Simultaneous analysis of total and phospho proteins in single cells for accurate assessment of intracellular cell signaling events

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Abstract

Monitoring the activation (or inhibition) of key signaling pathways have been used as a viable method to assess cell signaling events and their implications in various diseases. Phospho-specific antibodies used for the detection of cellular activities have been highly useful in understanding these events in greater detail, and the method most commonly implemented to date is the Western blot. Although often seen as the "gold standard", this method can be very laborious, low throughput, and not very quantitative. Moreover, results usually represent data from a mixed cell population.

Here, we describe a dual detection method for measuring both the total and phospho-specific activation of a given target in intact cells simultaneously using an innovative approach. Simultaneous measurement of both total and phospho-specific antibodies by fluorescence-based detection confirms target specificity of the phosphorylation event. Moreover, all experiments are performed at the single cell level, providing an extra level of accurate quantitation and validation over traditional immunoblotting methods. Together, a total and phospho antibody duo applied in multiplex provides an enhanced and more reliable detection of the phospho: total ratio within a mixed cell population. A variety of cell signaling pathways have been assessed (MAPK, PI3K, and histone H2A.X for DNA damage response) by implementing this dual detection method for analysis.

The Muse® Cell Analyzer: Accelerating cell signaling analysis quantitatively and confidently

- A highly intuitive, compact instrument for fluorescent based analysis
- Uses miniaturized fluorescent detection to deliver truly accurate, precise, and quantitative cell analysis compared to other methods.
- Contains an integrated computer and software for data acquisition and analysis
- The user interface is specifically tailored for streamlined applications, so cell analysis progresses from sample setup to analysis and results in just a few minutes.

Muse® novel software interface Enhances and Simplifies the user experience

Introduction

Intracellular signaling cascades are a very complex highway of different events. And, for each pathway, its activation/deactivation status can greatly influence many biological functions ,such as cellular metabolism and cell growth. So, understanding signaling pathways is vital, since they are responsible for maintaining cellular homeostasis, and any disruption or changes in cell signaling can have many consequences as a result. Having the ability to monitor activation (or deactivation) can certainly provide greater insight into these complex pathways as researchers continue to dissect and understand how cells function.

We have evaluated effects of various compounds (and UV radiation) using the Muse® Cell Analyzer, implementing a dual detection approach.

- **Wortmannin**, which is a specific PI3K inhibitor, attenuated the constitutive activation of Akt signaling in Jurkat cells. We observed a drop in Akt activation via decrease in phosphorylation when comparing a treated 12% phosphorylated) versus untreated (90% phosphorylated) sample.
- **U0126** is a potent MEK1/2 inhibitor. By treating Jurkat cells, we were able to demonstrate the inhibition of the MAPK signaling pathway by measuring the activation of phospho ERK1/2.
- **Etoposide** effectively activated the DNA damage response as shown by the phosphorylation of histone H2A.X, where the number of cells which are specifically phosphorylated increased from less than 5% to over 90% upon drug treatment
- **UV radiation** of cells caused a substantial increase in histone H2A.X activation as well, where cells showed greater than a 50% increase in the level of DNA damage present when measured by the Muse® Analyzer.

In all cases, these examples clearly illustrate a simple, reliable, and accurate method to detect phospho-specific cellular activation for a variety of signaling events using both total and phospho-specific antibodies. And given the ease and simplicity for cell signaling pathway activation detection when paired with the Muse® Cell Analyzer, this method offers a viable alternative or complementary tool to Western blotting methods.

Methods

I. Cell Culture

Cells are either "Treated" or "Untreated" with a given compound (e.g. inhibitor). Following treatment, cells are then stimulated with a known agonist to cause activation

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II. Cell Fixation / Permeabilization / Staining

Fix cells immediately after stimulation, permeabilize, and stain with the appropriate total and phospho-specific antibodies.

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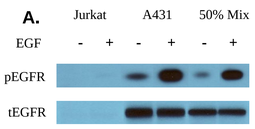
III. Muse ® Cell Analyzer Analysis:

Acquire cells using the Muse.

Template driven acquisition and analysis provides quantitative values including percentages (%) of inactivated, activated, and non-expressing cells

Results

I. Western blot analysis only provides population based data, whereas phospho-specific flow by Muse® Analyzer interrogates cells at the single cell level



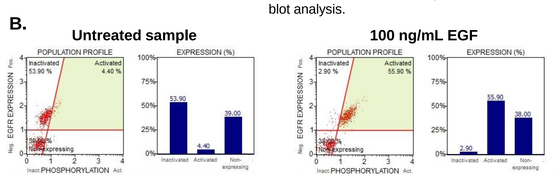


Figure 1. Quantitative vs. Qualitative assessment of cell signaling
Western blot analysis can only provide population based statistics. Moreover, the data generated is purely qualitative (A). Cell signaling analysis using the Muse® system clearly discriminates and can identify heterogenous cell populations (B). Also, it provides quantitative statistical measurements which cannot be achieved by traditional Western blot analysis.

II. PI3K Cell Signaling Analysis: Wortmannin attenuates the constitutive activation of Akt in Jurkat cells

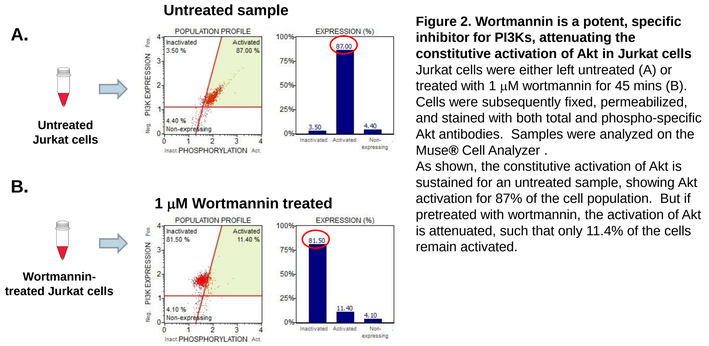
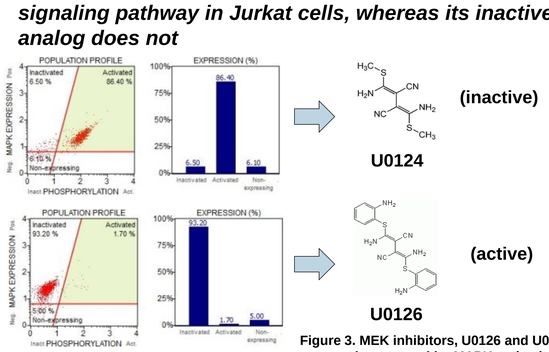


Figure 2. Wortmannin is a potent, specific inhibitor for PI3Ks, attenuating the constitutive activation of Akt in Jurkat cells
Jurkat cells were either left untreated (A) or treated with 1 µM wortmannin for 45 mins (B). Cells were subsequently fixed, permeabilized, and stained with both total and phospho-specific Akt antibodies. Samples were analyzed on the Muse® Cell Analyzer .
As shown, the constitutive activation of Akt is sustained for an untreated sample, showing Akt activation for 87% of the cell population. But if pretreated with wortmannin, the activation of Akt is attenuated, such that only 11.4% of the cells remain activated.

III. Specific MEK inhibitor, U0126, inhibits the MAPK signaling pathway in Jurkat cells, whereas its inactive analog does not



Cell Treatment (jurkat)	Activated?	Percentage (%) of ERK1/2 expression
Untreated	No	7.1%
100 ng/mL PMA treated	Yes	88.9%
10 µM U0126	No	1.7%
10 µM U0124	Yes	86.4%

Figure 3. MEK inhibitors, U0126 and U0124, are accurately assessed by MAPK activation assay.
U0126 is a specific MEK inhibitor, and after pretreatment with this compound, PMA stimulation yields no MAPK activation of this pathway, yielding an activation of less than 2%, confirming previous results of U0126's antagonist activity.
However, when Jurkat cells were pretreated with its inactive analog, U0124, PMA stimulation resulted in ERK phosphorylation, evidenced by shifts in the dot plots shown and corresponding bar graph, indicating that this compound is truly inactive since 86% of cells were activated upon PMA treatment.

IV. Topoisomerase inhibitor activity (anti-cancer drugs) and exposure to UV can be accurately assessed by the phosphorylation of histone H2A.X

A. Drug treatment exposure,

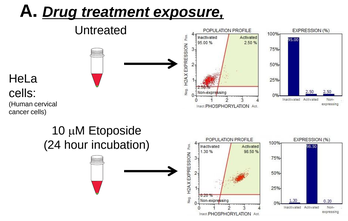


Figure 4. Both chemical (drug treatment) and physical (UV radiation) factors can contribute to DNA damage and histone H2A.X activation
HeLa cells chemically treated with a topoisomerase inhibitor, etoposide, undergo re-ligation of DNA strands which, in effect, will cause DNA strands to break (A). And as a result, this will cause errors in DNA synthesis resulting in damage, which will promote apoptosis for cancer cells, making etoposide a viable cancer therapy.
In (B), we exposed HeLa cells to varying doses of UV light and measured the extent of histone H2A.X activation for each. As the levels of UV dosage increased the level of histone H2A.X activation increased as detected by the Muse® Analyzer.

B. UV irradiation exposure,

According to Tanaka, et al. (2007), the increase in intensity of γH2AX after cell irradiation was strongly correlated with the dose of radiation

Summary

- Phospho-specific activation is a valuable and reliable method for detection of intracellular cell signaling events.
- The "Dual Detection" approach (e.g. use of total and phospho-specific antibodies for the same target) confirms target specificity of the phosphorylation event, eliminating false positives for greater accuracy and confidence in data.
- Key signaling pathways such as EGFR, Akt, and MAPK have all been closely associated with cancer research; a simple method for accurately monitoring cell signaling activation of these pathways be a very valuable tool for assessment of anti-cancer drugs.
- DNA damage can occur by either physical (e.g. UV irradiation) or chemical (e.g. anti-cancer drugs) means. The DNA damage signaling pathway and its activation can be accurately measured by analyzing the phosphorylation of histone H2A.X using the approach described.
- The Muse® Cell Analyzer delivers real-time, quantitative assessment of protein phosphorylation by laser-based fluorescence detection of each cell event, and, with built-in intuitive software and touchscreen interface, enables rapid setup and analysis.

References

1. English, J.M., *et. al.* (2002). *Trends Pharmacol Sci.;*23(1):40-5.
2. Walker, E.H., *et. al.* (2000). *Mol Cell*;6(4):909-19.
3. Tanaka, T., *et. al.* (2007). *Cell Cycle;*6(3):371-6.

Related Products

0500-3115 Muse® Cell Analyzer

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MCH200102	Muse® EGFR-RTK Activation Dual Detection Kit
MCH200103	Muse® PI3K Activation Dual Detection Kit
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MCH200105	Muse® Bcl-2 Activation Dual Detection Kit
MCH200107	Muse® Multi-Color DNA Damage Kit
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