Journal of Immunological Methods 436 (2016) 58–63

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Technical note

An optimized multiplex flow cytometry protocol for the analysis of intracellular signaling in peripheral blood mononuclear cells

Richard Davies [a,*], Petra Vogelsang [a,1], Roland Jonsson [a,b], Silke Appel [a]

[a] Broegelmann Research Laboratory, Department of Clinical Science, University of Bergen, Jonas Lies vei 87, 5021 Bergen, Norway
[b] Department of Rheumatology, Haukeland University Hospital, 5021 Bergen, Norway

ARTICLE INFO

Article history:
Received 18 March 2016
Received in revised form 16 June 2016
Accepted 27 June 2016
Available online 29 June 2016

Keywords:
Flow cytometry
Signaling
Phosphorylation
Multiparameter
Barcoding
PBMC

ABSTRACT

Phosphoflow cytometry is increasingly being used as a tool for the discovery of biomarkers used in the treatment and monitoring of disease and therapy. The ability to measure numerous phospho-protein targets simultaneously at a single cell level accurately and rapidly provides significant advantages over other methods. We here discuss important considerations required to successfully implement these methods. Three different blood collection tubes (lithium-heparin tubes, CPT with sodium citrate and CPT with sodium heparin) were evaluated, with PBMC isolated through lithium-heparin tubes/lymphoprep displaying reduced basal and increased stimulation induced phosphorylation compared to the other two methods. Further, we provide a protocol outlining an 8 color assay developed for the study of intracellular signaling in peripheral blood mononuclear cells. The assay allows for the quantitative measurement of the phospho-proteins ERK1/2, NF-κB p65, Stat1 (Y701), Stat1 (S727), Stat3 (Y705), Stat3 (S727), Stat4 (Y693), p38 and Stat5 (Y694), as well as the identification of T cells, B cells, natural killer cells and monocytes. The assay additionally incorporates fluorescent cell barcoding, reducing assay costs and increasing throughput while increasing data robustness. Inter-assay precision was assessed over a month long period for all experimental variables (phospho-protein measured, cell type and stimulant). Coefficient of variations (CVs) calculated from process triplicates of normalized median fluorescence intensity (MFI) of the phospho-proteins displayed median CVs under 10% when grouped according to cell type, stimulation agent and phospho-protein measured, while the CV for each triplicate did not exceed 20%.

1. Introduction

By using highly specific fluorochrome conjugated antibodies, flow cytometry allows for quantitative multi-parameter analysis of single cells within complex cell populations. The additional use of fluorochrome conjugated antibodies to the phosphorylated forms of molecules phosphorylated during signaling cascades allows for the analysis of signaling networks within complex cell populations such as peripheral blood mononuclear cells (PBMCs). This provides quantitative data of phosphorylation events at a single cell level; without the need for prior separation of the cells of interest (Krutzik and Nolan, 2003). The pathway of interest can be further investigated through its activation either in vivo or in vitro. This technique termed phospho-flow cytometry has shown significant promise in stratification of disease (Irish et al., 2004, Brown et al., 2015), monitoring disease progression (Cesano et al., 2013) and identification of biomarkers for monitoring therapeutic intervention (Gavasso et al., 2014). Additionally the method can be used in conjunction with fluorescent cell barcoding to enable a higher throughput while minimizing reagent consumption and maximizing data robustness through the multiplexing of samples prior to staining; making this method ideal for profiling of multiple samples (Krutzik et al., 2011).

We here provide a protocol outlining an 8 color assay developed for the study of intracellular signaling in PBMCs. In the procedure, PBMCs are stimulated, fixed, permeabilized and stained with 3 different antibody panels to identify various cell types - T cells, B cells and natural killer (NK) cells, and phospho-proteins-NF-κB p65 (pS529), ERK 1/2 (pT202/pY204), P38 (pT180/pY182), Stat4 (pY693), Stat1 (pY701), Stat1 (pS727), Stat3 (pY705), Stat5 (pY694) and Stat3 (pS727). Additionally, we utilize 2 amine reactive dyes, Pacific Orange™ (PO) and Pacific Blue™ (PB) to barcode 9 different samples, allowing for a higher

* Corresponding author at: Broegelmann Research Laboratory, Department of Clinical Science, University of Bergen, The Laboratory Building, 5th floor, Jonas Lies vei 87, N-5021, Norway.
E-mail address: Richard.Davies@uib.no (R. Davies).
[1] Present address: Department of Immunology and Transfusion Medicine, Haukeland University Hospital, Bergen, Norway.

http://dx.doi.org/10.1016/j.jim.2016.06.007

throughput while minimizing antibody consumption. Further, we discuss important issues related to the testing and development of in-house assays such as the choice of the blood collection tube.

2. Materials and methods

A schematic representation of the work flow and gating strategy is shown in Fig. 1.

2.1. Antibodies

The following phospho-specific monoclonal antibodies were used in 3 different panels during the flow cytometry protocol: Alexa Fluor ®647 conjugated anti-Stat4 (pY693, clone 38/p-Stat4, panel 1), anti-Stat 1 (pS727, clone K51-856, panel 2) and anti-Stat3 (pS727, clone 49/p-Stat3, panel 3); PerCP-Cy™5.5 conjugated anti-ERK1/2 (pT202/pY204, clone 20A, panel 1), anti-Stat1 (pY701, clone 4a, panel 2) and anti-Stat3 (pY705, clone 4/P-STAT3, panel 3); and PE-Cy™7 conjugated anti-p38 MAPK (pT180/pY182, clone 36/p38, panel 2), and anti NF-κB p65 (pS529, clone K10-895.12.50, panel 1), anti-Stat5 (pY694, clone 47/Stat5(pY694), panel 3) (all from BD Biosciences, San Jose, CA, USA). Cell surface markers incorporated in the assays were BV786 conjugated anti-CD3 (clone SK7, BD Horizon™), Alexa Fluor® 488 conjugated anti-CD20 (clone H1 (FB1), BD Biosciences) and PE conjugated anti-CD56 (clone N901, Beckmann Coulter, CA, USA).

2.2. Blood collection

Blood was collected at the blood bank at the Haukeland University Hospital in Bergen, Norway. All blood donors provided written informed consent. Peripheral blood was collected in Lithium-heparin tubes (BD diagnostics) and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation with lymphoprep™ (Axis-Shield, Oslo, Norway) as per manufactures instructions. For the experiments analyzing the effect of the isolation method, BD Vacutainer® CPT™ with Sodium Citrate or Sodium Heparin (BD diagnostics) were utilized. PBMCs were washed twice with phosphate buffered saline (PBS; Lonza, Basel, Switzerland) before being resuspended in ice cold 50% X-vivo 20™ and 42.5% Profreeze™-CDM non-animal origin (NAO) chemically defined freeze medium (both from Lonza) and 7.5% DMSO (Hybrid max, Sigma D2650) at 3 to 5 × 10^6 cells/ml. PBMCs were then frozen in a CoolCell® (Biocision; San Rafael, CA, USA) freezing chamber at −70 °C overnight before being moved to a −150 °C freezer for approximately 6 months.

2.3. Cell culture and stimulation

Before stimulation, cryopreserved PBMCs were rapidly thawed using a water bath set to 37 °C and washed once in prewarmed X-vivo 20™ by centrifugation at 300*g* for 5 min. The cells were then resuspended in X-vivo 20™ and rested at 37 °C at 5% CO_2 for 30 min before the cell

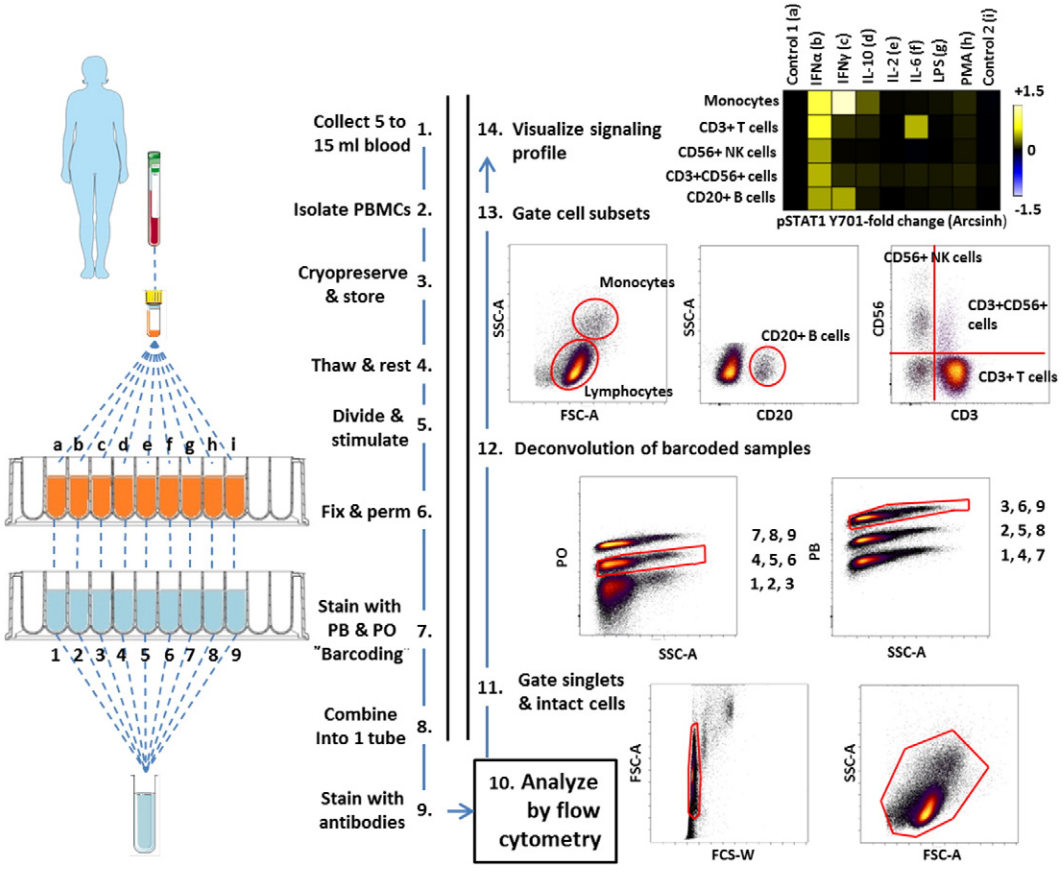

Fig. 1. Schematic representation of the work flow and gating strategy used in the analysis of intracellular signaling pathways in PBMCs. Blood was collected in Lithium-heparin tubes (BD diagnostics), and PBMCs were isolated by density gradient centrifugation before cryopreservation. At a later date PBMCs samples were thawed and rested in serum free media and then divided into 9 wells and stimulated as described in materials and methods. After stimulation, samples were fixed, permeabilized and stained with different concentrations of pacific blue (PB) and pacific orange (PO) dyes. Samples were washed to remove excess dye and combined into one tube and stained with fluorochrome conjugated antibodies as given in Materials and methods. Samples were analyzed on a flow cytometer, single cells were gated based on their forward scatter area (FSC-A) and forward scatter width (FSC-W), followed by intact cells based on side scatter area (SSC-A) and FSC-A. The different stimulation conditions were then identified through the intensities of their PB and PO stains. Cell subtypes were identified based on their FSC-A and SSC-A scatter properties as either monocytes or lymphocytes. Lymphocytes were then subtyped as B cells (CD20+), T cells (CD3+CD56−), NK cells (CD3−CD56+) or CD3+CD56+ NKT cells based on surface antigen expression. Cells within each subtype were analyzed based on the change of MFI in each stimulation condition relative to the unstimulated reference sample. Data was analyzed, scatter graphs and heat maps were produced in cytobank (Kotecha et al., 2010).

concentration was adjusted to 1.25×10^6 cells/ml in X-vivo 20™ and 400 μl was dispensed into 9 wells of a Megablock® 96 well plate (Starstedt, Nümbrecht, Germany). The cells were rested at 37 °C with 5% CO_2 for 11/2 h to decrease basal phosphorylation levels. Following, the PBMCs were either left unstimulated (2 samples) or were stimulated for 15 min with cytokines (100 ng/ml), LPS (10 μg/ml; Sigma-Aldrich) or PMA (100 ng/ml; Sigma-Aldrich) at 37 °C with 5% CO_2. Recombinant human (rh) interleukin-10 CHO (IL-10), interleukin-2 (IL-2), interleukin-6 (IL-6), interferon-alpha 2 beta (IFNα2β), IFN-gamma (IFNγ) were from ImmunoTools (Friesoythe, Germany). Lyophilized cytokines and LPS were reconstituted in X-vivo 20™, while PMA was reconstituted in DMSO. All compounds were diluted in 100 μl X-vivo 20 ™ and added to 400 μl of cells resulting in a final cell concentration of 1×10^6 cells/ml during stimulation. All assays were performed in serum free media (X vivo-20™) to allow for a constant stimulation environment for the PBMCs and avoid unspecific stimulation.

2.4. Fluorescent cell barcoding and phospho-epitope staining for flow cytometry

PBMCs were fixed by adding 16% PFA (Electron Microscopy Sciences (Hatfield, PA, USA) warmed to 37 °C directly into the culture medium resulting in a final PFA concentration of 1.5%. The samples were mixed thoroughly by pipetting. The cells were fixed at RT for 10 min before pelleting at 1000 g for 5 min. The PBMCs were then vigorously resuspended by vortexing in 50 μl PBS before drop wise addition of 1 ml ice cold methanol and incubation on ice for 30 min. The permeabilized cells were kept overnight at −80 °C. After washing with PBS, the PBMCs were stained according to a 3 × 3 barcoding grid (9 stimulation conditions) using 3 levels of pacific orange (PO) and pacific blue (PB) succinimidyl ester dyes (PB 100, 25 and 6.3 ng/ml; PO 250, 70 and 0 ng/ml; Life Technologies, Grand Island, NY, USA) for 30 min in the dark at 4 °C in a volume of 1 ml. Barcoded PBMCs were then washed once and the 9 different dye concentration/combination samples were combined into one sample. The sample was washed and incubated with 1 μl Fc receptor block (Miltenyi Biotec, Bergisch Gladbach, Germany) per 1×10^6 cells for 10 min on ice. Following, the sample was subdivided into 3 volumes and incubated for 30 min at RT in the dark with the 3 different antibody staining panels. An aliquot of the barcoded cells was collected before addition of antibody as a barcoding only control. The samples were then washed twice and re-suspended in staining medium containing 2 mM EDTA (Sigma-Aldrich) prior to analysis.

2.5. Analysis

Samples were acquired on a LSRI Fortessa flow cytometer (BD Biosciences, San Jose, CA, USA) with BDFACSDiVa™ Software (BD Biosciences) at the Bergen Flow Cytometry Core Facility, University of Bergen, Norway. The flow cytometer was equipped with 407, 488, 561 and 635 nm lasers, and emission filters for PerCP-Cy5.5 (LP: 685, BP: 695/40), Alexa fluor-488 (LP: 505, BP: 530/30), PE-Cy7 (LP: 750, BP: 780/60), PE (LP: −, BP: 582/15), APC (LP: −, BP: 670/14), Pacific blue (LP: −, BP:450/50), Pacific orange (LP: 570, BP: 585/42) and BV 786 (LP: 750, BP: 780/60). The cytometer was routinely calibrated with BD cytometer setup and tracking beads (BD Biosciences). A minimum of 200,000 events in the intact cell gate was collected for each sample. Gating was conducted as shown in Fig. 1: First, single cells were gated based on forward scatter area (FSC-A) and forward scatter width (FSC-W) properties, followed by intact cells based on side scatter area (SSC-A) and FSC-A. The different stimulation conditions were then identified through the intensities of their PB and PO stains against SSC-A. Cell subtypes were identified based on their FSC-A and SSC-A scatter properties as either monocytes or lymphocytes. Lymphocytes were further subtyped as B cells (CD20+), T cells (CD3+CD56−), NK cells (CD3−CD56+) or CD3+CD56+ NKT cells based on surface antigen expression. The fold change (arcsinh) was calculated from median fluorescence intensity values (MFI) of unstimulated controls to MFI of each stimulated samples for each identified PBMC subtype. Fold change (arcsinh) was calculated through Cytobank or alternatively through Microsoft Excel using the formula (ASINH (MFI stimulated/cofactor)) - (ASINH (MFI unstimulated/cofactor)), with an assigned cofactor of 150. To assess the precision of the multiplexed assay complete process triplicates were run using cryopreserved PBMCs from 3 different donors over a 1 month period. MFI values of target phospho-proteins were normalized against their respective unstimulated samples and coefficient of variation (CV) values were calculated based on the normalized triplicate values. Additionally we cryopreserved PBMCs from a single donor with unstimulated and stimulated samples run in each assay for a positive control, inter-assay normalization, assessing assay to assay variability and calculating relative basal phosphorylation level in the unstimulated sample.

3. Results

3.1. The PBMC isolation method affects their signaling profile in a cell and pathway dependent manner

We first compared the effect of different PBMC isolation methods. The signaling profile of PBMCs separated using CPT sodium citrate or sodium heparin tubes as well as collection with lithium-heparin tubes followed by density gradient separation of PBMCs are shown in Fig. 2. The figure shows fold changes (arcsinh) for monocytes, T cells (CD3+), NK cells (CD3−CD56+), CD3+CD56+ NKT cells and B cells CD20+) measured in channels for pERK, pNF-κB, pP38, pStat1 S727, pStat1 Y701, pStat3 S727 pStat3 Y705, pStat4 Y693 and pStat5 Y694. Measurements were made for all 3 donor PBMCs and fold changes were calculated by comparing unstimulated samples to their respective stimulated samples for each donor and basal measurements were calculated against an unstimulated single donor control. The signaling profile was affected in a cell and pathway dependent manner. Differences among the groups were assessed through repeated measures one-way ANOVA, with the Greenhouse-Geisser correction and Holm-Sidak's multiple comparisons test, $p > 0.05$ was considered significant. Basal measurements of cells derived from different PBMC isolation methods differed significantly. Lithium-heparin tubes followed by density gradient separation of PBMCs showed significantly lower basal phosphorylation of Erk1/2, P38, Stat1 Y701, Stat3 S727 and Stat5 Y694 in multiple cell types than those isolated through CPT sodium citrate or sodium heparin tubes. PBMC samples showed significant differences in fold changes following stimulation between PBMC isolation methods. Fold changes of PBMCs isolated using tubes with heparin additives were comparable, but differed significantly from the citrate tube. In particular a decreased phosphorylation fold change for NF-κB, Stat1 Y701, Stat3 Y705 and Stat5 Y694 following stimulation when compared to their respective unstimulated controls was exhibited. These differences were shown in multiple cell types but were most notable in NK cells, where for example pStat4 had mean fold change of 1.29 when peripheral blood was collected in lithium-heparin tubes compared to 0.337 with CPT tubes with sodium citrate. These decreases were often not corresponding with increases in basal signaling (e.g. Stat4 Y693 and Stat3 S727 in NK cells) and therefore do not explain the observed repression

3.2. Phosphoflow distinguishes cell and treatment specific signaling profiles

Examples of results from following the described method is shown as a heat map in Fig. 3A. The figure displays a scale indicating fold change (arcsinh) relative to their respective unstimulated sample. Different cell subsets in PBMCs are activated to different degrees dependent on the agent used in cell stimulation. Interferon α was shown to activate multiple phospho-proteins, this was strongly shown in pStat4 and pStat1 (Y701) and to lesser degrees pStat3 (Y705), pStat3 (S727) and pStat5, with the level of activation dependent on cell type. While

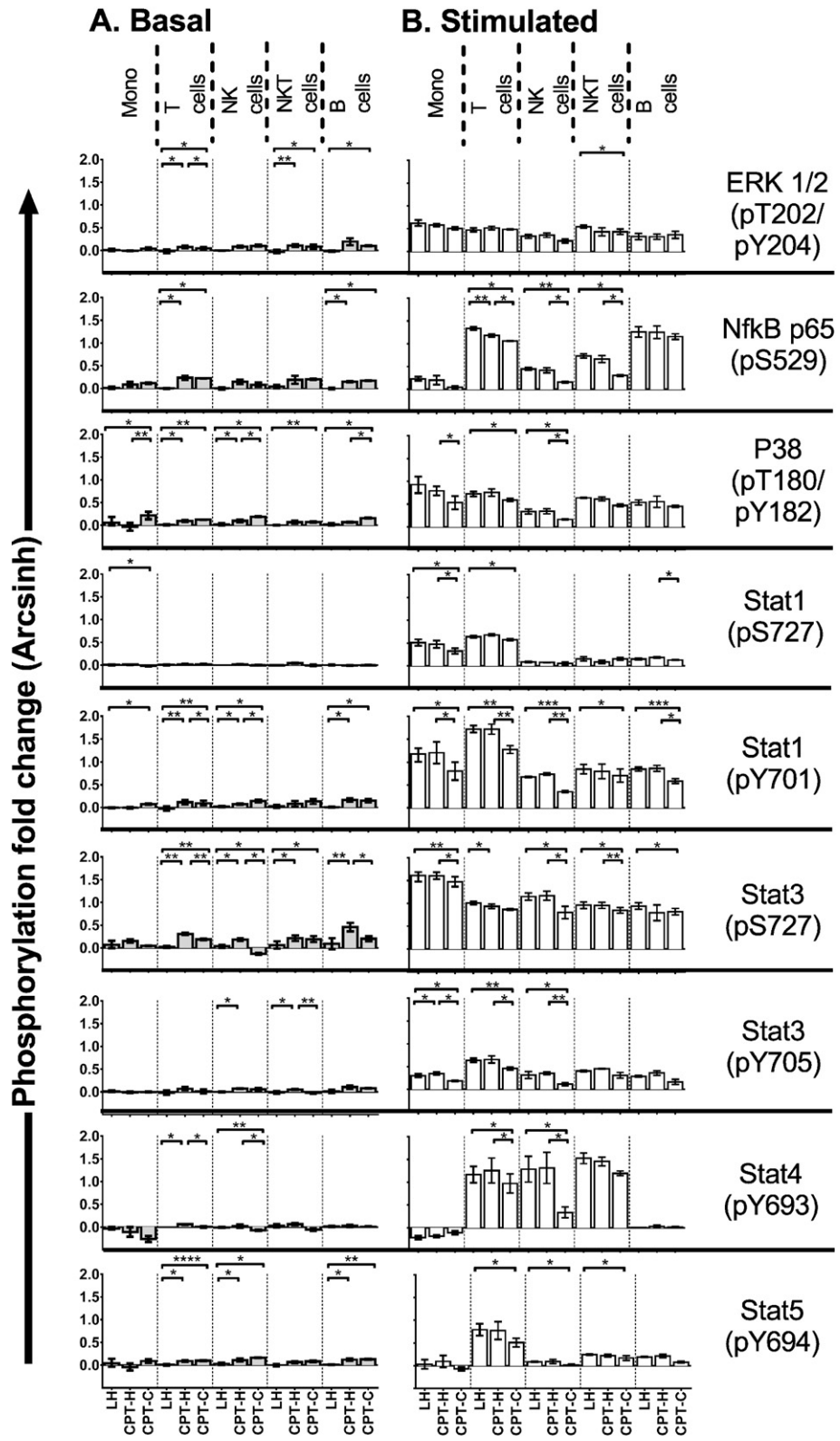

Fig. 2. Effect of PBMC isolation method on cell signaling. The Isolation method is displayed at the base of the figure with lithium-heparin (LH) tubes, CPT with sodium-citrate (CPT-C) or sodium-heparin (CPT-H) shown. Cell type is shown on top and phospho-protein measured on the right of the figure. The axis for phosphorylation fold change (arcsinh) following stimulation and basal fold differences (arcsinh) are shown on the left hand side of the figure. Basal fold differences are shown on the left hand side of the figure (A) and are represented by grey bars while fold changes following stimulation are shown on the right (B). The bars show the mean and standard deviation. The PBMC isolation method was shown to have an effect on degree of signaling in PBMCs after stimulation with PMA and interferon α for 15 min and basal levels. These effects varied with cell type and kinase measured. In most cases peripheral blood mononuclear cells isolated using CPT (CPT-H and CPT-C) showed higher degrees of basal phosphorylation then PBMCs isolated by lithium-heparin tubes and density gradient centrifugation. Heparin based isolation methods resulted in stronger post stimulation fold changes. A repeated measure one-way ANOVA, with the Greenhouse-Geisser correction and Holm-Sidak's multiple comparisons test was used to analyze differences between groups, significant p values are denoted as * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.001, **** ≤ 0.0001.

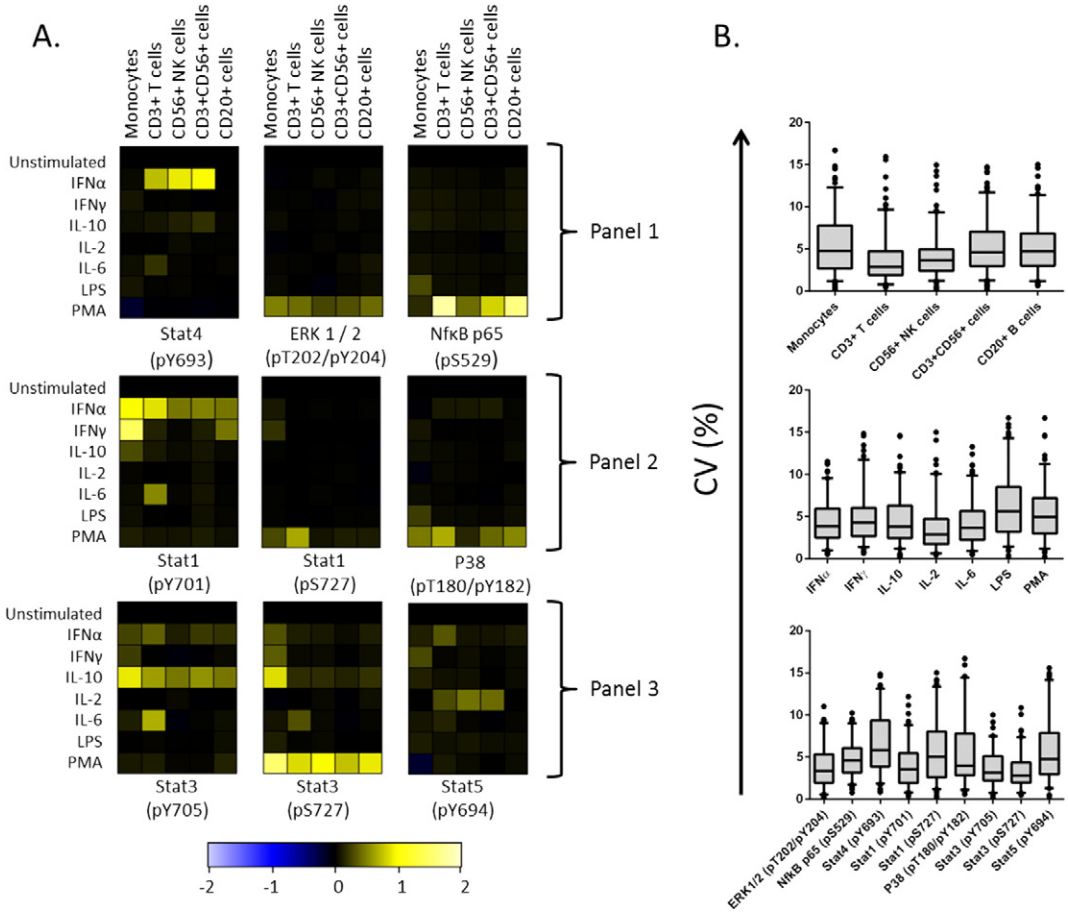

Fig. 3. Phosphoflow distinguishes cell and treatment specific signaling profiles. A. Heat map representation of the stimulation profile of PBMCs. Data was generated as outlined in the protocol with antibody panels used in the analysis shown to the right of the maps. All cells were gated according to the schematic shown in Fig. 1. The columns represent the cell subsets, T cells, B cells, NK cells, CD3 + CD56 + cells and monocytes. Each row represents a sample stimulated with a cytokine or chemical, with the phospho-specific antibody detected shown at the base of each map. The color of each block represents the fold change (arcsinh) in the channel corresponding to the analyzed phosphorylated protein relative to the un-stimulated sample, and the degree of change shown in the scale at the figure base. Data was analyzed and heat maps produced in cytobank (Kotecha et al., 2010). B. Precision of flow cytometry based phospho-protein measurements of PBMCs from 3 different donors. Analysis of PBMCs shows excellent precision of normalized MFI values of phospho-proteins. CV values for the multiplexed assays measured in complete process triplicates, grouped according to cell type analyzed (upper panel. $n = 189$), stimulation agent (medium panel. $n = 135$) and phospho-protein measured (lower panel. $n = 105$). Upper panel: The cell specific group median assay CVs were 4.76% (monocytes), 2.88% (CD3 + T cells), 3.64% (CD56 + NK cells), 4.61% (CD3 + CD56 + cells) and 4.73% (CD20 + B cells), with 95% of the results having CVs within 10% (CD3 + T cells and CD56 + NK cells) and 13% (monocytes, CD3 + CD56 + cells and CD20 + B cells). Medium panel: CV values grouped by stimulation agent showed medians of 3.84% (IFNα), 4.27% (IFNγ), 3.8% (IL-10), 2.88% (IL-2), 3.64% (IL-6), 5.62% (LPS) and 4.96% (PMA), with 95% of the results having CVs within 10% (IFNα and IL-6), 12% (IFNγ, IL-10, IL-2 and PMA), and 15% (LPS). Lower panel: CV values grouped by phospho-protein measured had median assay CVs of 3.36% (pERK 1/2), 4.62% (pNfκB), 5.81% (pStat4), 3.56% (pStat1(Y701)), 5.05% (pStat1(S727)), 3.98% (pP38), 3.16% (pStat3(Y705)), 2.80% (pStat3(S727)) and 4.79% (pStat5), with 95% of the results having CVs within 10% (pERK 1/2, pNfκB, pStat1(Y701), pStat3(Y701), pStat3(S727)) and 15% (pStat4, pStat1(S727), pP38, pStat5). Box plots show the medium value plotted as a line with each box displaying the distribution of the inner quartiles and vertical lines showing 95% of the data.

IFNγ activation profile displayed greater cell subset specificity, strong activation was limited to pStat1 (Y701), pStat1 (S727), pStat3 (Y705), pStat3 (S727) and pStat5 in monocytes; as well as pStat1 (Y701) in B cells. Interleukin-10 showed a greater degree of specificity in phospho-protein activation, strong induction was shown in pStat3 (Y705) in all cell types with relatively lower levels of activation of pStat3 (S727). Interleukin-2 activation was largely limited to activation of pStat5 in T cells, NK cells and CD3 + CD56 + NKT cells. IL-6 activation was more pronounced in T cells in particular pStat1 (Y701), pStat3 (Y705) and pStat3 (S727); while LPS displayed a higher levels of activation in monocytes of NF-κB and P38. PMA showed high phospho-protein activation in diverse cell types, with strong activation of ERK 1/2, NF-κB, P38 and Stat3 (S727).

3.3. Multiplex assay shows good reproducibility over phosphor-proteins, cell types and stimulations

To assess the precision of the established assay; PBMCs from 3 separate donors were isolated and cryopreserved. Subsequently on 3 separate occasions over a 1 month period, PBMCs for each donor were thawed and processed as described in the method. Data of measured MFI for each phospho-protein under each stimulation condition was normalized against the corresponding unstimulated controls for each phospho-protein, cell type and donor. The coefficient of variation (CV; 100 ∗ standard deviation/mean) was calculated for the 3 replicates for each normalized phospho-protein MFI measurement in each cell type and stimulation agent. The results generated were grouped for each phospho-protein, cell type and stimulation agent and are shown in Fig. 3B. The data indicates excellent precision of measurements; with robustness of the assay also shown to be dependent on cell type and phospho-protein measured, as well as stimulation agent. The assay was shown to be more precise in measurements of pERK 1/2, pNF-κB, pStat1 (Y701), pStat3 (Y705) and pStat3 (S727) than pStat4, pStat1 (S727), pP38 and pStat5 with median CVs of 3.36%, 4.62%, 5.81%, 3.56%, 5.05%, 3.98%, 3.16%, 2.80% and 4.79%, respectively, with 95% of the results having CVs within 10% (pERK 1/2, pNF-κB, pStat1(Y701), pStat3(Y701), pStat3(S727)) and 15% (pStat4, pStat1(S727), pP38, pStat5). CVs for the triplicate phospho-protein measurements of T cells (2.88%) and NK

cells (3.64%) were lower than those of monocytes (4.76%), CD3 + CD56 + cells (4.61%) and B cells (4.73%). 95% of the results had CVs within 10% for CD3 + T cells and CD56 + NK cells and 13% for monocytes, CD3 + CD56 + cells and CD20 + B cells. Additionally, assay precision was shown to be greater using cytokine based stimulation (median CVs of 3.84% (IFNα), 4.27% (IFNγ), 3.8% (IL-10), 2.88% (IL-2), and 3.64% (IL-6)) than LPS (5.62%) or PMA (4.94%). Still, 95% of the results had CVs below 10% (IFNα and IL-6), 12% (IFNγ, IL-10, IL-2 and PMA) and 15% (LPS).

4. Discussion and conclusion

The monitoring of phosphorylation in signal transduction pathways is increasingly being seen as a relevant tool in the treatment of disease with multiple research groups showing its promise in the monitoring (Huang et al., 2011) and prognosis (Brown et al., 2015) of disease, as well predicting and monitoring therapeutic treatments (Everson et al., 2014, Gavasso et al., 2014). Phosphoflow cytometry which can quantitate the level of phosphorylation in numerous phospho-protein targets simultaneously at a cell type-specific, single cell level provides an ideal tool for these research endeavors. Accurate measures require well tested and validated panels showing consistent and reproducible results. Here we presented 3 panels for an optimized 8 color multiplexed phosphoflow assays for the analysis of cryopreserved PBMC samples. The assays incorporates monoclonal antibodies specific for phospho-proteins ERK 1/2, NF-κB p65, Stat1 (Y701), Stat1 (S727), Stat3 (Y705), Stat3 (S727), Stat4, Stat1 (S727), P38 and Stat5. These targets were chosen as their activation is involved in numerous immune responses. Moreover, associations have been shown with dysregulation of intracellular signaling molecules involved in immune responses and autoimmunity (O'Shea and Plenge, 2012). The assay incorporates markers for common cell lineages in peripheral blood - T cells, B cells and NK cells; while monocytes were identified using their scatter properties. Additionally the assays utilize a 9× barcoding matrix allowing up to 9 samples to be measured simultaneously as 1 sample. This significantly reduces antibody consumption and flow cytometry acquisition times, significantly reducing the cost and time required to process and measure multiple samples, while additionally increasing data robustness. The use of heparin as an anti-coagulant was shown to be superior in retaining PBMC responsiveness compared to citrate. Citrate strongly affected NK cell responses to stimuli in multiple kinases, while CPT based isolation methods were associated with higher basal phosphorylation. Because of the varied response in PBMC subtypes we recommend prior testing before committing on blood collection methodology. Care should be taken to assess the isolation procedures effect on responses by different cell type. Additionally the resting period prior to PBMC stimulation should be optimized with regards to isolation protocol used to minimize basal signaling. As an example to illustrate the strength of this method we measured multiple phospho-proteins under multiple stimulation conditions simultaneously within a single barcoded sample in triplicates for 3 different donors. The assay showed excellent inter-assay precision over a month long period with CVs calculated against process triplicates of normalized MFI of the phospho-proteins displaying median CVs of under 10% when grouped by cell type, stimulation agent and phospho-protein measured, while the CV derived from each normalized triplicate measurement did not exceed 20%.

Sources of funding

This work was supported by grants from the Bergen Research Foundation, the Broegelmann Foundation and the Meltzer Foundation.

Acknowledgements

We thank all blood donors who participated in the study. The flow cytometry analysis was performed at the Flow Cytometry Core Facility, Department of Clinical Science, University of Bergen.

References

Brown, R., Yang, S., Weatherburn, C., Gibson, J., Ho, P.J., Suen, H., Hart, D., Joshua, D., 2015. Phospho-flow detection of constitutive and cytokine-induced pSTAT3/5, pAKT and pERK expression highlights novel prognostic biomarkers for patients with multiple myeloma. Leukemia 29 (2), 483–490.

Cesano, A., Perbellini, O., Evensen, E., Chu, C.C., Cioffi, F., Ptacek, J., Damle, R.N., Chignola, R., Cordeiro, J., Yan, X.J., Hawtin, R.E., Nichele, I., Ware, J.R., Cavallini, C., Lovato, O., Zanotti, R., Rai, K.R., Chiorazzi, N., Pizzolo, G., Scupoli, M.T., 2013. Association between B-cell receptor responsiveness and disease progression in B-cell chronic lymphocytic leukemia: results from single cell network profiling studies. Haematologica 98 (4), 626–634.

Everson, R.G., Jin, R.M., Wang, X., Safaee, M., Scharnweber, R., Lisiero, D.N., Soto, H., Liau, L.M., Prins, R.M., 2014. Cytokine responsiveness of CD8(+) T cells is a reproducible biomarker for the clinical efficacy of dendritic cell vaccination in glioblastoma patients. J. Immunother. Cancer 2, 10.

Gavasso, S., Mosleth, E.F., Maroy, T., Jorgensen, K., Nakkestad, H.L., Gjertsen, B.T., Myhr, K.M., Vedeler, C., 2014. Deficient phosphorylation of Stat1 in leukocytes identifies neutralizing antibodies in multiple sclerosis patients treated with interferon-beta. PLoS One 9 (2), e88632.

Huang, X., Guo, Y., Bao, C., Shen, N., 2011. Multidimensional single cell based STAT phosphorylation profiling identifies a novel biosignature for evaluation of systemic lupus erythematosus activity. PLoS One 6 (7), e21671.

Irish, J.M., Hovland, R., Krutzik, P.O., Perez, O.D., Bruserud, O., Gjertsen, B.T., Nolan, G.P., 2004. Single cell profiling of potentiated phospho-protein networks in cancer cells. Cell 118 (2), 217–228.

Kotecha, N., Krutzik, P.O., Irish, J.M., 2010. Web-based analysis and publication of flow cytometry experiments. Curr. Protoc. Cytom. Chapter 10 (Unit10), 17.

Krutzik, P.O., Nolan, G.P., 2003. Intracellular phospho-protein staining techniques for flow cytometry: monitoring single cell signaling events. Cytometry A 55 (2), 61–70.

Krutzik, P.O., Clutter, M.R., Trejo, A., Nolan, G.P., 2011. Fluorescent cell barcoding for multiplex flow cytometry. Curr. Protoc. Cytom. Chapter 6 (Unit 6), 31.

O'Shea, J.J., Plenge, R., 2012. JAK and STAT signaling molecules in immunoregulation and immune-mediated disease. Immunity 36 (4), 542–550.