV or Rapamycin (RP). Whole cell lysates were added to MSD MULTI-SPOT 4 Spot 96-well plates coated with capture antibody at four spatially distinct electrodes per well. Phosphorylated proteins were detected with MSD SULFO-TAG detection antibodies.

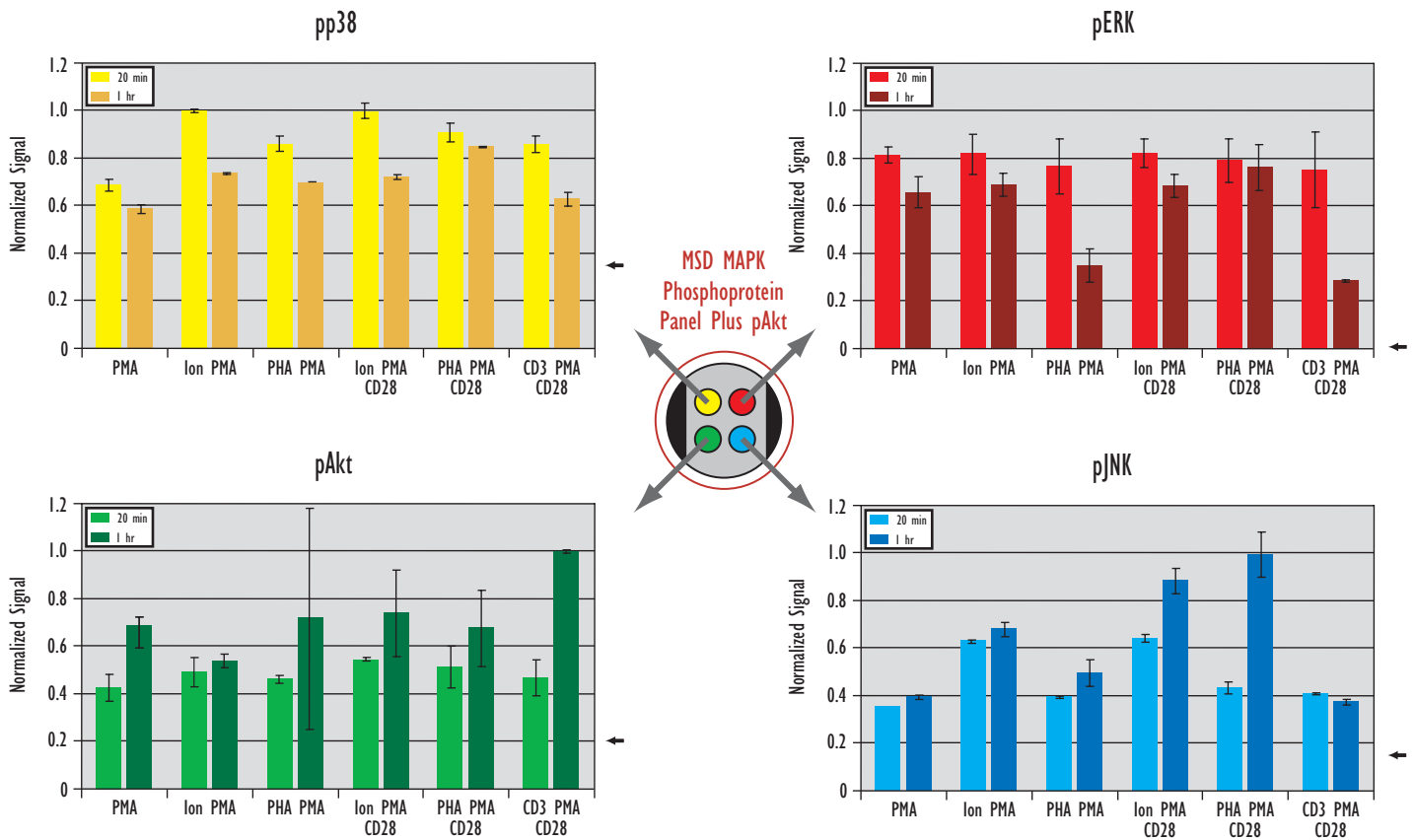Jurkat Cell Lysates

PMA-Treated
LY-Treated

Logarithmically growing Jurkat cells were treated with PMA or LY294002. Whole cell lysates were added to MSD MULTI-SPOT 4 Spot 96-well plates coated with capture antibody at four spatially distinct electrodes per well. Phosphorylated proteins were detected with MSD SULFO-TAG detection antibodies.

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❾ Phosphoprotein Data

Jurkat cells were treated with different mitogen combinations for 20 minutes and 1 hour. Cell lysates were prepared as described above and were assayed for phosphoprotein levels in an MSD 4-spot MULTI-SPOT plate at a level of 7 μg total protein per well. The phosphoprotein responses were normalized to the maximum signal for each phosphoprotein, and the normalized signal for the 20 minute unstimulated condition is indictated with an arrow (←).

❿ Conclusions

This poster described a high throughput, self-consistent method for the analysis of intra and inter cellular effects of stimulation on cells: In a new application of the MSD technology, cell supernatants and cell lysates from the same set of Jurkat cells were used to make parallel determinations of cytokine and phosphoprotein levels. This approach demonstrates the ability to quantitatively measure a complicated ensemble of biological processes using a single technology and provides information to better characterize the manifold of interdependent pathways from upstream (phosphoprotein) to downstream (cytokine) levels. Knowledge obtained from this model system is being used to develop testable hypotheses for the purpose of furthering the understanding of these systems in a more general way.

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