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Biochemical and Biophysical Research Communications 323 (2004) 355–360
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Profiling the expression of mitogen-induced T-cell proteins by using multi-membrane dot-blotting

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Received 5 August 2004
Available online 28 August 2004

Abstract

High throughput technologies are standard methods for analysis of the proteome. Multi-layer multi-well plate dot-blotting system (MLDot) technology is a high-throughput dot blotting system that provides a simple, cost-effective approach for protein expression profiling in multiple samples. In contrast to traditional dot blot, MLDot uses a layered stack of thin, sieve-like membranes in place of a single nitrocellulose membrane. Therefore, up to 10 membranes can be prepared from the samples arrayed in a single 96-well plate. We describe the ability of MLDot to detect the predicted changes in protein expression following multiple mitogen treatment of T-cells. We compare the levels of the phopshorylated forms of CREB, Jun, and Akt in Jurkat T-cells as detected by MLDot to those measured by a gel-based assay. We also describe the ability of MLDot to detect differences in the levels of phosphorylated Akt in Jurkat cells as compared to primary lymphocytes.

Keywords: Cell signaling; Multiplex; Protein expression; Phosphorylation; Neoplasia; Drug discovery

To provide a simple, cost-effective alternative approach for protein expression profiling in multiple samples, a multi-layer multi-well plate dot-blotting system (MLDot) was developed [1]. MLDot is similar to traditional dot blot, but uses a layered membrane stack in place of a single nitrocellulose membrane. Therefore, up to 10 membranes can be prepared from a single transfer of samples arrayed in one 96-well plate. Thus, MLDot increases the efficiency and throughput of the traditional protein dot blot for high throughput molecular profiling.

MLDot technology is based on protein function and identification layered membranes (P-FILM). The thinness of these sieve-like membranes (approximately 10 μm as compared to approximately 100 μm of conventional membranes) and high affinity and low protein binding capacity (10–30 ng/cm^2) allow samples to be transferred simultaneously to multiple individual layers [1–3].

To perform MLDot, cell lysates contained in a multi-well plate are transferred to the P-FILM stack using a vacuum apparatus [1]. The levels of total proteins and specific proteins of interest can be quantitated on each membrane by using fluorescent detection methods. In this way MLDot can simultaneously profile multiple proteins in multiple samples. For instance, MLDot provides the ability to test the effects of multiple drug combinations on the proteome and thus is a useful tool for high throughput analysis of drug targets.

We have previously demonstrated that MLDot is a novel technology capable of detecting nuclear, cytoplasmic, and membrane proteins within a size range of 10–200 kDa [1]. Furthermore, we found that 15–20 μg

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doi:10.1016/j.bbrc.2004.08.093

is the recommended amount of input protein to provide the optimal balance between maintaining the ability to detect concentration changes in a linear fashion and achieving low inter-membrane variability [1]. In this report we apply MLDot for high throughput proteomic profiling of biological changes in Jurkat cells treated with multiple mitogen combinations and test the ability of MLDot to detect changes in protein expression predicted by transcriptional responses. Additionally, we validate the MLDot results by using a gel-based assay (MLWestern). Analogous to MLDot of samples in a multi-well plate format, protein samples separated by PAGE can also be transferred to P-FILM membranes in a Western blot assay (MLWestern).

Jurkat cells were chosen because they provide a good in vitro system for studying lymphocyte related diseases including cancers, autoimmune disorders, and autoinflammatory disorders. Mitogens such as phorbol myristate acetate (PMA), phytohaemagglutinin (PHA), and Ionomycin, as well as activation of the CD3 and CD28 receptors via crosslinking receptor specific antibodies, have been shown to activate several signal transduction proteins in both Jurkat cells and primary blood lymphocytes [4–8]. For example, the activity of the CREB and c-Jun proteins is affected similarly in Jurkat cells and primary T-cells [4–8]. One important difference between Jurkat cells and primary lymphocytes is the activity of the Akt protein [9–11]. Since Akt is constitutively activated in Jurkat cells, mitogens result in greater changes in Akt activity in primary lymphocytes than in Jurkat cells [9–11]. Therefore, we chose to profile the activities of CREB, c-Jun, and Akt in T-cells treated with multiple mitogens by MLDot analysis. In addition to further understanding basic mechanisms of signaling, these types of studies have clinical relevance for elucidating the molecular mechanisms of therapeutic agents.

Materials and methods

Cell culture and protein extraction. The Jurkat cell line was cultured in RPMI media with 10% FCS at 37 °C. Primary blood lymphocytes (PBL) were processed as previously described [4]. Jurkat cells and PBL were treated with the indicated mitogen/drug combinations at the following concentrations: 50 ng/ml PMA (Sigma, St. Louis, MO, USA), 1 μg/ml PHA (Sigma, St. Louis, MO, USA), 720 ng/ml Ionomycin (Calbiochem, San Diego, CA, USA), 1.5 μg/ml anti-CD28 (Research Diagnostics, Flanders, NJ, USA), and 1:1000 dilution anti-CD3 ascites fluid (ATCC) for 5 h. Jurkat cells were lysed with RIPA buffer, centrifuged (12,000 rpm), and the supernatant was collected and stored at −20 °C. PBL were harvested, washed twice with PBS, and lysed in a modified RIPA buffer.

Transfer of proteins by MLDot. Lysates containing 15 μg of total protein (100 μl volume) were loaded in each well of a 96-well plate and transferred onto a 5-membrane stack of P-FILM membranes (20/20 GeneSystems, Rockville, MD, USA) by vacuum transfer through the Bio-Dot Microfiltration Apparatus (Bio-Rad, Hercules, CA, USA). After transfer, membranes were rinsed in deionized water, separated, and air-dried.

Detection of total protein. Biotinylation was performed by incubating membranes in 0.001% EZ-Link Sulfo-NHS-Biotin (Pierce, Rockford, IL, USA) solution in 1× PBS buffer for 10 min, followed by 3 × 3 min washes in TBST (50 mM Tris, 150 mM NaCl, and 0.05% Tween 20) buffer, air-drying, and incubation with a streptavidin-Cy5 conjugate.

Detection of specific protein. Membranes were incubated overnight at 4 °C in TBST + 0.1% BSA solution of antibodies against the following proteins: Phospho-Jun (1:1000, Cell Signaling, Beverly, MA, USA), phospho-CREB (1:1000, Cell Signaling, Beverly, MA, USA), and phospho-Akt (1:1000, BD, San Diego, CA). After three washes (3 min each) in TBST buffer, membranes were incubated for 1 h at room temperature in Fluorescein-goat anti-mouse IgG (1:1,000) (Molecular Probes, Eugene, OR, USA), washed three times (3 min each) in TBST buffer, and air-dried.

Fluorescent detection of protein signal. Membranes were scanned on the Typhoon scanner (Amersham Biosciences, Piscataway, NJ, USA). Signal intensity was determined by Image Quant software (Amersham Biosciences, Piscataway, NJ, USA) and data were graphed and analyzed by Excel (Microsoft) and JMP (SAS Institute).

Quantitation and normalization of protein signal. For total protein on each membrane, the signal intensity of the uppermost left sample was arbitrarily set at 1.0. The signal intensity of each of the other 95 samples was then normalized to this reference sample. The intensity of each specific protein signal was normalized to its corresponding total protein signal.

MLWestern. Total protein concentrations of Jurkat lysates were determined by using the BCA Protein Assay Kit (Pierce, IL, USA). Twenty micrograms of each sample was separated in a 4–20% Tris–HCl polyacrylamide gel in Mini-Cell Module (BioRad, Hercules, CA, USA) and transferred onto a 5-membrane stack of P-FILM membranes (20/20 GeneSystems, Rockville MD, USA). The membranes were rinsed in TBST buffer and incubated overnight at 4 °C in TBST + 0.5% BSA solution of the same antibodies and dilutions used for MLDot. The membranes were washed three times (5 min each) in TBST and incubated with secondary antibody (1:2000 dilution in 0.5% BSA) conjugated to AP (Rockland, Gilbertsville, PA, USA) for 30 min at room temperature. The membranes were next washed once for 5 min in TBST, then 2 times (5 min each) in TBS. After washing, the membranes were incubated with Duolux chemiluminescent substrate AP (Vector, Burlingame, CA, USA) according to manufacturer's protocols. The resultant chemiluminescent signal was detected on KODAK BIOMAX MR X-ray film.

Results and discussion

MLDot assay detects proteome changes in mitogen activated T-cells

We performed MLDot experiments to measure the response of Jurkat to various mitogen treatments. Specifically, the activities of the CREB, Jun, and Akt signal transducing proteins were assayed. Jurkat cells were treated in triplicate experiments with the following mitogen combinations: PMA/PHA, PMA/Ionomycin/CD28, PMA/Ionomycin, PMA/CD28, PHA, CD3/CD28, and CD3. These mitogens were chosen because in Jurkat cells PMA and Ionomycin, as well as activation of the CD28 receptor, have previously been shown to increase the activity of CREB and c-Jun proteins [4,5]. Furthermore, PHA as well as the combination of CD3/CD28 increased CREB-mediated transcription [6,7]. In combination with other mitogens, PHA has also been shown to increase pro-

tein levels of c-Jun as well as the activity of c-Jun kinase in Jurkat cells [5,8]. Although the CREB and Jun targets downstream of Akt signaling are modified, changes in the phosphorylation state of Akt are not readily discernable due to the constitutive phosphorylation of this protein in Jurkat [9].

Cells were lysed and part of each sample was used for MLDot while the remainder was saved for MLWestern validation experiments. First, MLDot to a 5-membrane stack was performed. The levels of the phosphorylated (p) forms of CREB, Jun, and Akt were measured by fluorescent detection of a Cy5-secondary antibody conjugate and quantitated by using the Typhoon scanner and ImageQuant software. The levels of expression of all of these specific proteins were normalized to the corresponding amount of total protein for each sample. Total protein was assayed by biotinylation of the membranes followed by incubation with a streptavidin-FITC conjugate. Fig. 1 shows the percent differences in expression of p-CREB, p-Jun, and p-Akt after selected mitogen treatments as compared with the unstimulated cells. The coefficient of variability (CV) values were calculated for each triplicate set of treatments and were plotted as error bars for each treatment. CV values ranged from ±0.85% to 13.5%. Eleven of the 14 samples had CV values less than ±10%, which indicates the low variability and high reproducibility of MLDot.

Jurkat cells showed the highest induction of p-CREB and p-Jun in response to the mitogen combinations of PMA/Ionomycin/CD28 and PMA/CD28. In comparison with unstimulated cells, p-CREB was induced 61% in response to PMA/Ionomycin/CD28 and 77% in response to PMA/CD28. p-Jun was induced 39% in response to PMA/CD28 and 50% in response to PMA/Ionomycin/CD28. Therefore, MLDot detected the expected mitogen-induced changes in the activities of CREB and c-Jun. Furthermore, as expected Jurkat cells showed slight overall decreases (10–20%) in the levels of p-Akt in response to the mitogen combinations of PMA/PHA, PMA/Ionomycin, and CD3.

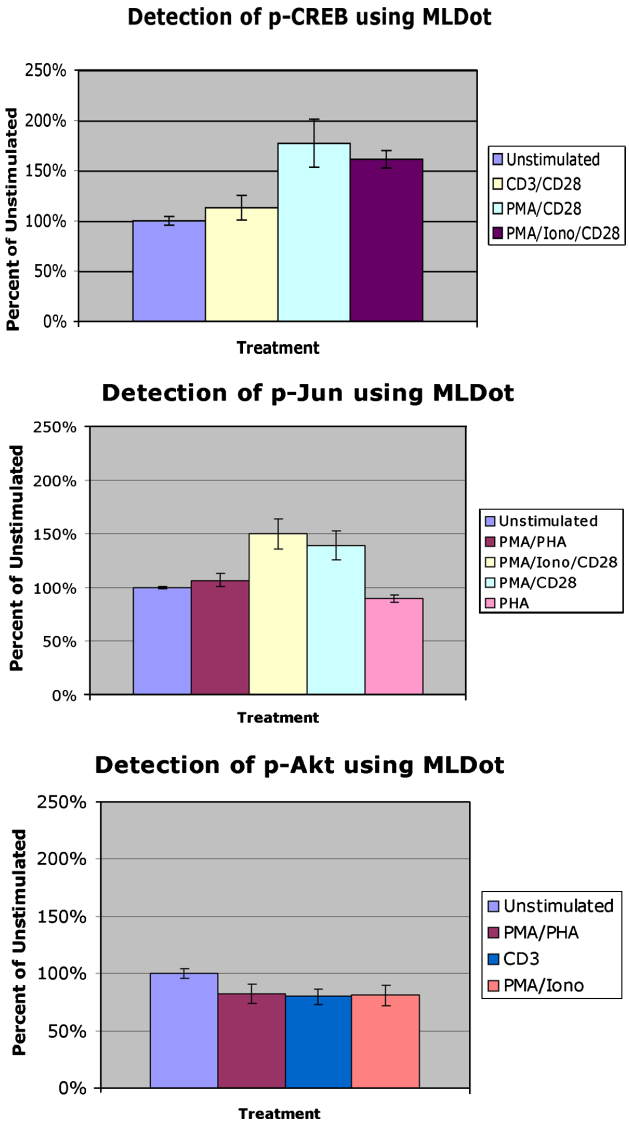

Fig. 1. MLDot assay of the expression of p-CREB, p-Jun, and p-Akt in Jurkat following treatment with various mitogens. Triplicate experiments were performed and each colored bar represents the mean value of three experiments. The expression levels of each protein were designated as 100% in the unstimulated cells. The expression levels of each protein following mitogen treatment are represented as a percentage of the levels in unstimulated cells. CV values are shown as a black bar on each of the colored bars.

MLWestern assay detects proteome changes in mitogen-stimulated T-cells

We performed MLWestern to verify the changes in expression of p-CREB, p-Jun, and p-Akt that were determined by MLDot. MLWestern was performed on the same samples that were used for MLDot and the results are shown in Fig. 2. Fig. 2A is a graphical representation of the signal intensities of p-CREB, p-Jun, and p-Akt proteins shown in Fig. 2B. MLWestern confirmed the mitogen-induced protein changes detected by MLDot (compare Figs. 1 and 2A). For example, p-CREB was induced 61% in response to PMA/Ionomycin/CD28 and 78% in response to PMA/CD28. p-Jun was induced 49% in response to PMA/CD28 and 54% in response to PMA/Ionomycin/CD28. Although MLDot and MLWestern detected comparable changes in protein expression levels for most of the samples, MLWestern did detect greater changes in protein levels in some samples than were detected by MLDot. This is expected because of the higher sensitivity of the fluorescent detection used in MLDot and because the proteins of interest are clearly separated from background proteins in MLWestern. For example, MLWestern detected a 51% increase in p-Jun upon PMA/PHA treatment compared to a 7% increase detected by MLDot.

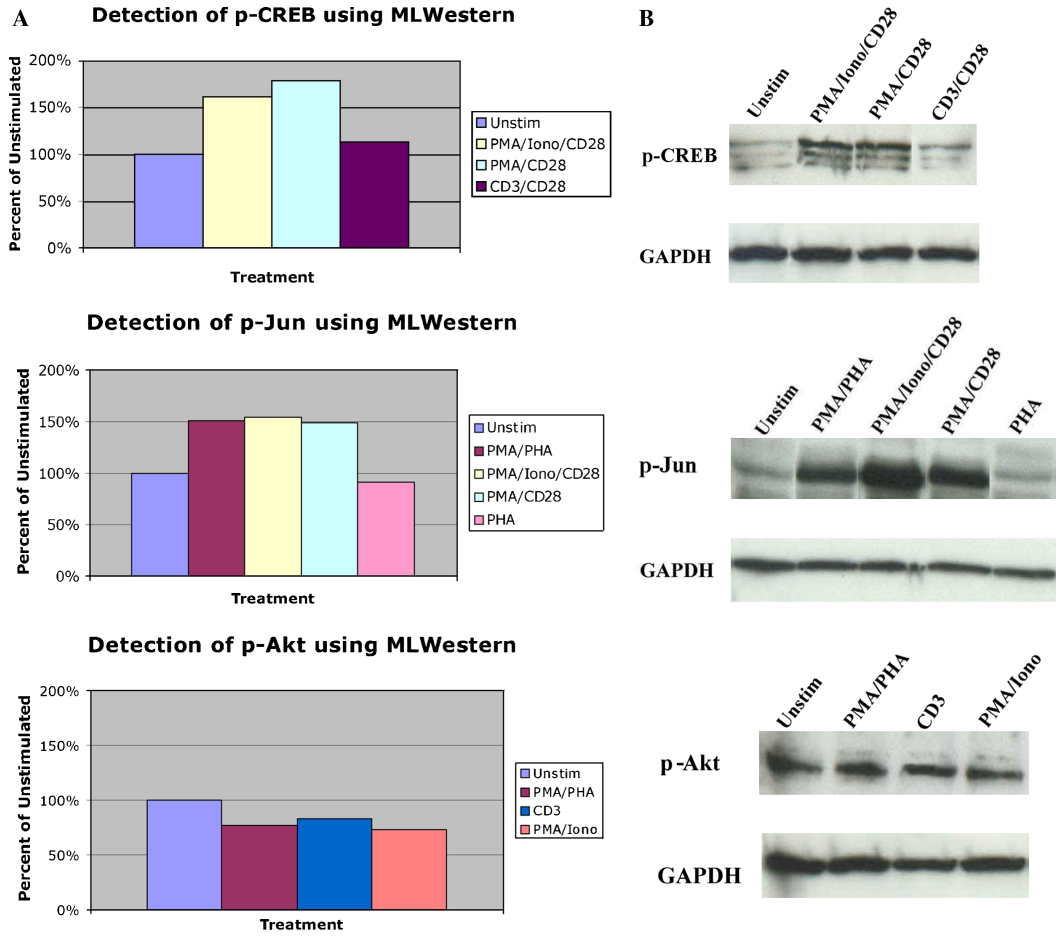

Fig. 2. MLWestern assay of the expression of p-CREB, p-Jun, and p-Akt, in Jurkat following treatment with various mitogens. (A) Bar graphs of the expression level of each protein following mitogen treatment. The expression levels of each protein were designated as 100% in the unstimulated cells. The expression levels of each protein following mitogen treatment are represented as a percentage of the levels in unstimulated cells. (B) MLWestern detecting expression of p-CREB, p-Jun, and p-Akt in Jurkat following the indicated mitogen treatments.

Comparative analysis between MLDot and MLWestern

The data from Figs. 1 and 2 were graphed in a comparative analysis between MLDot and MLWestern. Selected mitogen treatments are shown in the *X*-axis. The levels of p-CREB, p-Jun, and p-Akt as compared to the corresponding levels in unstimulated cells are shown. Similarities between the slopes of the curves indicate that MLDot and MLWestern are providing comparable and reproducible data. For each of the three proteins, the slopes of the curves between each mitogen treatment point were very similar (Fig. 3). The same mitogen treatments (PMA/Ionomycin/CD28 and PMA/CD28) resulted in the greatest changes in protein expression as detected by both MLDot and MLWestern.

MLDot to measure p-Akt in primary T-cells and Jurkat cells

We next tested whether MLDot could detect differences in p-Akt expression in primary lymphocytes and the Jurkat T-cell line. Since Akt is constitutively phosphorylated on Ser 473 in Jurkat cells [9], little change in Akt activity is expected in response to mitogen treatment. However, activation of CD28 has been shown to result in increased Akt activity in Jurkat cells [10]. In contrast to Jurkat cells, mitogen activation of primary human peripheral blood T cells induces Akt activation [11]. PBL and Jurkat were treated with the mitogen combinations of P/I/CD28 and CD3/CD28 and MLDot was performed. The mitogen combination of P/I/CD28 induced a 0.77-fold increase in p-Akt in PBL compared to only a 0.19-fold increase in p-Akt in Jurkat cells (Fig. 4). The mitogen combination of CD3/CD28 resulted in a 0.38-fold increase in p-Akt in PBL and a 0.28-fold increase in p-Akt in Jurkat cells (Fig. 4). Therefore, MLDot detected the expected differences in the amounts of mitogen-induced p-Akt between primary T-cells and a T-cell line.

Statistical analysis of MLDot and MLWestern

We performed statistical analysis to address two questions. The first question was whether the changes in protein expression levels between the unstimulated

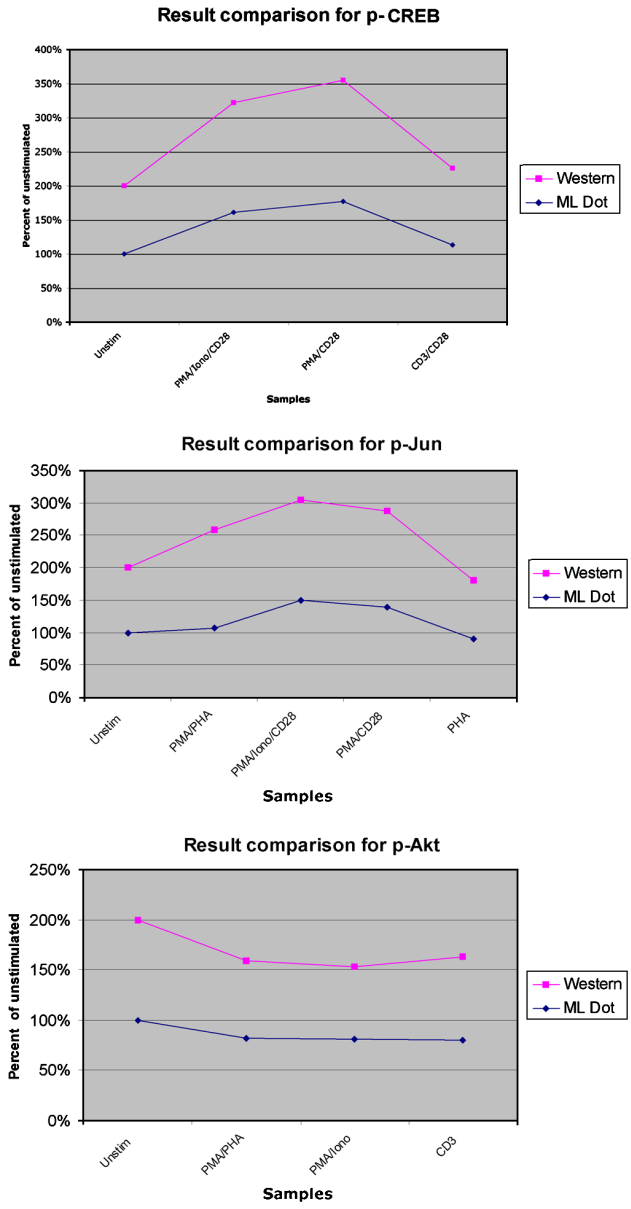

Fig. 3. Comparison of MLDot and MLWestern. A comparative analysis of the MLDot and MLWestern data presented in Figs. 1 and 2 is shown. Mitogen treatments are shown in the *X*-axis. The levels of the indicated protein (p-CREB, p-Jun, and p-Akt) following mitogen treatment in relation to the levels in unstimulated cells are shown in the *Y*-axis. Note that the degree of similarities between the slopes of the curves correlates with the reproducibility of data determined by MLDot and MLWestern assays.

and stimulated cells are statistically significant with the MLDot system. The second question was whether there are statistically significant differences between expression level changes measured by MLDot versus MLWestern.

To answer the first question, we determined the probability that the expression levels of p-CREB, p-Jun, and p-Akt following treatment with mitogen as detected by MLDot differed significantly from those levels in untreated cells. A one-tailed distribution Student's *t* test

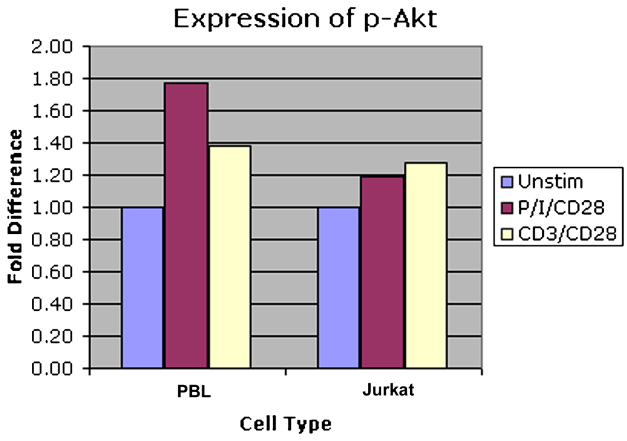

Fig. 4. Changes in expression of proteins in PBL and Jurkat in response to mitogen treatment. Shown are the fold differences in protein expression of p-Akt in PBL and Jurkat. Blue bars represent unstimulated cells, red bars represent cells treated with P/I/CD28, and yellow bars represent cells treated with CD3/CD28.

Table 1
Statistical analysis comparing MLDot results between unstimulated and mitogen-stimulated cells

Protein	Treatment	*P* value
pCREB	PHA	0.05
	CD3	0.09
	PMA/CD28	0.05
	PMA/IONO/CD28	0.01
p-Jun	PHA	0.06
	CD3	0.08
	PMA/CD28	0.06
	PMA/IONO/CD28	0.05
p-Akt	PHA	0.01
	CD3	0.06
	PMA/CD28	0.01
	PMA/IONO/CD28	0.05

Summary of one-tailed distribution *t* test comparing the expression of p-CREB, p-Jun, and p-Akt in Jurkat cells between unstimulated and mitogen-treated cells. Jurkat cells were treated with the mitogen combinations of PHA, CD3, PMA/CD28, and PMA/Ionomycin/CD28, and protein expression was detected by MLDot.

of type 3 two-sample unequal variance was performed by using Microsoft Excel software. The expression levels of each protein in unstimulated cells were designated as 100% and the protein levels in stimulated cells were compared to this baseline value. *P* values of <0.1 indicate that there is a statistically significant difference between the compared data sets, i.e., a statistically significant difference in protein expression between unstimulated and stimulated cells. Table 1 shows that upon stimulation with the mitogens PHA, CD3, PMA/CD28, and PMA/Ionomycin/CD28, Jurkat cells showed statistically significant changes in expression of p-CREB, p-Jun, and p-Akt as detected by MLDot. Therefore, the protein changes detected by MLDot are statistically significant.

Table 2
Statistical analysis comparing detected changes in protein expression levels between MLDot and MLWestern

Protein	Treatment	*P* value
p-CREB	PMA/IONO/CD28	0.49
	PMA/CD28	0.48
	CD3/CD28	0.49
p-Jun	PMA/IONO/CD28	0.42
	PMA/CD28	0.30
	PHA	0.40
p-Akt	PMA/PHA	0.31
	PMA/IONO	0.25
	CD3	0.37

Summary of one-tailed distribution *t* test comparing the expression level changes of p-CREB, p-Jun, and p-Akt as detected by MLDot vs MLWestern.

To answer the second question, we determined the probability that the changes in expression levels of p-CREB, p-Jun, and p-Akt following treatment with mitogen as detected by MLDot did not differ significantly from the corresponding expression changes as measured by MLWestern. A one-tailed distribution Student's *t* test of type 3 two-sample unequal variance was performed by using Microsoft Excel software. For both MLDot and MLWestern the expression levels of each protein in unstimulated cells were designated as 100% and the protein levels in stimulated cells were compared to this baseline value. Expression level changes were then calculated and the corresponding changes were compared between MLDot and MLWestern. For example, the expression level change for p-CREB following treatment with PMA/Ionomcyin/CD28 was determined by both MLDot and MLWestern and then these values were compared by the *t* test. *P* values of >0.1 indicate that there is no statistically significant difference between the compared data sets, i.e., the expression level changes detected by MLDot are statistically similar to those changes detected by MLWestern. Table 2 shows that the detected expression level changes of p-CREB, p-Jun, and p-Akt were statistically similar between MLDot and MLWestern. Therefore, there is no statistically significant difference between protein expression level changes measured by MLDot and MLWestern.

In summary, MLDot can be used to profile proteomic changes in T-cells. We demonstrate that MLDot has the ability to detect predicted changes in the expression of p-CREB, p-Jun, and p-Akt following treatment with combinations of the mitogens PMA, PHA, Ionomycin, anti-CD3, and anti-CD28. These results were confirmed by the gel-based MLWestern assay. Furthermore, MLDot has the ability to detect the expected differences in the amounts of mitogen-induced p-Akt between primary T-cells and a T-cell line. MLDot provides a straightforward and cost-effective method for profiling proteomic changes in multiple samples treated with mitogens and drugs.

Acknowledgments

We thank Dr. Michael Emmert-Buck of the National Cancer Institute and Dr. Dan-Paul Hartmann of Georgetown University for excellent scientific discussion. This work was supported by the National Cancer Institute contract N43-CO-31044.

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