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White Paper

Signal Transduction Analysis of Pediatric Leukemia with a Spectral Flow Cytometer

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1. Introduction

While cure rates for childhood leukemia have improved steadily over the past several decades, those patients who experience relapse remain difficult to cure. Cancer remains the number one cause of death due to disease in children in developed countries. Acute lymphoblastic leukemia (ALL) is the most common cause of cancer in children worldwide. Currently, 1 in 5 children diagnosed with ALL will relapse from their disease and the majority of those who relapse will die. The central tenet of modern therapeutic approaches in childhood ALL is to risk stratify therapy so that children likely to survive are exposed to less toxic therapy to avoid late effects while children at high risk of treatment failure are treated with intensive therapies to improved their outcomes. In addition, the incorporation of novel therapeutics aimed at improving outcome is being tested in early phase clinical trials. Distinguishing populations of children at diagnosis who are destined to fail currently available therapies remains a challenge. In addition, it has been challenging to assess the cellular consequences of using therapeutic agents for those patients who are being treated with novel drugs. Such assessments remain important and critical steps in determining whether specific drugs or drug combinations should move forward in the clinic.

Phosphoflow cytometry (Figure 1) represents a highly powerful tool for directing therapeutic decision-making in childhood leukemia. Genetic and epigenetic alterations underlie the malignant phenotype. The biochemical consequences of these alterations manifest as aberrations in the activity of signal transduction networks and cellular responses to growth factors and cytokines. Importantly, these biochemical aberrations represent potential novel drug targets. Phosphoflow cytometry enables interrogation of these signaling alterations at the single cell level. Identification of these signaling alterations enables testing of targeted therapeutic agents. Phosphoflow cytometry can also be used to test, in real time, whether a patient sample is sensitive to a given chemotherapeutic agent. If the leukemia cell is resistant, phosphoflow cytometry can also be used to interrogate the mechanisms of this resistance and to unmask potential therapeutic means of overcoming this resistance. These features make phosphoflow cytometry an exciting tool in the emerging field of personalized medicine.

2. Cellular Autofluorescence

A significant problem encountered in current phosphoflow cytometry studies is the inherent autofluorescence (AF) present in primary patient samples, which occurs frequently after exposing cells to drugs in vitro. Variable levels of AF can be measured and removed using the unique features of the spectral flow cytometer developed by Sony Corporation. To observe cellular AF of samples, we measured unstained cells with the spectral flow cytometer. Figure 2 shows comparison of spectrum charts in some cell types as indicated. Xeno1, Xeno2 and Xeno3 are three different lines of cryopreserved xenograft samples derived from ALL patients generously provided to us by our colleague Dr Stephan Grupp at the Children's Hospital of Philadelphia. The spectrum chart shows wavelength in the horizontal axis, signal intensity in the vertical axis and population density in color scale. These figures indicate that cellular AF has high variability for all cell types, and the spectrum pattern is different depending on cell type.

In conventional methods, cellular AF spectral intensity variance is not considered, and only the average intensity of unstained cells is subtracted from multicolor data. In that method, deviation of AF is not considered, so the variance in AF intensity is addedto fluorescent dye signals such as FITC and PE. The spectral flow cytometer can distinguish cell AF against these dye signals spectrally, estimate the AF intensity in every cell, and remove it. The intensity dispersion among cells is controlled, so reduced deviation of dye signals are expected. Analyzing data is difficult with conventional flow cytometers because the cellular autofluorescence spectrum is similar to dyes such as FITC and PE, and thus, it is challenging to distinguish them.

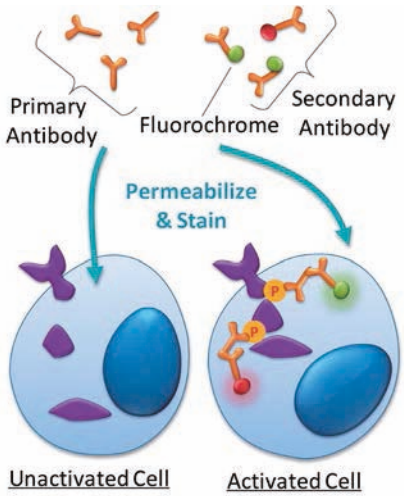

Figure 1. Phosphoflow cytometry

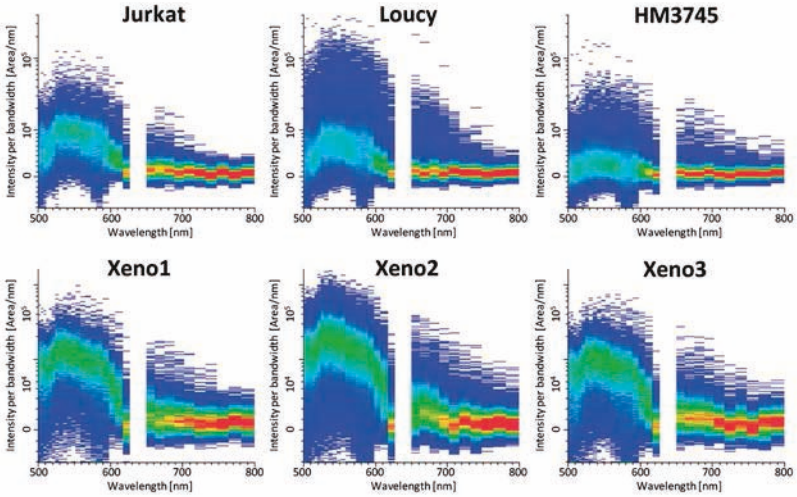

Figure 2. Comparison of cellular autofluorescence

3. Multi-color staining and spectrum compensation

We prepared T-ALL cell lines (Jurkat and Loucy) and a primary patient sample (HM3745) pre-treated with Vehicle (negative control), 4-Hydroxycyclophosphamide (4HC), Dexamethasone (Dex), BEZ235 (a dual PI3K/mTOR inhibitor) and phorbol 12-myristate 13-acetate (PMA) for 1 hour, and stained them with a 7 color antibody panel (Table 1). Then we measured these samples with the spectral flow cytometer, as well as unstained cell samples and single stain beads.

The spectrum compensation algorithm used here is based on a least square method (LSM). Here, we analyzed data two different ways; (1) subtraction of the mean intensity of unstained samples (similar to conventional compensation algorithms), then performance of 7 color LSM spectrum compensation, and (2) calculation of AF spectrum from unstained data, and together with 7 dye spectra, performance of 8-color LSM spectrum compensation. A portion of the analysis results is presented in Figure 3 (Horizontal axis: Caspase-3 Intensity (Area), Color scale: median value). The histograms in the top row show results from (1) normal AF subtraction with 7-color LSM, and the bottom 3 histograms are from (2) AF-included 8-color LSM. At a glance, we can see that AF-included 8-color LSM shows much sharper histograms with low variance, indicating that spectral removal of AF has a positive effect on the compensation calculation. A slight increase in Caspase-3 activation is reflected by the color scale in 1 hour Dex and PMA-treated samples.

Caspase-3	FITC
Survivin	Alexa Fluor 700
pERK	PE
pS6	APC
CD3	APC-Cy7
CD5	PE-Cy7
CD7	PE-Cy5

Table 1. Antibody panel

4. Phosphoflow and Survivin results

Next, we prepared Loucy samples treated with Vehicle, Dex, 4HC, BEZ235+Vehicle, BEZ235+Dex, and BEZ235+4HC for 24 hours and stained with the same antibody panel. Then the CD3+/CD7+/Caspase-3-negative population was gated and histograms of pERK, pS6 and Survivin were generated (Figure 4). We can clearly confirm the treatment dependent regulation of these intracellular molecules.

5. Conclusion

Assessing the biochemical consequences of exposing leukemic blasts to different drugs has been a technical challenge in the field due to the presence of autoflurescence using conventional flow cytometric techniqes. The cellular AF level differs depending on cell species and condition, and also has high variance among cells. In conventional compensation methods, such variability is not taken into account and deviation of cellular AF is addedto the variance of fluorescent dye intensities. The spectral flow cytometer has the capability to spectrally distinguish cellular AF from other dyes. We confirmed that by handling cellular AF as one color, and by running spectral compensation, we can obtain data with much lower variability. This feature helps us to analyze signal transduction molecules quantitatively, and we expect it will enable us to perform stable and reproducible analyses in large clinical investigations.

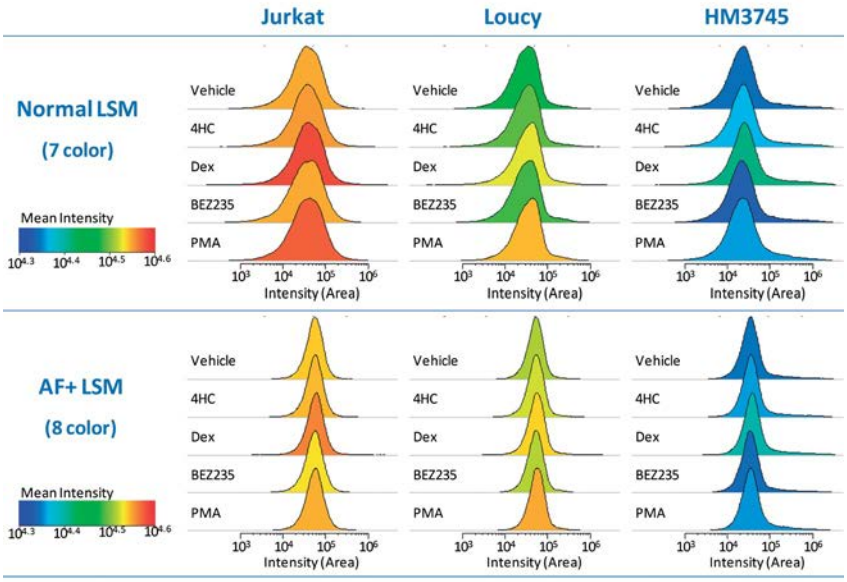

Figure 3. Caspase 3 (FITC) histograms with and without AF spectrum separation

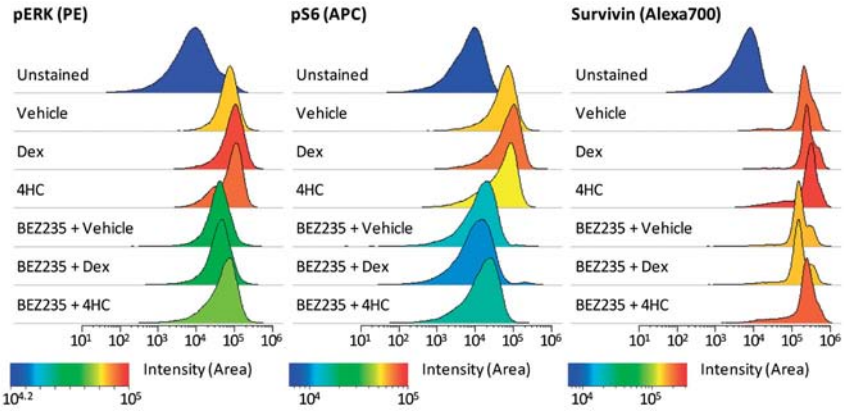

Figure 4. Analysis of signal transduction molecules in loucy samples

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