# Alternative Fixation Method Improves Flow Cytometry - Assisted Phospho-Detection Competence

## Competența fosfo-detecției flow-citometrice este îmbunătățită substanțial prin utilizarea unei metode alternative de fixare

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### Abstract

*Analysis of protein phosphorylation within intracellular signaling pathways may clarify the functional status and biological responses of cells to various stimuli, reflecting an enhanced enzymatic activity. Phospho-specific antibody (p-Ab)-based detection methods that have been used to date, including flow cytometry, require extensive optimization for the achievement of accuracy and rational specificity. Objective. We sought to optimize a phospho-specific flow cytometry detection method, based on the premise that fixation is a critical step of such protocols. Materials and methods. We compared two different methods for phospho-detection in phorbol myristate acetate (PMA)-activated peripheral blood mononuclear cells (PBMCs) and assessed how they distinctly impacted on cell viability, surface staining intensity and phospho-detection competence, aiming to develop an optimized protocol with balanced efficiency in surface and intracellular phospho-labeling. PMA-activated PBMCs were used for testing the detection efficiency of five p-Abs: pERK(T202/Y204), pp38MAPK(T180/Y182), pAKT(T308), pSTAT-3(S727), pSTAT-1(Y701) by flow cytometry. Results. 1. Fixation prior to surface staining led to an improved signal-to-noise ratio for all p-Abs evaluated; 2. cell suspension integrity was not affected by the fixation method used; 3. reduced efficiency of surface marker detection levels remained within acceptable ranges; 4. one freeze-thaw cycle did not significantly impair the cell suspension integrity or staining efficiency. Conclusion. These results provide a practical approach for phospho-detection in heterogeneous tissue samples. The clinical relevance of our technical approach appears substantial, as frequent dysfunctions within signaling pathways are linked to various diseases and consequently require proper identification methodology.*

***Keywords:*** *phosphorylation, cell signaling, flow cytometry*

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## Rezumat

*Evaluarea nivelului de fosforilare al proteinelor aparţinând căilor de semnalizare intracelulară reflectă statusul funcţional şi biologic celular ca răspuns la variaţi stimuli prin intensificarea activităţii enzimatice. Metodele consacrate de detecţie bazate pe anticorpi monoclonali fosfo-specifici (p-Ab=phospho-specific antibodies), inclusiv citometria în flux, necesită parcurgerea unor etape riguroase de optimizare care să garanteze acurateţea şi specificitatea detecţiei. Obiectiv. Plecând de la premiza că fixarea este o etapă critică în cadrul protocoalelor de procesare a probelor, ne-am propus să optimizăm detecţia proteinelor fosforilate, prin elaborarea unui protocol echilibrat în ceea ce priveşte intensitatea markerilor de suprafaţă şi intracelulari. Material si metodă. Au fost utilizate două metode de fosfo-detecţie în suspensii de celule mononucleate sanguine (peripheral blood mononuclear cells=PBMCs) activate cu phorbol myristate acetate (PMA). A fost comparat efectul lor distinct asupra viabilităţii, intensităţii de suprafaţă şi capacităţii de fosfo-detecţie. Prin citometrie în flux au fost testaţi următorii p-Ab: pERK(T202/Y204), pp38MAPK(T180/Y182), pAKT(T308), pSTAT-3(S727), pSTAT-1(Y701). Rezultate. 1.Utilizarea unei metode alternative de fixare asigură o eficienţă superioară a fosfo-detecţiei pentru toţi p-Abs evaluaţi, în comparaţie cu metoda clasică de fixare. 2.Viabilitatea celulară nu este afectată de metoda de fixare utilizată. 3.Metoda alternativă de fixare asigură o eficienţă acceptabilă a marcării de suprafaţă. 4.Integritatea suspensiei celulare sau eficienţa de marcare nu sunt afectate de îngheţ-dezgheţ. Concluzii. Relevanţa clinică a unor astfel de protocoale tehnice este substanţială, întrucât o gamă din ce in ce mai largă de patologii se asociază cu disfuncţii în cadrul căilor de semnalizare intracelulară, necesitând aşadar metodologii sensibile şi precise de investigare.*

***Cuvinte cheie :*** *fosforilare, semnalizare celulară, flow citometrie*

## Introduction

The phosphorylated state of a particular protein correlates with its biological condition (1, 2), reflecting an enhanced enzymatic activity and an ongoing signal-propagation event downstream to the transcription machinery. Therefore, the analysis of intracellular protein phosphorylation may clarify the functional status and biological responses of cells to various stimuli. With the increasingly advanced technology available, researches have begun to identify more clinical applications of the analysis of phosphorylated proteins. Nevertheless, this area of research with immediate prospects for clinical applications is still in development and considerable efforts are under way to optimize the accuracy and specificity of phosphorylated protein detection.

Phospho-antibody-based detection methods that have been used to date are subject to particular technical challenges generated by the transient, reversible nature of the phosphorylation event, difficult antigen accessibility and frail stability of the phospho-epitope. One of the most difficult technical issues of such methods is providing information on complex functional responses within heterogeneous cell samples. Cell types that appear homogeneous by surface markers have been found to be heterogeneous based on their intracellular signaling profile (3). Western blot and ELISA quantify phospho-epitopes from bulk populations of cells (4, 5). To obtain results characteristic to a certain cell type, these methods would require either cell sorting, or depletion of irrelevant cell types prior to analysis. Both ways of cell enrichment procedures put cells through artificial conditions and can induce changes within their phospho-protein status during the process. In contrast, flow cytometry and fluorescent microscopy are able to provide measurements within single cells (6-11). Microscopy, however, is limited in the number of cells that can be analyzed (12-13), while flow cytometry allows a much higher throughput. Western blot enables the measurement of phospho-proteins in denatured-detergent soluble cell lysates, while flow cytometry allows the study of phospho-proteins trapped in detergent-insoluble fractions (such as lipid membrane rafts or cytoskeleton) which may be missed by western blot. Therefore, measuring the phosphorylation state of proteins by flow cytometry may become a method of choice in clinical applications. The technique is very rapid, informative and allows various statistical evaluations, such as population means, medians, standard deviations, coeffi-

cient of variation and/or peak shapes (3). Nevertheless, measuring intracellular antigens by flow cytometry is a complex process and requires extensive optimization in order to obtain best accuracy, rational specificity and semi-quantitative results.

Peripheral blood mononuclear cells (PBMCs) can be rapidly brought into an activated state by phorbol myristate acetate (PMA) treatment, and thus have the potential to provide an instrument for testing the efficiency of p-Abs detection. Here, we compared two different methods for phospho-detection in PMA-activated PBMCs and discuss how they distinctly impacted on cell viability, surface staining intensity and phospho-detection competence, aiming to develop an optimized protocol with balanced efficiency in surface and intracellular phospho-labeling.

## Material and methods

### *Sample acquisition and PBMCs isolation*

Peripheral blood samples collected on EDTA were obtained from five normal donors according to institutionally approved protocols. The study and analysis were carried out according to the guidelines of the Declaration of Helsinki and good clinical practice. All donors gave their written informed consent for institutional-initiated research studies. PBMCs were obtained within two hours from venipuncture by density gradient centrifugation on Ficoll-Histopaque (Sigma). In one case the cells were adjusted to $1x10^6$/mL and stored at -80°C for several weeks before stimulation and staining.

### *Reagents, solutions, and antibodies*

PMA (Sigma) was prepared as 400µM in 100% ethanol. Para-(p-) formaldehyde was stored as a 4% solution in phosphate buffered saline (PBS) at room temperature (RT). Wash buffer consisted of PBS, 1% fetal calf serum (FCS), 0.1% sodium azide. Freezing medium consisted of 20% FCS, 10% Dimethyl sulfoxide (DMSO) in RPMI-1640 medium (Sigma). 90% methanol was stored at -20°C for at least one hour before use as a permeabilization reagent. For surface staining, the following fluorochrome (fluorescein isothiocyanate-FITC and allophycocyanin-APC) conjugated monoclonal antibodies were used: CD45-APC (BD Bioscience) and CD64-FITC (BD Pharmingen). For intracellular staining, the following BD Phosflow phycoerythrin- (PE)- conjugated p-Abs were used: pERK(T202/Y204)-PE, pp38MAPK(T180/Y182)-PE, pAKT(T308)-PE, pSTAT-3(S727)-PE, pSTAT-1(Y701)-PE. These antibodies are specifically directed against phosphorylated threonine (T), tyrosine (Y) or serine (S) residues. Isotype matched controls were used.

### *PMA activation of PBMCs*

$1x10^7$ PBMCs were stimulated at a final concentration of 50nM PMA in 1ml FCS-free RPMI-1640 medium, 10 min, 37°C. Stimulation was blocked with 1% FCS in PBS.

### *Flow cytometry*

Data acquisition was performed using a FACS Canto II flow cytometer (BD Bioscience). Up to 10 000 events were acquired and saved as data files. The same settings for gains, voltages and spectral overlap corrections were used for all measurements. Data acquisition was performed with FACS Diva software (version 6.1). Data analysis was performed with FlowJo software (Tristar Inc., kind gift from Prof. Mertelsmann, Hematology & Oncology Department, University of Freiburg, Germany).

### *Surface and intracellular staining*

***Method 1***. For each donor, six FACS tubes containing each 50 µL of PMA-stimulated PBMCs suspension were incubated for 30 min at RT, in darkness, with CD45-APC (1:25) and CD64 FITC (1:25). Samples were washed once in FCS-free PBS (300xg, 5 min, RT), supernatant discarded and cell pellets resuspended in a final concentration of 2% p-formaldehyde for 10 min at 37°C. Permeabilization was carried out with 90% methanol for 30 min on ice. For intracellular staining, surface-labeled, fixed and permeabilized cells were washed once, stained immediately with 1:5 p-Abs, and incubated on ice for 1 hour. Completely stained cells were washed once, resuspended in 200 µL wash

buffer and analyzed by flow cytometry.

***Method 2.*** The same steps as in method 1 were performed, with the difference that PBMCs suspensions were fixed immediately after PMA activation, then stained for surface markers, permeabilized and stained with p-Abs. All steps were performed on ice in order to keep any intra-cellular phosphatases inactive.

***Statistical analysis***

For flow cytometry results, FlowJo-software assisted calculations of median fluorescence intensity (MFI), median fluorescence intensity ratio (MFIR, as the ratio between MFI of sample versus control), and percentages of positive cells were performed. MFIR values greater than 2 were associated with a positive expression and efficient staining. When appropriate, statistical analyses were performed by means of paired Student's t tests. A p value of $<0.05$ was considered to indicate statistical significance.

## Results

***Fixation prior to surface staining leads to an improved signal-to-noise ratio for all p-Ab evaluated***

In order to evaluate the expression level of both surface and intracellular markers, lymphocytes and monocytes found within PBMCs suspensions were identified based on CD45 and CD64 expression and following the software-assisted exclusion of cell doublets and artifacts. *Figure 1* depicts the gating strategy used for the flow cytometry analysis in complex mixtures of cell populations, such as PBMCs.

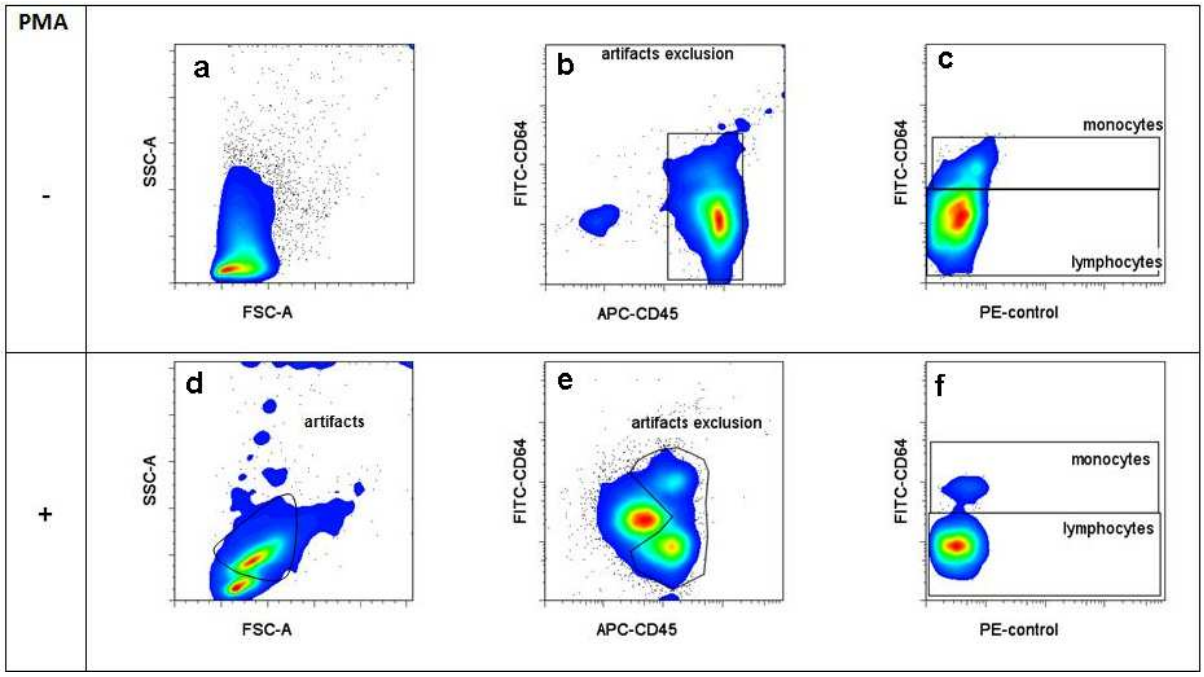


**Figure 1. Gating strategy used for the flow cytometry analysis of individual cell populations within PBMCs complex cell mixtures.** Figure illustrates density plots (generated by flow cytometry) of unstimulated (a-c) and PMA-activated (d-e) PBMCs from donor 3. Lymphocytes and monocytes within the PBMCs suspensions are identified by gating on surface CD45 and CD64 (b, e), following the software-assisted exclusion of artifacts. Artifacts are defined as those cells that have partly lost the intensity of surface markers and display compromised right-angle light scatter. These artifactual cells have the characteristics of apoptotic cells on a FSC/SSC graph (low FSC values, variable SSC) (a, c). Data acquisition was performed with FACS Diva software (version 6.1) and data analysis with FlowJo software (Tristar Inc).

**Table 1. Phosphorylation levels within activated lymphocytes measured by flow cytometry**

| | MFIR pERK | | MFIR pAKT | | MFIR pp38 | | MFIR pSTAT-3 | | MFIR pSTAT-1 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | method 1 | method 2 | method 1 | method 2 | method 1 | method 2 | method 1 | method 2 | method 1 | method 2 |
| **Donor 1** | 66.80 | 81.58 | 29.14 | 32.70 | 4.38 | 6.90 | 9.52 | 9.20 | 3.06 | 3.86 |
| **Donor 2** | 10.80 | 17.40 | 4.20 | 5.80 | 1.45 | 3.20 | 4.85 | 7.90 | 1.94 | 4.20 |
| **Donor 3** | 3.79 | 5.80 | 1.54 | 4.80 | 0.86 | 4.80 | 18.63 | 23.50 | 0.82 | 2.70 |
| **Donor 4** | 14.45 | 22.60 | 1.95 | 7.90 | 1.02 | 2.70 | 4.32 | 7.60 | 1.01 | 3.20 |
| **Donor 5** | 8.77 | 12.90 | 11.40 | 21.60 | 3.80 | 5.10 | 4.11 | 5.80 | 5.45 | 6.80 |
| **mean** | 20.92 | 28.06 | 9.65 | 14.56 | 2.30 | 4.54 | 8.29 | 10.80 | 2.46 | 4.15 |
| **SD** | 25.93 | 30.55 | 11.60 | 12.20 | 1.66 | 1.67 | 6.20 | 7.20 | 1.89 | 1.59 |
| **p** | 0.0153 | | 0.0151 | | 0.0045 | | 0.0223 | | 0.0018 | |

In *Table 1*, the phosphorylation level of five key proteins involved in intracellular signaling within activated lymphocytes is summarized.

The table illustrates the mean fluorescence intensity ratio (MFIR) values of the phospho-proteins pERK(T202/Y204), pAKT(T308), pp38MAPK(T180/Y182), pSTAT-3(S727) and pSTAT-1(Y701) as detected by intracellular staining of PMA-activated PBMCs using both fixation methods. The analyses were performed with gating on activated lymphocytes. Data of mean MFIR values as assessed from five donors, standard deviations and p-values (t Student's test) of both method 1 and 2 are given. The table thereby reveals the increased efficiency of phospho-staining with method 2 as compared to method 1, as MFIR values for all five investigated proteins were up to 5.6 greater with method 2 (when fixation was used) as compared to method 1. In addition, with method 2, we were able to detect levels of protein-phosphorylation previously found below the detection limit with method 1: while method 1 was inefficient for the phospho-detection of pAKT (in 2 out of 5 cases), of pp38 (in 3 out of 5 cases) and of pSTAT-1 (in 3 out of 5 cases), method 2 yielded positive MFIR values in the same samples. *Figure 2* depicts a representative example of efficient staining of all five p-Abs as analyzed.

***Cell suspension integrity is not affected by the fixation method used***

Different sample processing procedures may substantially impact on the viability of cells, with the risk of introducing misleading results in final data interpretation. In our experiment, as the viability of fixed cells is practically zero, the integrity of the cell suspension was evaluated based on the percentages of artifacts generated following each fixation method. We applied the term of "artifact" to those cells which lost surface markers and displayed compromised right-angle light scatter. These artificial cells have the characteristics of apoptotic cells on a FSC/SSC graph (low FSC values, variable SSC) and are excluded from analysis, in order to obtain an accurate evaluation of surface and intracellular markers. Percentages of artifacts generated following fixation by our two methods were compared and results are displayed in *Table 2*.

The table shows mean MFIR values and standard deviations. Data were recorded from six different replicates of each of the five donors (n=30) after their PBMCs were treated with or without PMA. Cell suspension integrity was not significantly affected by any of the fixation method used ($p > 0.05$) and both methods generated comparable percentages of artifacts (with an increase in PMA-treated PBMCs regardless of the fixation method used) showing an equivalent impact on the cell suspension integrity. Therefore, method 2 did not negatively impact on the cell suspension integrity when compared to method 1.

To assess whether the fixation method selectively affected any of the two cell types present within the suspension, ratio between lymphocytes and monocytes in each case was also calculated, again showing no significant differences between both methods (*Table 2*). Therefore, method 2 as compared to method 1 did not selectively deplete any of the cell types presented within the analyzed PBMC suspension.

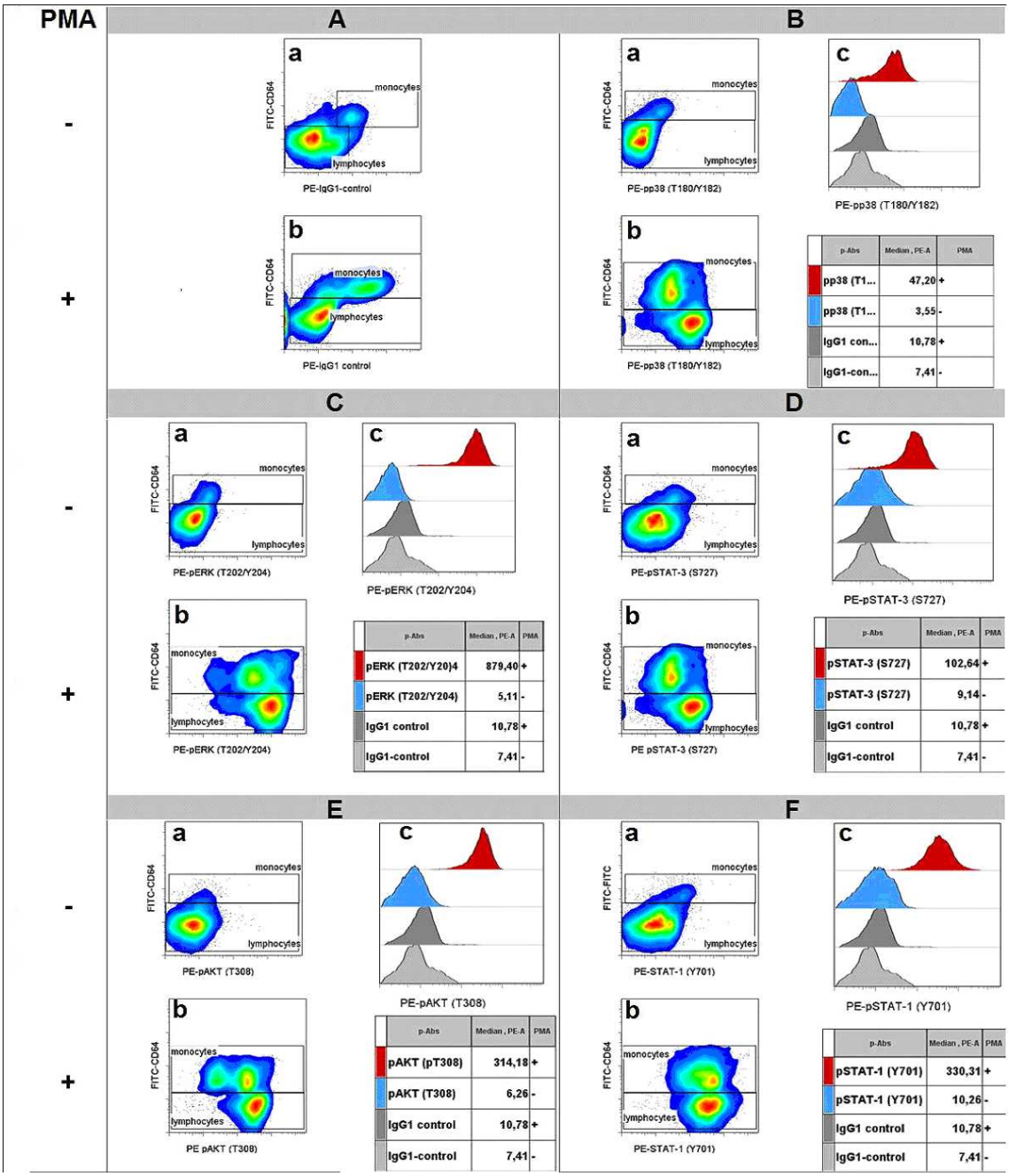


**Figure 2. Representative example illustrating efficient intracellular phospho-staining when method 2 of fixation is used.** Figure depicts two dimensional flow cytometric graphs of p-Abs versus CD64 density plots (a and b). Flow images from both, unstimulated (a) and PMA-activated (b) PBMCs from donor 1 are displayed. Histograms overlays for each antibody show efficient phospho-staining of PMA-treated PBMC versus appropriate controls (c). Gating is set on lymphocytes. Figures c present the flow cytometric analysis of five phospho-proteins: A: isotype (negative) control; B: pp38MAPK(T180/Y182); C: pERK(T202/Y204); D: pSTAT3(S727); E: pAKT(T308); F: pSTAT1 (Y701). Data acquisition was performed with FACS Diva software (version 6.1) and data analysis with FlowJo software (Tristar Inc).

**Table 2. Cell suspension integrity measured by percentages of artifacts and lymphocyte/monocyte ratios**

| | -PMA | | | | | | +PMA | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | method 1 | | | method 2 | | | method 1 | | | method 2 | | |
| **% of artifacts** | 13.64 | +/- | 4.82 | 6.87 | +/- | 5.24 | 29.91 | +/- | 15.46 | 26.18 | +/- | 12.36 |
| **lymphocyte/monocyte ratio** | 19.16 | +/- | 8.41 | 22.94 | +/- | 11.83 | 18.37 | +/- | 5.90 | 27.47 | +/- | 6.82 |

**Table 3. The impact of a freeze-thaw cycle on cell suspension integrity and detection level of surface/ intracellular markers**

| | method 1 | method 2 | method 2+freezing | p (method 2 versus method 2+freezing) |
|---|---|---|---|---|
| MFIR CD45 | 650.05+/-148.89 | 164.24+/-61.36 | 169.56+/-37.23 | 0.41 |
| MFIR CD64 | 11.45+/-2.93 | 5.70+/-1.18 | 4.27+/-1.19 | 0.06 |
| % artifacts | 27.41+/-8.31 | 30.74+/-10.62 | 35.69+/-7.28 | 0.13 |
| MFIR pERK | 8.77 | 12.92 | 9.61 | - |
| MFIR pAKT | 11.24 | 21.66 | 13.42 | - |
| MFIR pp38 | 3.80 | 5.11 | 3.22 | - |
| MFIR pSTAT-3 | 4.11 | 5.83 | 4.62 | - |
| MFIR pSTAT-1 | 5.45 | 6.81 | 5.95 | - |

***Fixation prior to surface staining leads to a reduced efficiency of surface marker detection levels***

The impact of the fixation method on surface staining efficiency is shown in *Figure 3.*

We examined the effect of both fixation methods on the intensity of surface markers (CD45 for lymphocytes and CD64 for monocytes). The level of the surface staining as measured by the MFIR values was assessed in six different replicates for each of the five donors (n=30), with PBMCs being treated with or without PMA. In comparison to method 1, method 2 of fixation led to significant reduction in both CD45 [in the absence (p=0.006) and presence (p=0.011) of PMA] and CD64 [in the absence (p=0.004) and presence (p=0.016) of PMA]. However, the expression values of both markers remained well above the detection limit (MFIR >2). This suggests that method 2 - as compared to method 1 - did not completely impair the efficiency of cell surface staining.

***One freeze-thaw cycle did not have a major impact on cell suspension integrity or detection level of surface/ intracellular markers***

As most of the clinical samples are usually stored at deep-freezing temperatures and in order to develop an optimized protocol for phosphorylated proteins detection with clinical relevance, we evaluated the impact of these storage conditions on cell suspension integrity. PBMCs from one donor were frozen at -80°C for several weeks, PMA-stimulated PBMCs were processed by method 2 for flow cytometric analysis. In *Table 3*, MFIR values of the five phospho-proteins detected by intracellular staining of PMA-activated PBMCs are depicted.

For the surface markers and percentages of apoptosis, MFIR values from six replicates, their standard deviations, and p values (analyzing the impact of freezing [t Student's test]) were assessed. For intracellular markers, one experiment was performed. Fixation of these cells using method 2 had an insignificant impact (p >0.05) on cell suspension integrity (as measured by the percentages of artifacts) and cell surface marker intensity (as measured by the expression of CD45 and CD64). However, the surface staining was still maintained high above the lower detection limit for CD45 on lymphocytes (marker representative for any high-density surface marker) and still within the detection area for CD64 on monocytes (representative for any low-density surface marker). As for the intracellular phospho-staining, although the detection level was reduced in comparison to method 2 alone, method 2 combined with a freeze-thaw cycle was more effi-

cient than method 1 in four out of five p-Abs tested (*Table 3).* Therefore, the storage conditions seemed to minimally affect the improved phospho-detection efficiency of method 2 of fixation.

## Discussion

The protein phosphorylation status has been traditionally evaluated by laborious biochemical methods such as in vivo labeling, peptide mapping, phospho-amino-acid analysis and purification of kinase activities (14). Nowadays, modern techniques are being validated to identify substrates of a certain, purified, active kinase of interest (15-17), to investigate functional kinases (18-22) or to measure the phosphorylation status of various proteins with biological functions (4-13, 23).

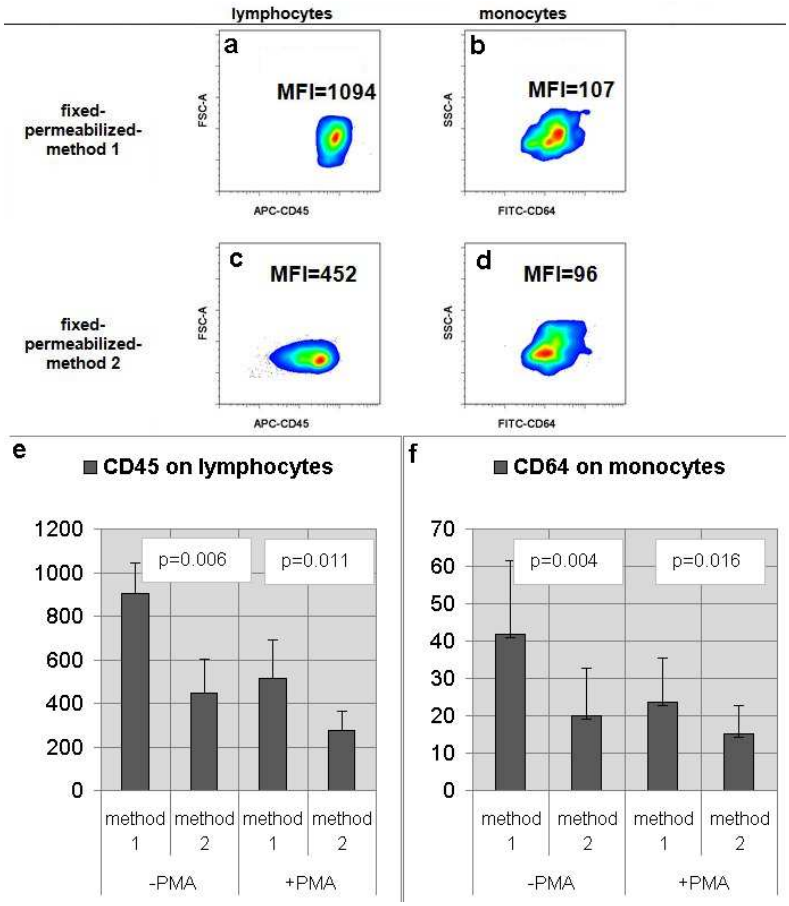


**Figure 3. Detection levels of surface marker expression depend on the fixation method used.** The flow cytometry plots from the upper side (a to d) illustrate one representative example showing the impact of the fixation method on surface marker expression (unstimulated PBMCs from donor 3). Values of MFI (median fluorescence intensity) for CD45 on lymphocytes (a, c) and CD64 on monocytes (b, d) are also provided. In comparison to method 1 (a, b), method 2 of fixation (c, d) leads to significant reduction in both CD45 and CD64. Data acquisition was performed with FACS Diva software (version 6.1) and data analysis with FlowJo software (Tristar Inc). The graphs from the lower side illustrate mean values of median fluorescence intensity ratio (MFIR) for CD45 on lymphocytes (e) and CD64 on monocytes (f) as detected by flow cytometry, their standard deviations, and the significance of the difference between the two methods, as calculated by the t Student's test. Data represents the results of surface staining and flow analysis in six different replicates for each of the five donors (n=30) after their PBMCs were treated with or without PMA (phorbol myristate acetate).

We report here an optimized flow cytometry protocol aimed at balancing the staining efficiencies of surface and intracellular phospho-markers in order to obtain a practical tool for multiparametric comparison among various cell types within the same heterogeneous sample and within the same experiment.

Our study was based on the premise that fixation is a critical step within the phospho-specific detection protocols. Fixation is required for an efficient blockage of the phosphorylation status, preventing both de-phosphorylation and further, unwanted phosphorylation events. Although previous publications have recommended the use of p-formaldehyde as an efficient fixation reagent for phospho-protein detection, to our knowledge, none has been used fixation prior surface staining (3, 11, 24). It is also well-known that any fixation procedure may negatively impact the intensity of cell surface expression. When we used p-formaldehyde addition prior to the surface staining (method 2), major benefits in terms of the efficiency of phospho-protein detection were obtained in comparison to method 1. Although the intensity of surface markers significantly decreased, with method 2 the detection efficiency was still maintained within acceptable ranges for both markers, where "acceptable" signifies the preservation of their utility in identifying distinct cellular subsets (lymphocytes versus monocytes). We expect that this adequate surface detection efficiency applies for markers with a wide variability of their membrane density, as CD45 on lymphocytes may be representative for any high-density surface marker, while CD64 on monocytes is representative for any intermediate/ low-density surface marker. As no significant variations between the two methods 1 and 2 in terms of cell suspension integrity were recorded, we suggest that fixation prior to surface staining will become one of the methods of choice for phospho-protein detection in clinical samples.

The most frequently assessed technical parameters in optimization experiments are: phospho-antigen accessibility (optimal access to nuclear antigens have been tested under different permeabilization approaches), preservation of surface staining and light scatter properties, use of optimal cross-linking (fixation) reagents for preventing both the reversibility of the phosphorylation event and any further unspecific kinase activity (3, 6, 9, 24). While some published protocols provide excellent staining of phospho-epitopes, surface markers defining relevant cell subsets are suboptimal (9). Other protocols, focused on optimal preservation of surface epitopes, may risk inefficient phospho-protein staining.

Stability of both surface and intracellular antigens is also of particular concern when the protocol is applied to cryopreserved samples, as most clinically-derived specimens are stored at deep-freezing temperatures (either liquid nitrogen or -80/-150°C). Therefore, we evaluated the impact of one freeze-thaw cycle and report here of a superior preservation of phospho-protein levels when method 2 was used, in comparison with method 1 on freshly isolated PBMCs. The cryo-storage-related loss of cell marker detection efficiency is a complex process (24) and each epitope should be investigated individually before long-term storage. Albeit our results are of note, one may argue that it is less probable that a unique protocol would suffice for the majority of phospho-proteins and for the wide range of clinically derived cell types, so various protocols remain to be screened for different class of phospho-epitopes and protein families.

In conclusion, we describe a protocol that offers an optimal balance between surface and intracellular staining efficiency, with improved phospho-signal-to-noise ratio due to immediate fixation after PMA stimulation of PBMCs. PMA-activated PBMCs provide an instrument to rapidly test the efficiency of p-Ab detection and such approach may be further extended on a broader range of cells with pathological and pharmaceutical significance. Once the detection protocol is optimized, it may be used within areas of diverse clinical applications,

such as immune system characterization, pharmacodynamic monitoring, drug screening and stratification of oncologic patients into groups for targeted, personalized therapies.

## Abbreviations

APC = allophycocyanin,
DMSO = dimethyl sulfoxide,
FCS = fetal calf serum,
FITC = fluorescein isothiocyanate,
MFI = median fluorescence intensity,
MFIR = median fluorescence intensity ratio,
p-Ab = phospho-specific antibody,
PBMCs = peripheral blood mononuclear cells,
PBS = phosphate buffered saline,
PE = phycoerythrin,
PMA = phorbol myristate acetate,
RT = room temperature,
S= serine,
T = threonine,
Y = tyrosine.

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**Conflicts of interest:** none to declare.

## References

1. Bordignon J, Pires Ferreira SC, Medeiros Caporale GM, Carrieri ML, Kotait I, Lima HC et al. Flow cytometry assay for intracellular rabies virus detection. J Virol Methods 2002;105:181-6.
2. Dorn I, Schlenke P, Mascher B, Stange EF, Seyfarth M. Lamina propria plasma cells in inflammatory bowel disease: intracellular detection of immunoglobulins using flow cytometry. Immunobiology 2002;206: 546-57.
3. Krutzik PO, Irish JM, Nolan GP, Perez OD. Analysis of protein phosphorylation and cellular signaling events by flow cytometry: techniques and clinical applications. Clin Immunol. 2004 Mar;110(3):206-21.
4. Khanna KK, Gatei M, Tribbick G. Phospho-specific antibodies as a tool to study in vivo regulation of BRCA1 after DNA damage. Methods Mol Med. 2006;120:441-52.
5. Versteeg HH, Nijhuis E, van den Brink GR, Evertzen M, Pynaert GN, van Deventer SJ et al. A new phosphospecific cell-based ELISA for p42/p44 mitogen-activated protein kinase (MAPK), p38 MAPK, protein kinase B and cAMP-response-element-binding protein. Biochem J. 2000 Sep 15;350 Pt 3:717-22.
6. Chow S, Hedley D, Shankey TV. Whole blood processing for measurement of signaling proteins by flow cytometry. Curr Protoc Cytom. 2008 Oct;Chapter 9:Unit 9.27.
7. Bardet V, Tamburini J, Ifrah N, Dreyfus F, Mayeux P, Bouscary D et al. Single cell analysis of phosphoinositide 3-kinase/Akt and ERK activation in acute myeloid leukemia by flow cytometry. Haematologica. 2006 Jun;91(6):757-64.
8. Perez OD, Mitchell D, Campos R, Gao GJ, Li L, Nolan GP. Multiparameter analysis of intracellular phosphoepitopes in immunophenotyped cell populations by flow cytometry. Curr Protoc Cytom. 2005 May;Chapter 6:Unit 6.20. Aug 20;4(8):e6703.
9. Krutzik PO, Nolan GP. Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events. Cytometry A. 2003 Oct;55(2):61-70.
10. Perez OD, Nolan GP. Simultaneous measurement of multiple active kinase states using polychromatic flow cytometry. Nat Biotechnol. 2002 Feb;20(2):155-62.
11. Chow S, Patel H, Hedley DW. Measurement of MAP kinase activation by flow cytometry using phospho-specific antibodies to MEK and ERK: potential for pharmacodynamic monitoring of signal transduction inhibitors. Cytometry. 2001 Apr 15;46(2):72-8.
12. Gulmann C, Sheehan KM, Conroy RM, Wulfkuhle JD, Espina V, Mullarkey MJ et al. Quantitative cell signaling analysis reveals down-regulation of MAPK pathway activation in colorectal cancer. J Pathol. 2009 Aug;218(4):514-9.
13. Kase S, Yoshida K, Jin XH, Koyama Y, Kitaichi N, Ohgami K et al .Phosphorylation of p27(KIP1) in lens epithelial cells after extraction of fiber cells. Int J Mol Med. 2006 Dec;18(6):1187-91.
14. Hunter T. Signaling--2000 and beyond. Cell. 2000 Jan 7;100(1):113-27.
15. Campbell M, Lie WR, Zhao J, Hayes D, Mistry J, Kung HJ et al .Multiplex analysis of Src family kinase signaling by microbead suspension arrays. Assay Drug Dev Technol. 2010 Aug;8(4):488-96.
16. Ansuini H, Meola A, Gunes Z, Paradisi V, Pezzanera M, Acali S et al. Anti-EphA2 Antibodies with Distinct In Vitro Properties Have Equal In Vivo Efficacy in Pancreatic Cancer. J Oncol. 2009;2009:951917.
17. Zou J, Friesen H, Larson J, Huang D, Cox M, Tatchell K et al .Regulation of cell polarity through phosphorylation of Bni4 by Pho85 G1 cyclin-dependent kinases in Saccharomyces cerevisiae. Mol Biol Cell. 2009 Jul;20(14):3239-50.
18. Ueda T, Sasaki M, Elia AJ, Chio II, Hamada K, Fu-

kunaga R et al. Combined deficiency for MAP kinase-interacting kinase 1 and 2 (Mnk1 and Mnk2) delays tumor development. Proc Natl Acad Sci USA. 2010 Aug 10;107(32):13984-90.
19. Sangle GV, Zhao R, Mizuno TM, Shen GX. Involvement of RAGE, NADPH oxidase, and Ras/Raf-1 pathway in glycated LDL-induced expression of heat shock factor-1 and plasminogen activator inhibitor-1 in vascular endothelial cells. Endocrinology. 2010 Sep;151(9):4455-66.
20. Li J, Rix U, Fang B, Bai Y, Edwards A, Colinge J et al. A chemical and phosphoproteomic characterization of dasatinib action in lung cancer. Nat Chem Biol. 2010 Apr;6(4):291-9
21. Biondi RM, Nebreda AR. Signalling specificity of Ser/Thr protein kinases through docking-site-mediated interactions. Biochem J. 2003 May 15;372(Pt 1):1-13.
22. Gavin AC, Bösche M, Krause R, Grandi P, Marzioch M, Bauer A et al. Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature. 2002 Jan 10;415(6868):141-7.
23. Sun J, Masterman-Smith MD, Graham NA, Jiao J, Mottahedeh J, Laks DR et al. A microfluidic platform for systems pathology: multiparameter single-cell signaling measurements of clinical brain tumor specimens. Cancer Res. 2010 Aug 1;70(15):6128-38.
24. Chow S, Hedley D, Grom P, Magari R, Jacobberger JW, Shankey TV. Whole blood fixation and permeabilization protocol with red blood cell lysis for flow cytometry of intracellular phosphorylated epitopes in leukocyte subpopulations. Cytometry A. 2005 Sep;67(1):4-17

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