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Application Note

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A kinase inhibitor phenotypic screen using a novel multiplex T cell activation assay

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Introduction

T cells play a critical role in adaptive immune responses including pathogen elimination and tumor immunosurveillance. The binding of the T cell receptor (TCR) to peptides complexed with major histocompatibility complex (MHC) on antigen presenting cells, along with engagement of co-receptors such as CD4 or CD8 and co-stimulatory molecules (i.e. CD28), triggers an intricate signaling mechanism. TCR signal initiation is mediated by cytosolic tyrosine kinases such as LCK and ZAP70, leading to signal amplification through a network of serine-threonine kinases.[1, 2] Activation of the TCR pathway in naive and effector T cells leads to T cell activation, proliferation, and cytokine production.

Modulating TCR engagement and signaling pathway using biologics, small molecules or genetic engineering is highly relevant to many therapeutic areas including cancer immunotherapy, adoptive cell therapy, and vaccine development. Perturbations leading to increased hyper responsive TCR signaling and enhanced T cell activation is a major cause of autoimmune disease. Genetic defects, mutations and other mechanisms resulting in increased T cell kinase activity are involved in many autoimmune pathologies making them attractive targets for the direct inhibition of T cell activation.[3, 4]

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Introduction (continued)

Currently there are 37 FDA approved Kinase Inhibitors (KI), mostly for oncology indications, with approximately 250 in clinical testing.[5] KI drugs specifically targeting early signaling events during T cell activation are a new focus for the treatment of autoimmune diseases with recent FDA approvals for KIs in treating rheumatoid arthritis.[6]

The development of drugs and therapies regulating TCR activity require assays to profile T cell function and health. To address the need for rapid monitoring of T cell function, we developed an optimized, high-content, multiplexed assay using high throughput flow cytometry to measure T cell activation. The Intellicyt® Human T Cell Activation Cell and Cytokine Profiling Kit (TCA Kit) collapses the traditional workflow by evaluating cell phenotype, T cell activation markers, cell proliferation, cell viability, and quantitates secreted cytokines in a single assay using a 96 or 384-well plate format.

In recent years, there has been renewed interest in using phenotypic screens for drug discovery. This screening methodology does not require a specific drug target or knowledge of its role in the pathology of disease, but uses a specific, relevant biological model or signaling pathway to identify appropriate hits.[7] Phenotypic screens are being used with novel compound libraries as wells as drug repurposing and chemogenomic libraries.[8, 9] The use of these known libraries allow for signature and phenotypic matching where the characteristic of a drug with desirable properties can be matched to another drug with an unknown clinical profile.

To illustrate the value of the Intellicyt platform for T cell function in phenotypic screening, we used the TCA Kit to screen a 152 small molecule library of KI for their ability to inhibit human primary T cell activation in peripheral blood mononuclear cells (PBMCs) stimulated with anti CD3/CD28 beads. Samples were acquired on the Intellicyt® iQue Screener PLUS platform and the early/late activation markers CD69, CD25 and HLA-DR, and cell proliferation were assessed in viable CD4 and CD8 lymphocytes. To assess T cell function, the levels of secreted IFN-γ and TNF-α were quantitated. Data was analyzed and heat maps, IC50 curves and cytokine quantitation was generated using the integrated ForeCyt software. Profile maps, a unique analysis tool of ForeCyt software, was used to integrate assay metrics with Boolean logic to quickly locate hits using defined multiplexed criteria. This application note demonstrates the insight provided by the use of Intellicyt platform for phenotypic screening of small molecules affecting T cell activation.

Materials

Cells and Reagents

Cryopreserved PBMCs from healthy donors (Astarte Biologicals) were cultured in PBMC media (RPMI 1640, supplemented with 10% fetal bovine serum, 10 ng/mL of human IL-2, non-essential amino acids, sodium pyruvate and penicillin-streptomycin (all purchased through VWR)). CD3/CD28 DynaBeads (ThermoFisher Scientific), phytohemagglutinin (PHA, Sigma) and enterotoxin type B from Staphylococcus aureus (SEB, List Biological Laboratories) were used for T cell activation. The 152 compound chemogenomic KI library was purchased from Cayman Chemicals. T cell activation was assessed using the Intellicyt Human T Cell Activation Cell and Cytokine Profiling Kit, which measures cell proliferation and viability, cell surface early/late activation markers (CD69, CD25 and HLA-DR) and secreted cytokines (IFN-γ and TNF-α).

Methods

Profiling T Cell Activation

For proof of concept (POC) studies to test assay robustness, PBMCs from a single donor were cultured in a 96-well plate at a final concentration of 106 cells/ml. Different activation reagents (DynaBeads, PHA, and SEB) were added to the cells using an 11 point, 2-fold dilution series and each series was done in duplicate wells. Media without activation compounds were used as a control. T cell activation was measured by the TCA kit from a 10 µl cell/supernatant mixture sample transferred to the assay well from each culture well at 1, 3 and 6 days post-stimulation. Data were acquired on the Intellicyt iQue Screener PLUS and analyzed with ForeCyt software using the T cell activation kit data template (See data acquisition section below).

T Cell Activation Kinase Inhibitor Screening and Dose Response Assays

Cryopreserved PBMCs were cultured overnight in PBMC media. Cells were cultured in a 96-well plate in PBMC media with 20 µM of the indicated KI for 1 hour and stimulated with CD3/CD28 DynaBeads for 24 hours. The final concentration of cells were 106 cells/ml with a final concentration of 10 µM for the KI and a final culture volume of 100 µl. Negative controls were cells cultured without any drug, and positive assay control were cells cultured with 10 µM of the phosphatase inhibitor Cyclosporine A, a known inhibitor of T cell activation.

Twenty-four hours later, T cell activation was assessed following the TCA kit protocol. Briefly, INF-γ and TNF-α standard curves were generated in a separate standard-alone plate, and 10 µl of each sample culture from each well of the original 2 culture plates were transferred to 2, 96-well assay plates. Sequential addition of cytokine beads and cytokine detection antibodies were followed by a cocktail of viability dye and fluorescent CD antibodies. The total assay time was approximately 4 hours.

Dose response studies were performed on select KI using the same basic protocol as the initial kinase screening assay. An 11 point, 2-fold serial dilution was used for each kinase inhibitor with a concentration range of 10 µM to 10 nM. Media only was used for each compound as a negative control for the study.

Data Acquisition and Analysis

Samples were acquired on the Intellicyt iQue Screener PLUS. The panel design of the TCA kit dictated the violet, blue and red (VBR) laser configuration. The acquisition protocol and data analysis, including event gates and gating strategy, activation metrics, heat maps, standard curves and IC/EC 50 curves were auto-generated using the TCA template and ForeCyt software. Standard curves to quantitate the levels of secreted IFN-γ and TNF-α were generated using a 4-parameter curve fit with 1/Y2 weighting factor. The linear range for each standard curve was calculated using ForeCyt software. Profile maps were created in the multi-plate analysis Panorama feature in the ForeCyt software. Profile maps were used to identify compound hits with specific criteria, including inhibition of all activation metrics and select markers.

Results and discussion

The discovery and development of small molecules and antibodies targeting T cell function, as well as T cell-based cell therapies and cell manufacturing, require assays to rapidly and reliably profile T cell activation and cell health. To address this need, Intellicyt developed the TCA Kit to rapidly deliver high-content T cell activation data. Figure 1 shows the assay biochemistry. The assay discriminates between live and dead cells by using a membrane integrity dye, which stains only dead cells by DNA intercalation. Viable T cell subsets are identified using CD3, CD4 and CD8 antibodies and the cell surface activation markers measure early activation (CD69+), late activation (CD25+) and even later activation (HLA-DR+) in the different T cell subpopulations. The levels of secreted IFN-γ and TNF-α are quantitated in the same sample well using a bead-based assay. For long-term studies, cells are stained using proliferation dye (provided in the TCA kit) prior to culture to quantitate cell division during the study time course.

For POC studies, cryopreserved PBMCs from a single donor were cultured for 24 hours and then stained with the Cell Proliferation and Encoder Dye B/Green (included in the TCA Kit). Cells were stimulated with three different well-characterized T cell activators (CD3/28 DynaBeads, phytohemagglutinin (PHA), or Staphylococcal enterotoxin B (SEB)) using a 12 point, 2-fold serial dilution series (SEB used a 4-fold serial dilution). A 12 point, 3-fold standard curve to quantitate the levels of secreted INF-γ and TNF-α were generated. The plate set-up, concentration of cytokine standards, and concentration range of T cell activators are shown in Figure 1.

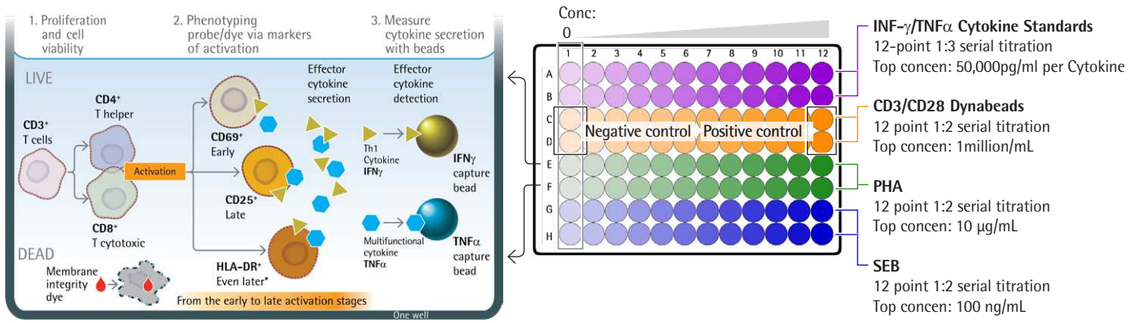

Figure 1: Assay Biochemistry and Proof of Concept Study Plate Set-up. The different T cell identifiers, and phenotypic and functional activation markers, measured in each well are seen in the left panel. A typical assay plate set-up used for POC studies is seen in the right panel. This plate includes the standard curves for cytokine quantitation and positive and negative controls.

To perform the assay, 10 µL of sample containing cells and supernatant were transferred from the culture plate to an assay plate on days 1, 3 and 6 after stimulation, and analyzed using the TCA Kit. The data were acquired on the iQue Screener PLUS VBR. The time-to-results for the assay, including data acquisition and analysis, is approximately 4 hours. The assay workflow is shown in Figure 2A. The gates and gating strategy are shown in Figure 2B. To increase the ease of use, all gates are pre-drawn, data metrics and visualizations are auto-generated, and color compensation is not required when using the TCA kit template. The seven decade dynamic range of the iQue Screener PLUS allows for easy discrimination between cytokine capture beads and cells using basic forward and side scatter plots. Due to differences in the capture bead's intrinsic fluorescence, the single cytokine capture beads are resolved into INF-γ and TNF-α populations for quantification. For the cell gates, viable single cells are determined, T cell subsets are identified and activation markers are assessed. Cells that proliferated during the experimental time course are identified by a decrease in fluorescent intensity.

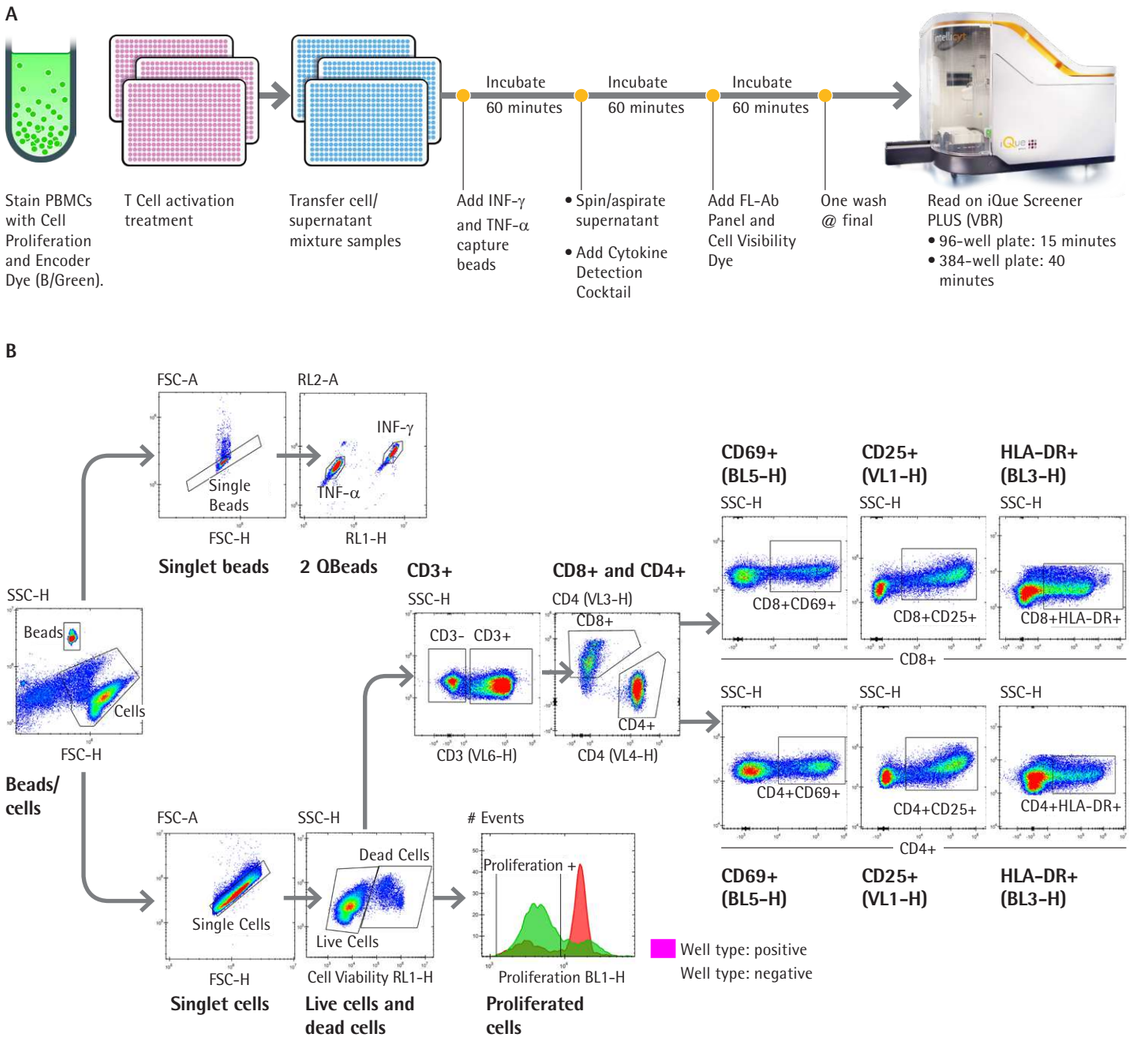

Figure 2. TCA Assay Workflow and Gating Strategy. A) To perform the TCA assay an aliquot of cells/supernatant mixture (10 µl or 5 µl for 96 or 384-well plate respectively) is transferred to an assay plate. Sequential additions of the pre-mixed reagents are added followed by a final wash before sample acquisition on the Intellicyt iQue Screener PLUS. B) The gating strategy is included in the TCA template that comes with the kit. Cells and cytokine capture beads are initially separated based on size and granularity. Cytokines capture beads are then resolved while viable cells are determined and T cell subsets and activation status for each subset is assessed.

The T cell time course activation data were analyzed and line graphs from the POC studies generated using the multi-plate Panorama feature in the ForeCyt software. Figure 3 shows cytokine secretion (pg/ml), proliferation (% of CD8+ proliferating cells) and phenotypic activation markers (% of CD8+ cells expressing the indicated markers) for each of the three T cell activation reagents with the blue line, red line and green line representing 1, 3 and 6 day post stimulation respectively. The x-axis shows the dose response for each compound. The data show a temporal and dose response for the various activation metrics with differences observed between the three compounds. For example, TNF-α secretion peaks at day 3 with DynaBeads and SEB treatment and significantly decreases at day 6, whereas little temporal difference in TNF-α secretion is observed with cells cultured in PHA. Further analysis show differences in multiple metrics in cells treated with SEB compared to other activating compounds. Taken together, these data show how the TCA Kit can rapidly generate high content data that can identify different mechanisms of action (MOA).

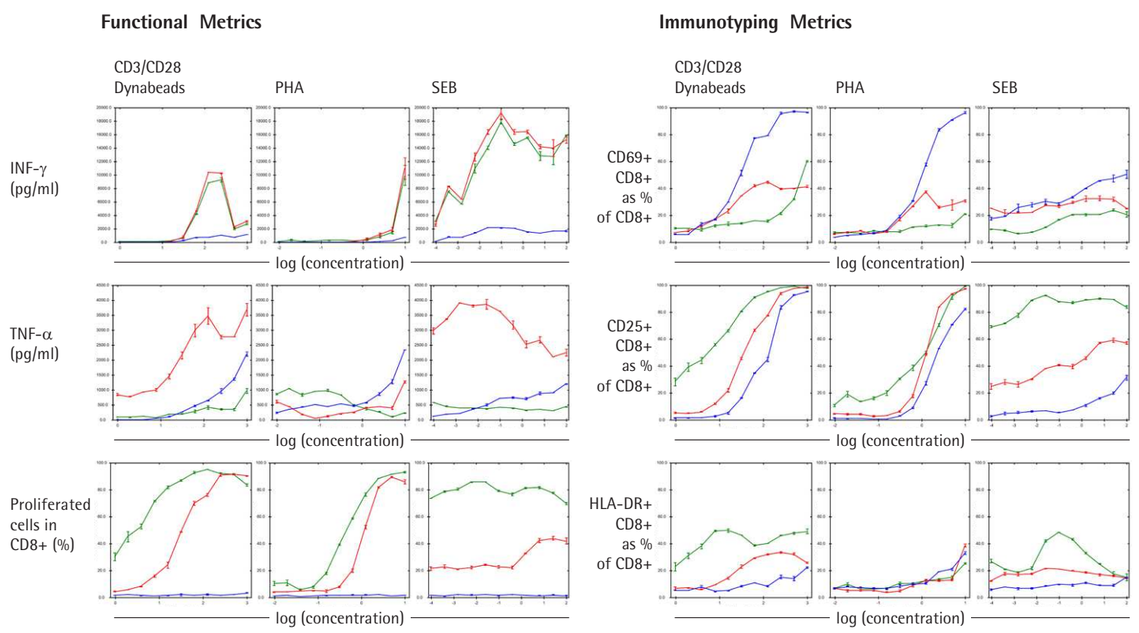

Figure 3. Selected T cell Activation Data. Line graphs showing the amount of cytokine secretion, % of proliferating CD8+ cells and the % of CD8+ cells co-expressing the various activation markers were generated in ForeCyt software. The blue, red and green lines represent 1, 3 or 6 days post stimulation respectively. The x-axis shows increase compound dose (PHA and SEB) or number of DynaBeads per cell. The error bars represent the standard deviation of duplicate measurements. This is representative of an experiment done multiple times.

For the initial KI library screen, PBMCs were cultured in IL-2 containing media and treated for 1 hour with 20 µM of each inhibitor and then cells were activated using anti-CD3/CD28 beads (Figure 4). Twenty-four hours later, the TCA assay was performed and samples were acquired on the iQue Screener PLUS VBR. For each of the 2 culture plates, a series of negative controls (media only) were used to determine T cell activation metrics in the absence of compound treatment. For positive controls, cells were treated with 10 µM of cyclosporine A, a known inhibitor of T cell activation (10).

Library 152: Kinase Inhibitors (Cayman Chemical)

Prepare Cells
- Thaw cryopreserved PBMCs
- Cultured overnight
- Washed and placed in IL-2 supplemental media

Treat with Kinase Inhibitors
- Treat for one hour using Kinase Inhibitor library
- Positive and negative controls
- Use 20µM of each compound

Activate Cells
- CD3/CD28 Dynabeads
- Incubate for 24 hours

Run TCA Assay
- Acquire data on iQue PLUS Screener VBR
- Visualize data in ForeCyt software including profile maps

Plate 1

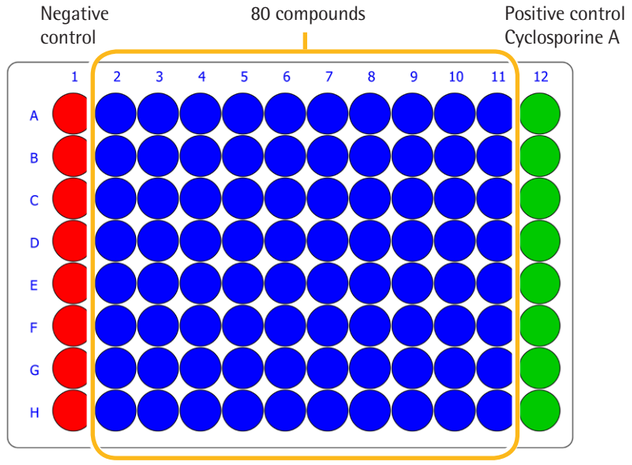

Plate 2

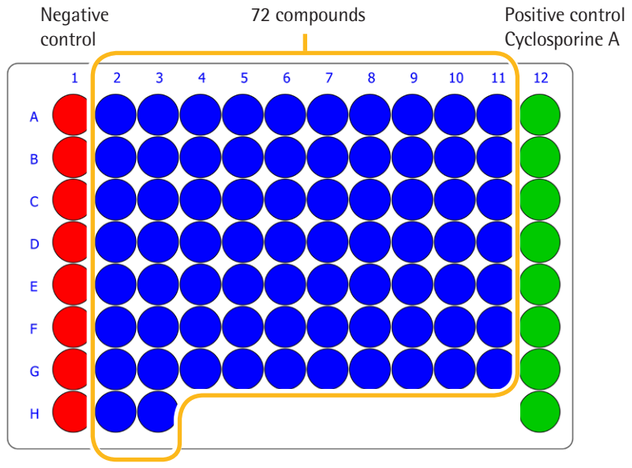

Figure 4. Kinase inhibitor screening experimental setup. PBMCs were cultured overnight and added to wells containing individual KI (final concentration of 20µM). One hour later, anti CD3/CD28 DynaBeads were added to each culture to activate T cells and TCA assay was performed 24 hours later.

A plate-level analysis showing the percentage of viable CD4 T cells that express the early activation marker CD69 from plate 1 is found in Figure 5. Using this visualization tool, we can quickly identify compounds that have inhibited expression of CD69 (CD69+ cells are in the rectangular gate) as well as KI that drastically reduced CD4+ T cell viability (wells with no cells, i.e. well H2). Below, in the plate-level view, are examples of inhibitors that affect different kinase families. In wells containing media alone, 86% of the CD4 cells are activated as assessed by CD69 expression and treatment with the FDA-approved Jak 1/2 inhibitor Ruxolitinib which reduced the percentage of CD4+CD69+ cells down to 55%. The Src inhibitor PP2 dramatically inhibited CD69 expression as only 3% of the CD4 T cells expressed CD69. Other compounds showed a range of CD69 inhibition.

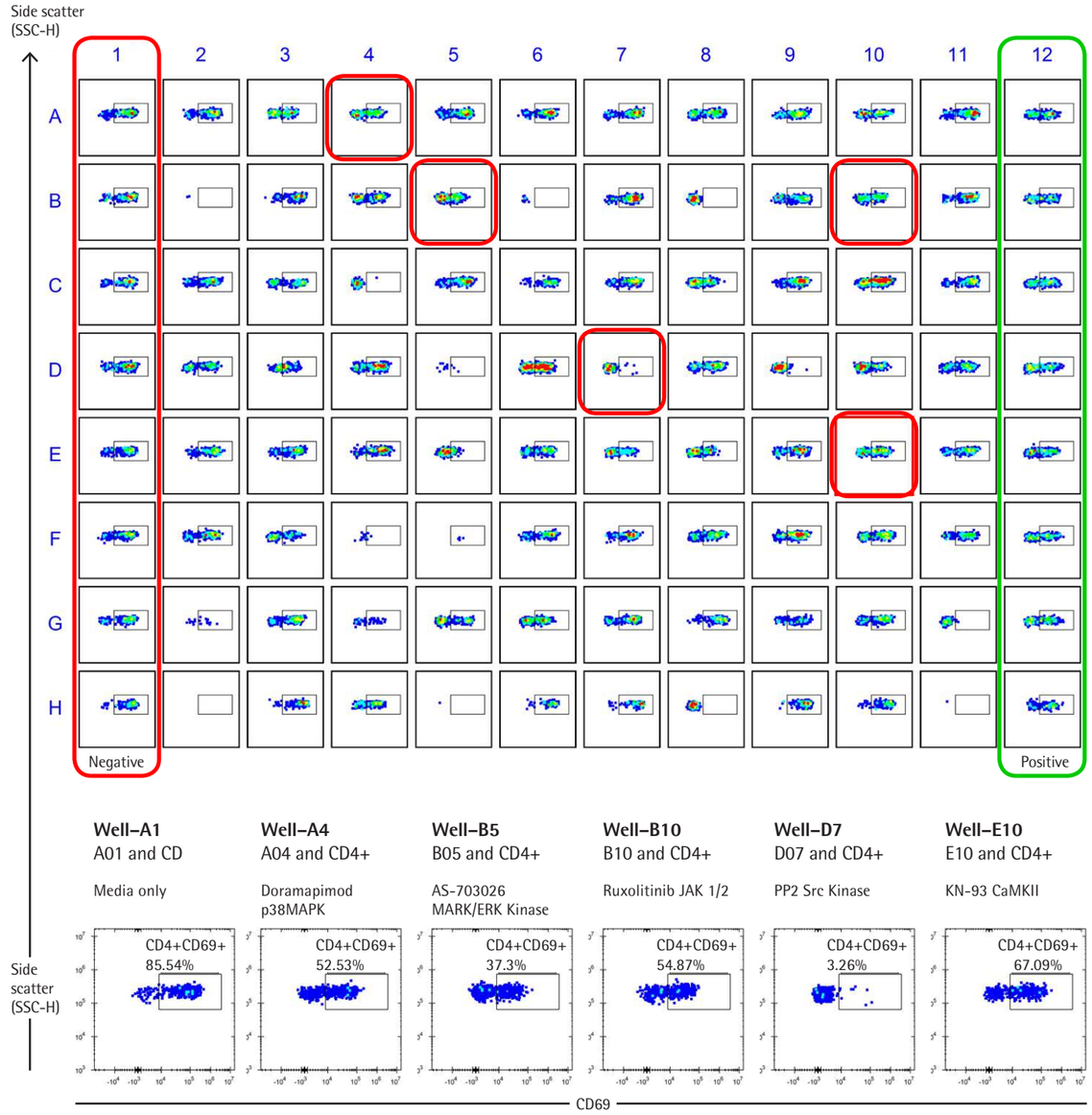

Figure 5. Compounds that inhibit CD69 expression. A plate-level view (from plate 1) of CD69 expression in T-helper cells was generated in ForeCyt software. The CD69 expressing cells are found in the rectangular gate. Wells highlighted in red are shown below with the indicated KI and the % of CD69 CD4+ cells found in the inset of each dot blot.

In Figure 5, we show a single activation metric (CD69) in the T-helper subset, whereas the TCA kit generated 15 different parameters requiring integration to provide a more complete and insightful picture of the role that different KI have in T cell activation. To integrate the data, we used ForeCyt Software's Profile Map data tool in the Panorama feature (Figure 6). The left panel shows the 11 parameters from the screening study that were integrated and the user-defined threshold for each of the metrics. Using the Profile Map, we identified 27 different KI that inhibited expression of all T cell activation markers and cytokine secretion (wells highlighted in blue in Figure 6). The overlay line graph in Figure 6 ranks all of the hits and the level of inhibition, providing easy visualization for each metric. For example, treatment with some KI completely inhibits all activation metrics, whereas other compounds have a greater impact on specific phenotypic markers or cytokines.

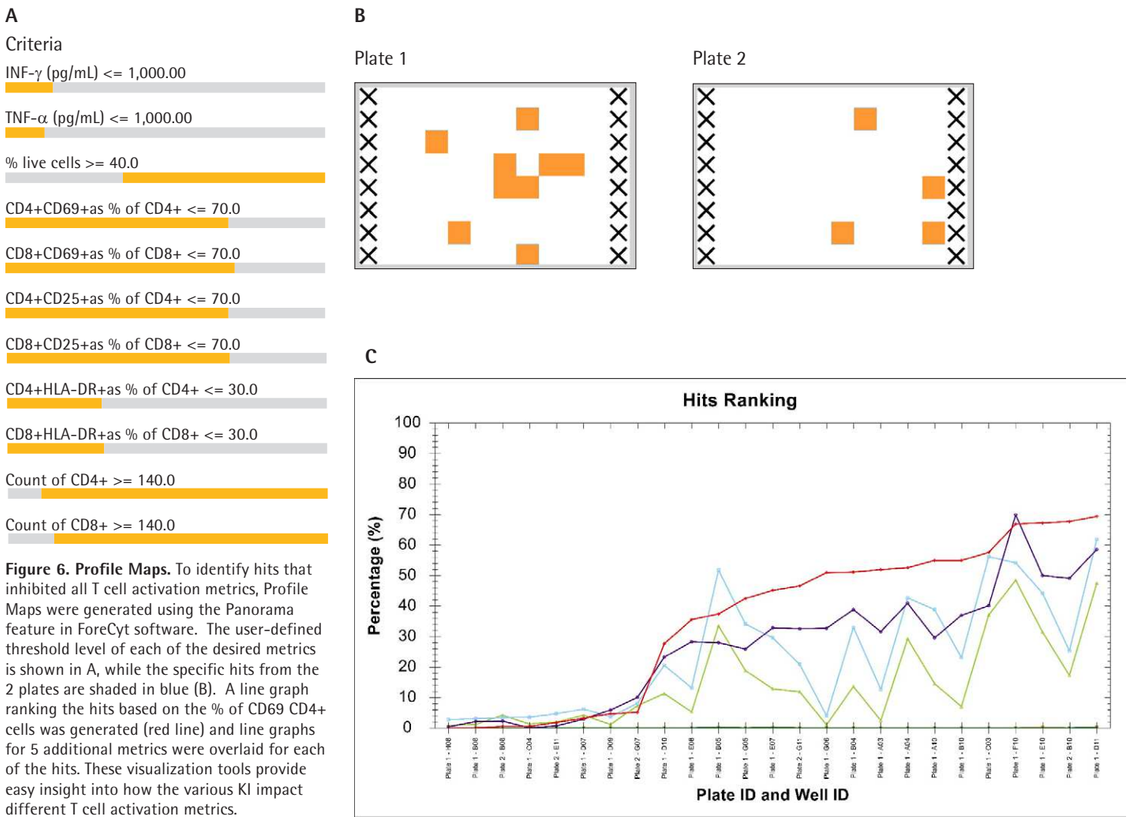

Figure 6. Profile Maps. To identify hits that inhibited all T cell activation metrics, Profile Maps were generated using the Panorama feature in ForeCyt software. The user-defined threshold level of each of the desired metrics is shown in A, while the specific hits from the 2 plates are shaded in blue (B). A line graph ranking the hits based on the % of CD69 CD4+ cells was generated (red line) and line graphs for 5 additional metrics were overlaid for each of the hits. These visualization tools provide easy insight into how the various KI impact different T cell activation metrics.

By changing the threshold of each parameter, Profile Maps can quickly identify KI that have unique MOA. Shifting the threshold of CD69 expression allowed us to identify two hits that did not affect CD69 expression, but inhibited all other T cell activation markers (Figure 7 and data not shown). This is of importance since CD69 is often the only marker used in many traditional T cell activation panels and these hits would not have been identified. The same strategy was used to identify three KI that did not affect CD25 expression, but inhibited all other metrics and additional compounds that showed differential cytokine secretion (data not shown). Finally, Profile Maps can be used to identify treatments that have similar phenotypes, which is important when trying to compare unknown compounds with drugs that have high safety profiles in the clinic. Using this signature mapping feature in the Profile Map tool, we identified three drugs that showed the same level of inhibition of all metrics as the FDA-approved RA drug Rixinitimab (data not shown). These data illustrate the value of the high-content data generated by Intellicyt's TCA kit and suggest KI can inhibit multiple pathways to inhibit T cell activation.

To confirm the MOA of the KI, and to determine inhibitory concentration curves, a set of compounds were chosen for dose response studies. The protocol for these studies were the same as the initial screen and the concentration ranged from 20 uM to 20nM for all of the compounds (Figure 7). The concentration dependent inhibition of activation and functional markers in the CD4+ population is shown in Figure 7, and all dose response curves and IC50 calculations (if required) were generated in ForeCyt software. The CHK inhibitor TG003 had no effect on T cell activation. In contrast the receptor tyrosine kinase/src kinase inhibitor PD166326 demonstrated a classic dose response inhibition of all T cell activation markers. The IC50 values ranged from 200 – 370 nM for each of the metrics and are shown in the inset of each graph. Tunicamycin, an EGFR/Erb B inhibitor, was a compound identified by the initial screen as a drug that did not affect CD69 expression, but did inhibit other phenotypic and functional markers. This was confirmed in the dose response study where CD69 expression remained unchanged, whereas the percentage of CD25 expressing CD4+ cells were ~50% and little to no INF- was detected at the high drug concentration. In contrast, the MEK1/2 inhibitor AS703026 showed only a slight decrease in the number of CD25 expressing cells, but a dose response decrease in the other activation metrics. These data show the differences in the MOA of various KI and illustrates the power of ForeCyt's visualization and analytic tools to increase biological insight of large data sets.

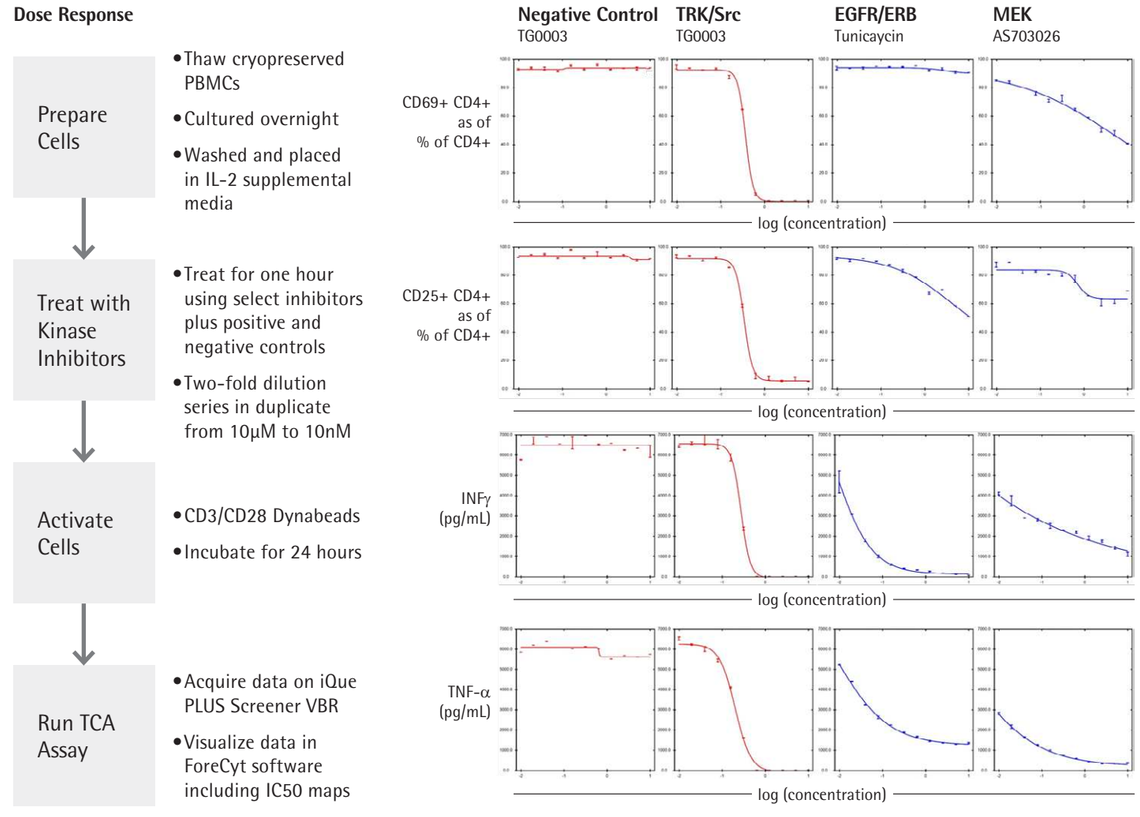

Figure 7. Dose Response Studies. Select KI were used for dose response studies to confirm MOA and determine IC50 concentrations for the various activation and functional markers.

Conclusion

This application notes shows the functionality of the Intellicyt TCA kit for long-term activation assays and in phenotypic drug discovery campaigns. Figure 8 shows the positioning of the TCA kit in the drug discovery workflow. In addition, the TCA kit is applicable to many functional assay workflows including development of checkpoint inhibitors and cell therapies and during cell manufacturing.

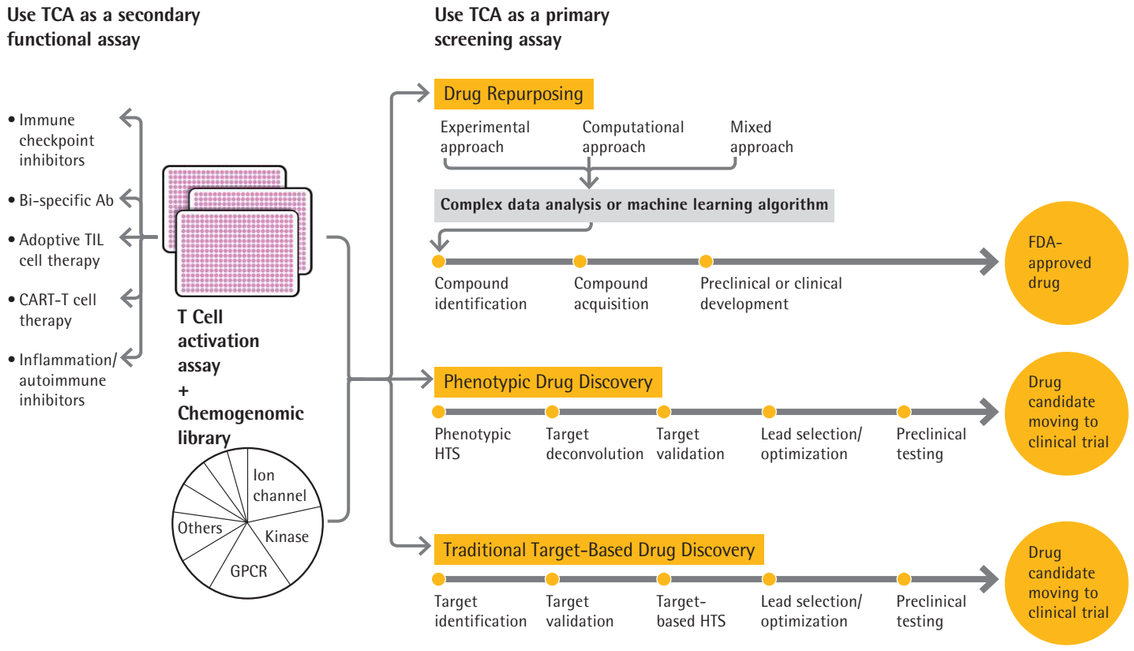

Figure 8. Position of T cell Activation Cell and Cytokine Profiling Kit in Drug and Biologic Discovery Workflow. The TCA assay can be used as a primary or secondary phenotypic screens in both the biologics and small molecule workflow. Additionally the TCA assay is used for functional studies in the immuno-oncology space and for characterizing T cells during cell manufacturing processes.

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