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Monitoring the immune responses to vaccination and pertussis: bordetella pertussis and beyond

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Chapter 2.1

Impact of blood storage and sample handling on quality of high dimensional flow cytometric data in multicenter clinical research

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Abstract
Obtaining reliable and reproducible high quality data in multicenter clinical research settings requires design of optimal standard operating procedures. While the need for standardization in sample processing and data analysis is well-recognized, the impact of sample handling in the pre-analytical phase remains underestimated. We evaluated the impact of sample storage time (≈ transport time) and temperature, type of anticoagulant, and limited blood volume on reproducibility of flow cytometric studies.

EDTA and Na-Heparin samples processed with the EuroFlow bulk lysis protocol, stained and stored at 4°C showed fairly stable expression of cell surface markers and distribution of the major leukocyte populations for up to 72h. Additional sample fixation (1% PFA, Fix & Perm) did not have any beneficial effects. Blood samples stored for <24h at room temperature before processing and staining seemed suitable for reliable immunophenotyping, although losses in absolute cell numbers were observed. The major losses were observed in myeloid cells and monocytes, while lymphocytes seemed less affected. Expression of cell surface markers and population distribution were more stable in Na-Heparin blood than in EDTA blood. However, storage of Na-Heparin samples was associated with faster decrease in leukocyte counts over time. Whole blood fixation strategies (Cyto-Chex, TransFix) improved long-term population distribution, but were detrimental for expression of cellular markers. The main conclusions from this study on healthy donor blood samples were successfully confirmed in EDTA clinical (patient) blood samples with different time delays until processing. Finally, we recognized the need for adjustments in bulk lysis in case of insufficient blood volumes.

Despite clear overall conclusions, individual markers and cell populations had different preferred conditions. Therefore, specific guidelines for sample handling should always be adjusted to the clinical application and the main target leukocyte population.

Keywords: Flow cytometry, Immunophenotyping, Clinical trial, Blood storage, Sample fixation, Anticoagulant

Introduction

Standardized sample processing, immunostaining procedures, and calibration of instruments assure quality and reproducibility of flow cytometric data [1, 2]. Still, limited guidelines are available for sample handling in the pre-analytical phase. Parameters such as sample volume, storage time and temperature, and type of anticoagulant seem crucial for optimal data quality, reproducibility, and correct data interpretation.

In multicenter studies transportation of samples is often required. Whenever granulocytes are not studied [3] and selective loss of leukocyte subsets can be afforded [4], batch shipment of cryopreserved mononuclear cells might be used. However, if precise quantification and detailed immunophenotyping of all leukocyte subsets is required, peripheral blood (PB) needs to be analyzed fresh prior to any substantial sample manipulation. Whenever sample collection and analysis cannot be performed at the same location, either fresh blood or stained blood samples are shipped for central data acquisition. Depending on the distance, availability of personnel and equipment, such shipment may cause serious delays.

Prolonged blood storage leads to selective loss of cell populations with a short half-life time, such as eosinophils and neutrophils [5, 6]. Additionally, biochemical changes, such as decrease in pH, changes in blood gases [7], alterations in the contents of amino acids, carbohydrates, lipids and cofactors [8, 9], might further influence cell viability. The magnitude of these changes partly depends on the temperature [10, 11]. Ng et al. reported that certain monocytes and natural killer (NK) cell subsets are relatively stable at 4°C [12], while Hodge et al. showed a delayed apoptosis of granulocytes in refrigerated blood [12, 13]. In contrast, lymphocyte stability is affected by low temperature, with optimal temperature for T cells being 14-16°C [14]. Thus, both time and temperature of blood storage need to be optimized for specific research questions.

Reagents like Cyto-Chex, TransFix and formaldehyde extend the storage time of whole blood prior to flow cytometric analysis [12, 15]. However, they might have some unwanted effects. Davis et al. and Ng et al. found that while Cyto-Chex is more effective in preserving cell counts, TransFix is more potent in preserving cell identity [12, 16]. The loss of signal intensity, especially in Cyto-Chex, was further confirmed by others [17].

The Fix & Perm reagent Kit (Nordic MUbio, Susteren, the Netherlands), Becton Dickinson Perm/Wash buffer (BD Biosciences, San Jose CA), paraformaldehyde (PFA), glutaraldehyde or ethanol are frequently used to stabilize processed and labeled samples [2, 18-21]. Still, these reagents may affect the detection of cell surface markers by reducing the availability of the targeted antigen epitopes [22, 23] and thereby hamper optimal discrimination between cell populations. So far, no extensive comparative study of the impact of these techniques on the quality of stained samples after storage has been performed.

Combining flow cytometry with functional assays may influence the choice of anticoagulant. Ethylene diamine tetra-acetate (EDTA) is generally preferred in immunophenotyping, but it retains calcium for enzymatic reactions which hampers functional studies; thus, for the latter assays EDTA should preferably be replaced by heparin [8]. Several studies have evaluated the impact of anticoagulant on sample stability. Heparin was found superior to EDTA in preserving granulocytes [13], while data on lymphocyte stability remains controversial [24, 25]. So far, little is known about the impact of anticoagulant on the stability of cellular markers over time.

Standardized protocols (e.g. the EuroFlow bulk lysis protocol [26]) are designed for predefined large blood volumes. If, for example in pediatric settings, sample volume is insufficient, protocol adjustments might improve the quality of the final data.

Here we evaluated the impact of different blood storage conditions and potential protocol adjustments on the quality of flow cytometric data in multicenter clinical research settings (**Figure 1**). For this purpose the highly standardized EuroFlow "primary immunodeficiency orientation tube" (PIDOT)[27], which allows identification of all major cell populations in blood, was used as a model to assess stability of cells and cell surface markers in healthy and clinical samples.

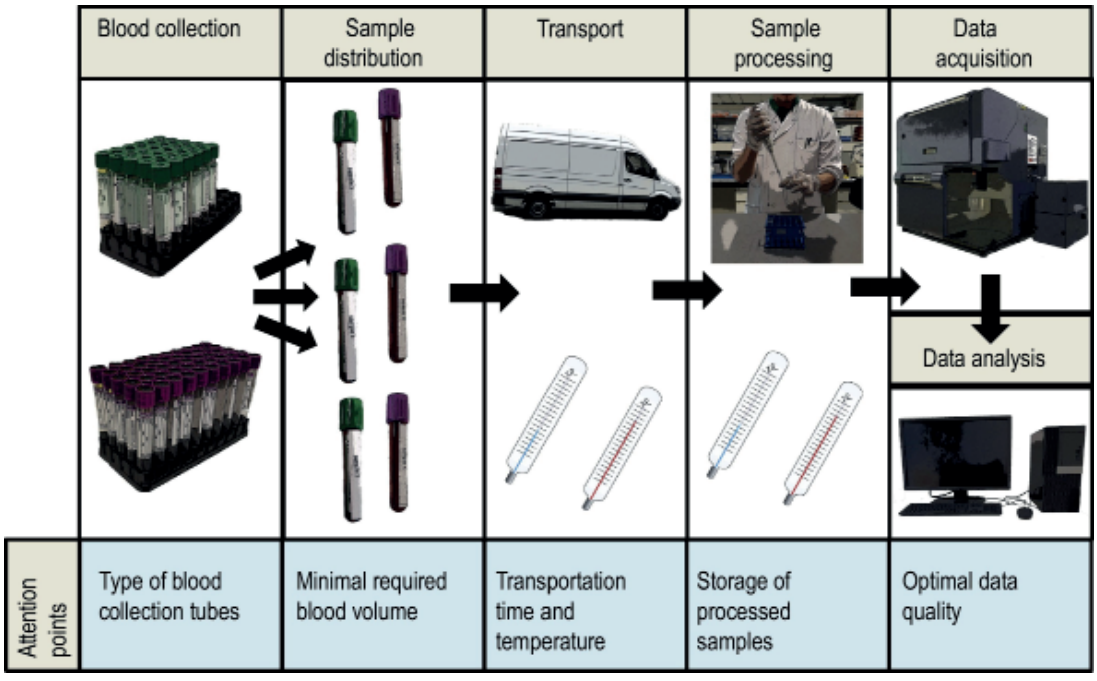


Figure 1. Critical decision steps involving the sample pre-analytical phase in clinical trial design. Depending on the design of the trial several decisions need to be made concerning (1) the choice of the blood collection tubes (anti-coagulant), (2) the distribution of samples based on the minimal volumes required per assay, (3) the time and temperature of sample transportation, and (4) storage of processed samples prior to data acquisition and data analysis.

Materials and methods

Samples

PB samples were obtained from healthy adult volunteers after they had given their informed consent to participate (n=15; m/f ratio: 5/10; age range: 23-62y, mean age: 36y). K3EDTA blood collection tubes (Vacuette®, Greiner Bio-One, Alphen aan den Rijn, the Netherlands) were used in all experiments, unless indicated otherwise. To evaluate the effect of anticoagulant on sample quality and stability, Na-Heparin blood collection tubes (Vacuette®, Greiner Bio-One) were used in parallel.

Blood processing and staining with PIDOT

All PB samples were processed according to the EuroFlow bulk lysis standard operating protocol (SOP) (available at www.EuroFlow.org). In brief, up to 2ml of blood was incubated with 50ml of ammonium chloride (NH_4Cl) at room temperature (RT) on a roller bank to lyse non-nucleated red blood cells. After a washing step, nucleated cells were counted on an automated hematological analyzer (Sysmex XP-300, Sysmex Europe GmbH, Norderstedt, Germany), and 2.5 x 10^6 cells were stained for 30min in the dark with the PIDOT antibody combination (Cytognos SL, Salamanca, Spain) according to the instructions of the manufacturer (www.cytognos.com)[27]. Finally, cells were incubated for 10min (in the dark; RT) with 2ml of BD FACS™ Lysing Solution (BD Biosciences), washed once and re-suspended in 200µl PBS prior to acquisition. A liquid format of the antibody combination was used [28], unless indicated otherwise. In the latter cases a dried version of PIDOT was used. Previous studies showed similar performance in samples stained using liquid and dried PIDOT [28].
In the experiments with limited sample volume, a modified version of the bulk lysis protocol was used where 100 or 200µl of K3EDTA PB sample was lysed in 3ml or 50ml of NH_4Cl without cell counting prior to acquisition on the flow cytometer.

Storage of stained samples (delayed acquisition)

Bulk-lysed PB samples stained with the PIDOT antibody combination were acquired directly or stored at 4°C for delayed acquisition (0h, 6h, 18h, 24h, 30h, 42h, 48h, 66h and 72h). In addition, part of the samples was treated with Reagent A of Fix & Perm® (F&P) or PFA (Merck, Darmstadt, Germany) to evaluate the effect of additional sample fixation on the stability of stained surface markers and leukocyte subset distribution. Treatment with Reagent A (F&P) was performed according to the manufacturer's protocol. In brief, after treatment with 1x BD FACS™ Lysing Solution and a washing step, 100µl of sample was incubated with 100µl of Reagent A for 15min in the dark (RT). Afterwards, samples were washed and stored in 200µl of PBS at 4°C for up to 72h. Treatment with PFA was performed in a comparable way, except for the incubation that was performed on ice. After initial evaluation of PFA concentration (0.5%, 1% and 2% final concentration), 1% PFA was used.

Storage of whole blood samples (delayed processing and staining)
To investigate the effect of prolonged blood storage on data quality, K3EDTA and Na-Heparin blood samples were stored at RT or 4°C for up to 72h prior to processing and staining. Additionally, treatment with TransFix (Cytomark, Leeds, UK) and Cyto-Chex blood collection tubes (Streck Cyto-Chex™, La Vista, NE) were evaluated.
TransFix was added to PB in K3EDTA collection tubes according to manufacturer's protocol (Revision 3 US, 2016-11). Both storage at RT (for up to 4 days) and at 4°C (for up to 14 days) were investigated. Cyto-Chex blood collection tubes were used according to manufacturer's protocol (Version 2017-02) and stored at RT for evaluation for up to 14 days.

Evaluation of absolute cell numbers
Two alternative approaches were used to determine absolute cell counts in (aged) K3EDTA and Na-Heparin blood samples. First, absolute leukocyte counts were measured on an automated hematological analyzer (Sysmex XP-300) at 0h, 6h, 12h, 24h, 30h, 36h, 48h, 54h, 60h and 72h after blood drawing. Then, analyses were repeated with Perfect-Count Microspheres™ (Cytognos) on BD LSRFortessa™ flow cytometer according to the EuroFlow SOP (www.EuroFlow.org). Leukocytes were discriminated based on the expression of CD45 (CD45-OC515, Cytognos).

Evaluation of clinical samples
To extrapolate the results of the storage experiments to diagnostic settings, flow cytometric data on 88 peripheral blood samples from primary immunodeficiency (PID) suspected individuals were evaluated retrospectively (age range: 0-79y, mean age: 30y). Flow cytometric data were obtained in a diagnostic context and approval for inclusion in the study was obtained by the Ghent University Hospital Ethics Committee (approval 2016/1137). In the diagnostic setting, samples were processed locally according to the EuroFlow guidelines. In all cases K2EDTA (BD Vacutainer®, Becton Dickinson Benelux, Erembodegem, Belgium) was used as anticoagulant, and whole blood was stored at RT for variable time periods before analysis with PIDOT (liquid format).

Flow cytometer set up and data acquisition
PIDOT stained samples were measured on FACSCanto™ II (BD Biosciences) instruments while Perfect-Count tubes were measured on LSRFortessa™ flow cytometer (BD Biosciences). Flow cytometers were calibrated daily according to the EuroFlow guidelines. In short, photomultiplier tube (PMT) voltages of the flow cytometer were set using BD™ Setup and Tracking (CS&T) beads (BD Biosciences) and SPHERO™ Rainbow calibration particles (Cytognos), as previously described)[2, 29].

Data analysis
Samples were mostly analyzed manually or manually checked after automated analysis with the EuroFlow PIDOT reference database (Infinicyt™ Software v2.0,

Cytognos) with respect to both marker stability and distribution of the major cell populations. Most positive and negative reference populations were defined as previously described [28]. For each reference population, mean fluorescence intensity (MFI) was determined. A deviation of 30% from the original MFI value (MFI-baseline) was set as the acceptance limit. The difference compared to MFI-baseline was determined by the following formula in which MFIx represents the MFI of a sample in a given condition: (MFIbaseline –MFIx)/MFIbaseline) *100. The distribution of a cell population was calculated as its relative frequency in the sample after excluding debris and doublets.

Determination of the degree of correlation between variables was performed using a Spearman correlation, after assessment of the normality of the distribution using the Shapiro Wilks test. For all statistical analyses the GraphPad Prism 8.0 software (GraphPad, San Diego, CA, USA), was used.

Results

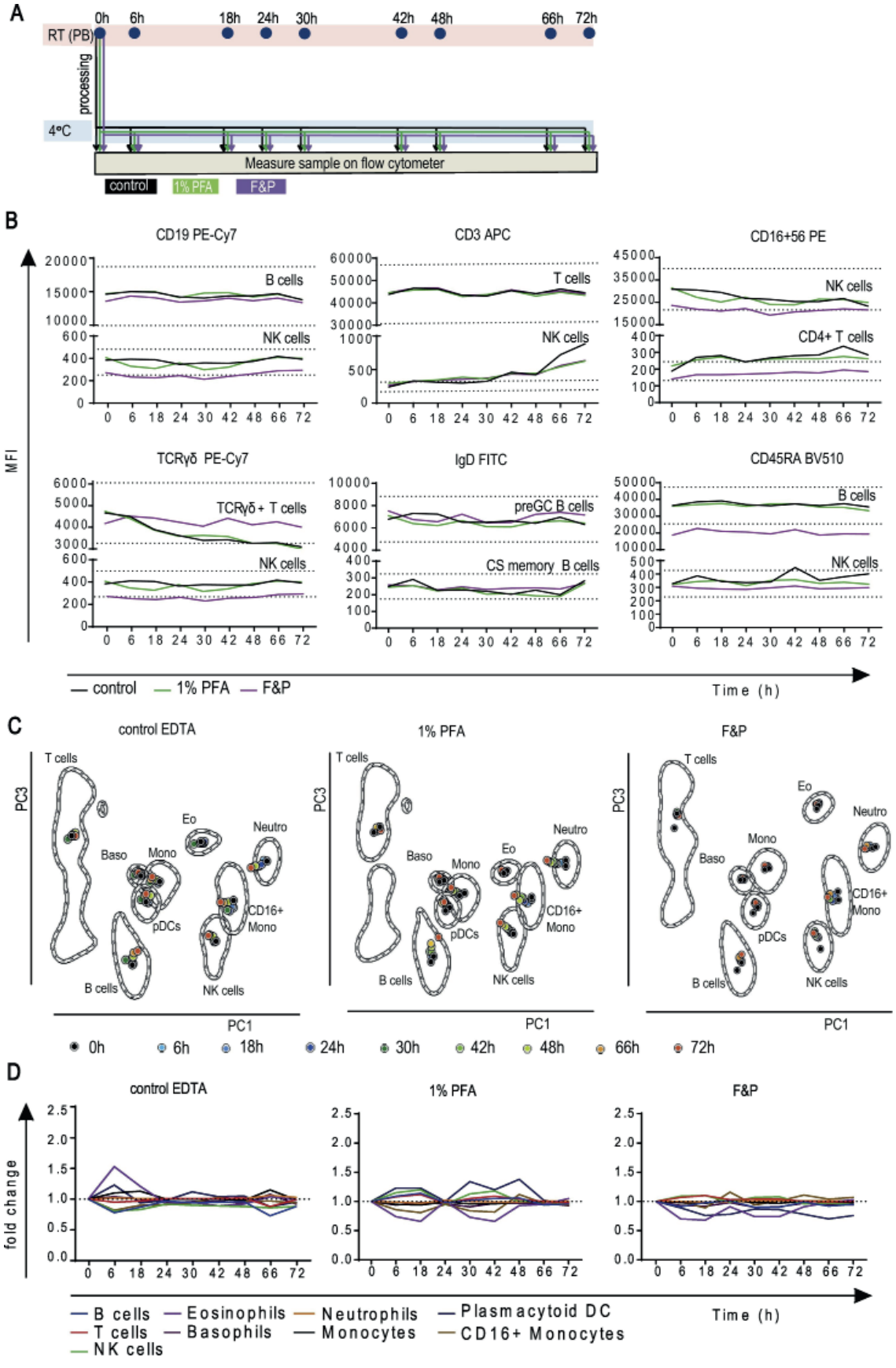
Stable expression of cell surface markers and distribution of major cell populations after delayed acquisition of stained samples

The PIDOT SOP (Cytognos PIDOT manual: revision 16-02-2018) recommends acquiring cells immediately after staining, or at most after 1h of storage at 4°C. To determine the effect of delayed acquisition of stained samples, PIDOT tubes were analyzed fresh or after they had been stored at 4°C for 6h, 18h, 24h, 30h, 42h, 48h, 66h and 72h (**Figure 2A**).

The impact of sample storage on the expression of cell surface markers was evaluated by analysis of their MFI on both positive and negative reference populations (**Supplemental Table 1, Figure 2B**). Most markers remained stable on positive reference populations for up to 72h with the exception of CD16+CD56 PE (> 10% loss of MFI after 24h), and TCRγδ PE-Cy7 (>10% loss of MFI after 18h, >30% loss after 48h). Despite the stable MFI on positive reference populations, background staining increased for most markers, in particular for CD16+CD56 PE (>30% after 6h), but in no case this hampered the discrimination of cell populations stained with PIDOT.

The Automatic Population Separator (APS) tool, based on the principal component analysis algorithms, was used to demonstrate overall segregation of populations in the multidimensional space. With the exception of neutrophils at 72h, all medians of populations in aged blood fell within one standard deviation (SD) line of the reference baseline population, and outside of one standard deviation lines of other populations (**Figure 2C**). Although basophils, monocytes and plasmacytoid dendritic cells (pDCs) were positioned closely together, they were clearly separable in other APS views (not shown).

To evaluate which of the major lymphoid and myeloid populations are the most affected by storage, their distribution was compared in stained samples stored for different time periods (**Supplemental Table 2**). Remarkably, the distribution


A
0h 6h 18h 24h 30h 42h 48h 66h 72h
RT (PB)
processing
4°C
Measure sample on flow cytometer
control 1% PFA F&P
B
CD19 PE-Cy7
CD3 APC
CD16+56 PE
B cells
T cells
NK cells
CD4+ T cells
TCRγδ PE-Cy7
IgD FITC
CD45RA BV510
TCRγδ+ T cells
preGC B cells
CS memory B cells
MFI
control 1% PFA F&P
Time (h)
C
control EDTA
1% PFA
F&P
PC3
PC1
T cells
Eo
Neutro
Baso
Mono
pDCs
CD16+ Mono
B cells
NK cells
0h 6h 18h 24h 30h 42h 48h 66h 72h
D
control EDTA
1% PFA
F&P
fold change
Time (h)
B cells
T cells
NK cells
Eosinophils
Basophils
Neutrophils
Monocytes
Plasmacytoid DC
CD16+ Monocytes

of the major cell subsets remained quite stable for up to 72h (**Supplemental Table 2, Figure 2D**). The largest variation from baseline was observed for the eosinophils, mainly due to substantial changes in one donor.

No beneficial effect of additional fixation of stained samples

The lysing solution utilized in the PIDOT SOP already contains a low concentration of fixative (<15% formaldehyde and <50% diethylene glycol in BD FACS™ Lysing Solution; 10x concentrated). To evaluate whether usage of an additional fixation step would have a positive impact on the quality of the data, freshly stained samples were treated with 1% PFA or with Solution A from F&P and evaluated after 0h, 6h, 18h, 24h, 30h, 42h, 48h, 66h and 72h storage at 4°C (**Figure 2A**).

Fixation with 1% PFA had no additional beneficial effect on the stability of cell surface markers (**Supplemental Table 1, Figure 2B**), whereas treatment with Solution A of F&P mainly had detrimental effects such as decreased MFI values for CD45RA BV510, IgM PerCP-Cy5.5 (>30%) and to a lesser extent for CD16+56 PE, CD4 PerCP-Cy5.5, CD27 BV421 and TCRγδ PE-Cy7 (>10%). After the initial decrease in MFI, signal remained relatively stable over time. Moreover, the discrimination among the distinct cell populations identified with PIDOT was not hampered by the additional fixation step. Evaluation of population phenotype in the APS view revealed that in stored samples fixed with 1% PFA, aged neutrophils (>42h), CD16+ monocytes (<48h) and NK cells (>66 h) showed the biggest variation from baseline, deviating >1SD line of the reference population (**Figure 2C**). F&P treated samples stayed within 1SD of the reference population at all times, but at the early time points fixed sample differed from baseline more than unfixed stored samples. In both PFA and F&P-treated samples, all populations were clearly separable.

The distribution of cell populations in fixed samples should reflect the distribution of the same cell populations in fresh samples and remain stable over time. However, directly after fixation with 1% PFA, the frequency of lymphocytes (B, T, NK cells) and CD16+ monocytes decreased by >10% (**Supplemental Table 2, Figure 2D**). Additionally, the frequency of basophils and eosinophils decreased by >10% after 6h. These changes were mainly compensated by the relative increase in neutrophils. Afterwards, the distribution of populations remained stable

Figure 2 (previous page). Longitudinal analysis of stained samples stored without or with additional fixative. Peripheral blood samples were collected into K3EDTA tubes stained and stored at 4°C for delayed acquisition. Additional stained samples were treated with 1% paraformaldehyde (PFA) or Fix&Perm Solution A (F&P) directly after staining and prior to the storage at 4 °C, n ≥ 3. (A) Flow chart showing the different storage conditions tested in this experiment (B) Mean fluorescence intensity (MFI) of selected cell surface markers on positive and negative reference populations. Each point indicates a mean value from ≥3 individual experiments. Dashed lines depict a 30% deviation from the MFI value at baseline (fresh sample without an additional fixative). (C) Individual populations from different time points separated by an automated population separator (APS) and visualized in 2D projections of the first and third principal components. Each dot represents a median value and each dashed line represent 1 standard deviation line of the indicated population at t = 0 h. Data of one representative donor is shown. Obtained data were analyzed with Infinicyt™ Software. (D) Changes in the frequency of individual cell populations over time relative to baseline.

over time. Fixation with Solution A resulted in a similar pattern, but with less impact on T cells and NK cells (**Supplemental Table 2**).

Hampered expression of cell surface markers and gradual loss of myeloid cells after prolonged whole blood storage

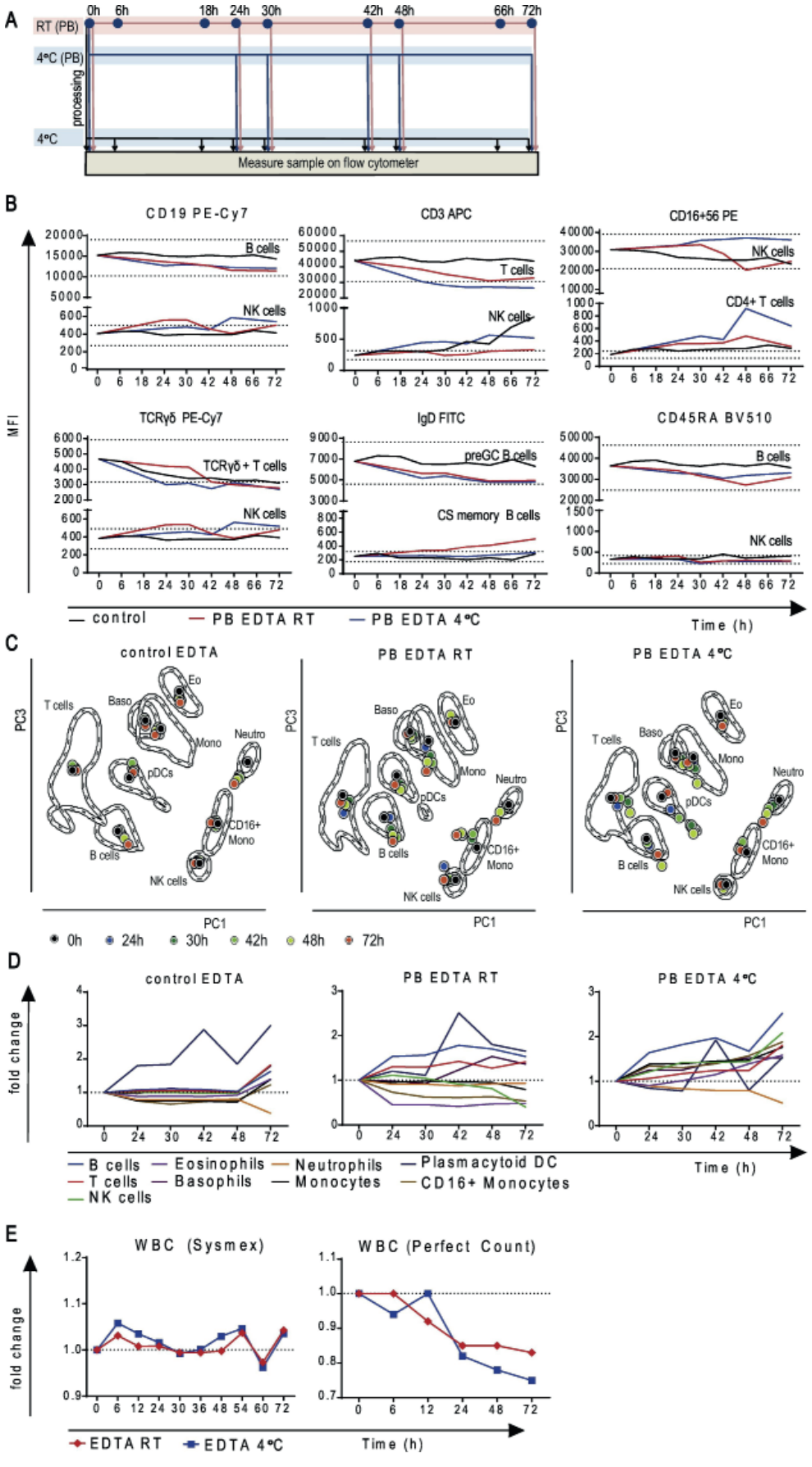
To mimic the delay caused by sample transportation, PB collected in EDTA tubes was analyzed at 0h or stored as whole blood either at RT or at 4°C, for delayed processing, staining and acquisition at 24h, 30h, 42h, 48h and 72h (**Figure 3A**).

In order to assess the impact of prolonged blood storage on detection of cell surface markers, MFIs of individual markers were analyzed on both positive and negative reference populations. Irrespective of storage temperature, already at 24h most markers showed >10% decrease in MFI on the positive reference cell populations, together with an increased unspecific staining on negative cells (**Supplemental Table 1, Figure 3B**). At RT the MFI signal was the least stable for CD27 BV421 and TCRγδ PE-Cy7 (>30% decrease in MFI from 42h onwards), while at 4°C the most affected markers were CD3 APC and TCRγδ PE-Cy7 (>30% decrease in MFI after 24h). Despite all the above, most populations could still be identified for up to 72h, but discrimination among them was clearly impaired over time.

In the APS view, (CD16+) monocytes, NK cells, neutrophils and B cells deviated >1SD of the reference population when blood was stored at RT from more than 30h (**Figure 3C**). Whereas in PB stored at 4°C the phenotype of B cells, T cells, and pDCs was impaired at later time points (>24h), CD16+ monocytes and NK cells showed limited deviation from baseline.

Several blood cell populations, mainly those of myeloid origin, have half-life times equal or shorter than 24h (6-8h for neutrophils, 18h for eosinophils, 1-2d for monocytes) [5, 6, 30]. Indeed, in blood stored for 24h at RT the frequency of eosinophils was reduced by 55% and the frequency of CD16+ monocytes by 27% (**Figure 3D, Supplemental Table 2**). Although the relative loss of neutrophils reached only 9%, a substantial part of these latter cells already showed early signs of apoptosis (lower FSC, SSC, CD45 and CD16 expression, without a clear separation from the viable population). The most overrepresented populations were long-living pDCs (+20%) and lymphocytes, with B cells being more overes-

Figure 3 (next page). Longitudinal analysis of blood samples stored in K3EDTA tubes. Peripheral blood samples were collected into K3EDTA tubes and stored either at RT or at 4°C for delayed staining. All results were compared to freshly stained K3EDTA samples, n ≥ 3. (A) Flow chart showing different storage conditions tested in this experiment (B) Mean fluorescence intensity (MFI) of selected cell surface markers on positive and negative reference populations. Each point indicates a mean value from ≥3 individual experiments. Dashed lines depict a 30% deviation from the MFI value at baseline. (C) Individual populations from different time points separated by an automated population separator (APS) and visualized in 2D projections of the first and third principal components. Each dot represents a median value and each dashed line represent 1 standard deviation line of the indicated population at t = 0 h. Data of one representative donor is shown. Obtained data were analyzed with Infinicyt™ Software. (D) Changes in the frequency of individual cell populations over time relative to baseline. (E) Changes in leukocyte count over time relative to baseline as measured by two independent methods.


A
0h 6h 18h 24h 30h 42h 48h 66h 72h
RT (PB)
4°C (PB)
processing
4°C
Measure sample on flow cytometer
B
CD19 PE-Cy7
CD3 APC
CD16+56 PE
B cells
NK cells
T cells
CD4+ T cells
TCRγδ PE-Cy7
IgD FITC
CD45RA BV510
TCRγδ+ T cells
preGC B cells
CS memory B cells
MFI
control
PB EDTA RT
PB EDTA 4°C
Time (h)
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PB EDTA RT
PB EDTA 4°C
PC3
PC1
T cells
Baso
Eo
Mono
Neutro
pDCs
CD16+ Mono
B cells
NK cells
0h 24h 30h 42h 48h 72h
D
control EDTA
PB EDTA RT
PB EDTA 4°C
fold change
Time (h)
B cells
T cells
NK cells
Eosinophils
Basophils
Neutrophils
Monocytes
Plasmacytoid DC
CD16+ Monocytes
E
WBC (Sysmex)
WBC (Perfect Count)
fold change
EDTA RT
EDTA 4°C
Time (h)

timated (+53%) than T cells (+29%) and NK cells (+11%). In blood stored at 4°C for 24h, both eosinophils and neutrophils were underestimated by around 11%, which was mainly compensated by a relative increase in both lymphocytes and monocytes (**Supplemental Table 2**). Thus, monocytes were better preserved at 4°C than at RT. Nevertheless, samples stored at RT were overall more stable than samples stored at 4°C.

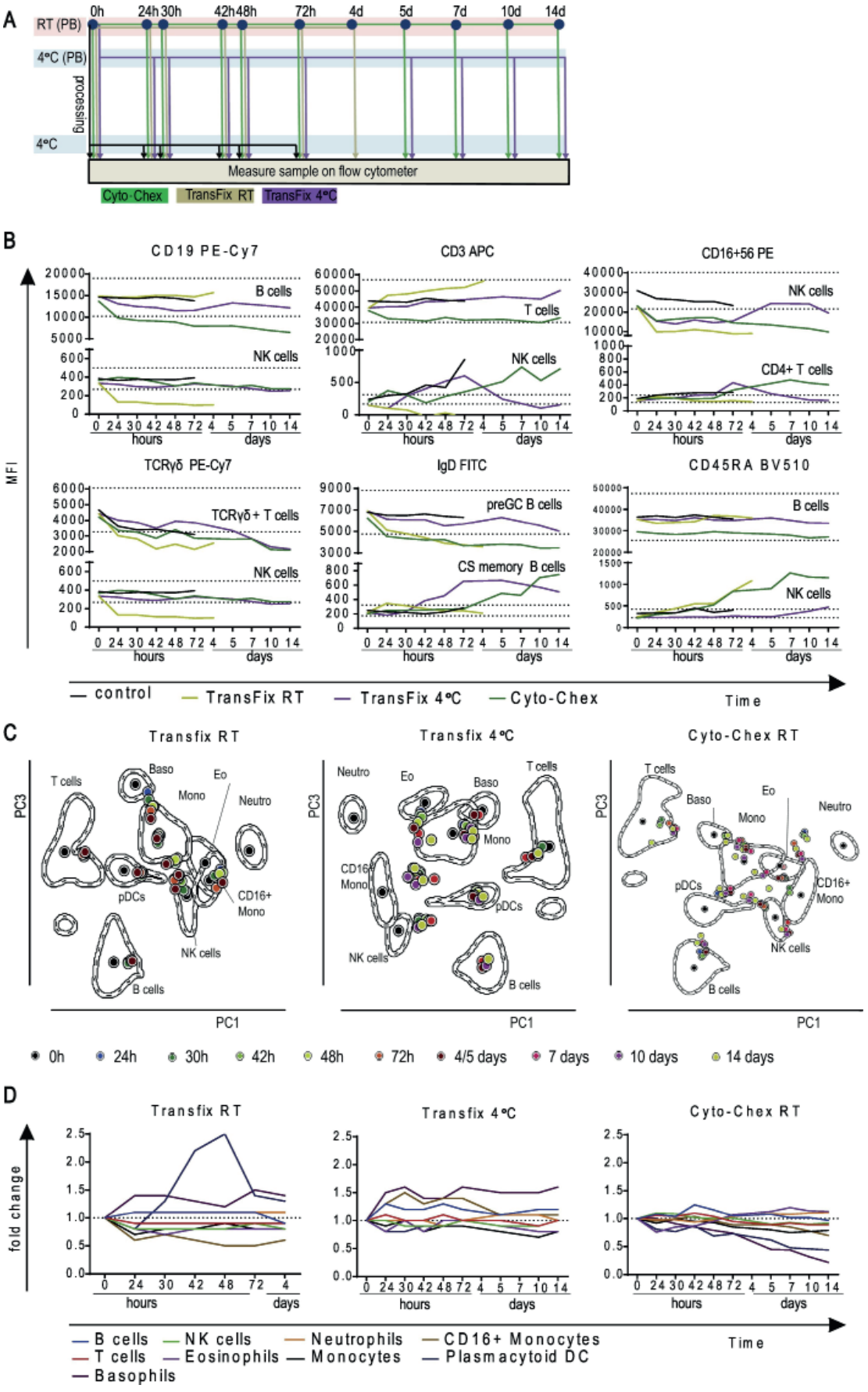
Cell death not only influences the distribution of cell populations in a sample, but also their absolute counts. Interestingly, at later time points, analysis on Sysmex XP-300 was more robust than analysis with the Perfect-Count Microspheres™, which showed progressive loss of cells of up to 17% (RT) and 25% (4°C) at 72h (**Figure 3E**). These differences may be caused by less extensive sample handling or the less stringent exclusion of dying cells on Sysmex XP-300.

Hampered expression of cell surface markers and initial changes in the distribution of cell populations after whole blood fixation

Two alternative whole blood fixation strategies were evaluated in parallel: Transfix for up to 4 days at RT and up to 14 days at 4°C and Cyto-Chex for up to 14 days at RT (**Figure 4A**). Both fixation methods resulted in an initial decrease in MFI (>10%) for approximately half of the markers tested (**Supplemental Table 1, Figure 4B**). In all cases the most affected markers were CD16+CD56 PE and CD27 BV421 (>30% MFI loss), followed by CD8 FITC and TCRγδ PE-Cy7 (>10% decreased MFI values). Additionally, TransFix led to changes in signal intensity of CD45RA BV510 (>10% increase at 4°C) and IgM PerCP-Cy5-5 (>10% decrease at RT), and Cyto-Chex fixation was associated with decreased signal intensity of CD4 PerCP-Cy5-5 (>10%). Further decrease in MFI over time occurred for all markers in Cyto-Chex tubes and for most markers in TransFix-treated samples. Decreased MFIs, increased background and affected cell scatter hampered discrimination of several cell subsets. Finally, the number of cell doublets and debris was increased, most likely due to an increased cell death and as a side effect of fixation (data not shown).

TransFix-treated samples stored at RT showed the best preservation of population phenotype of all whole blood-fixed samples. Nevertheless, in the APS view

Figure 4 (next page). Longitudinal analysis of blood samples stored in K3EDTA tubes with a whole blood fixative. Peripheral blood samples were collected into K3EDTA tubes, treated with TransFix and stored for delayed staining either at RT or at 4°C or collected into Cyto-Chex blood collection tubes and stored for delayed staining at RT. All results were compared to freshly stained K3EDTA samples, n ≥ 3. (A) Flow chart showing different storage conditions tested in this experiment (B) Mean fluorescence intensity (MFI) of selected cell surface markers on positive and negative reference populations. Each point indicates a mean value from ≥3 individual experiments. Dashed lines depict a 30% deviation from the MFI value at baseline (fresh sample without an additional fixative). (C) Individual populations from different time points separated by an automated population separator (APS) and visualized in 2D projections of the first and third principal components. Each dot represents a median value and each dashed line represent 1 standard deviation line of the indicated population at t = 0 h. Data of one representative donor is shown. Obtained data were analyzed with Infinicyt™ Software. (D) Changes in the frequency of individual cell populations over time relative to baseline.


A
0h
24h 30h
42h 48h
72h
4d
5d
7d
10d
14d
RT (PB)
4°C (PB)
processing
4°C
Measure sample on flow cytometer
Cyto-Chex
TransFix RT
TransFix 4°C
B
CD19 PE-Cy7
CD3 APC
CD16+56 PE
B cells
NK cells
T cells
CD4+ T cells
TCRγδ PE-Cy7
IgD FITC
CD45RA BV510
TCRγδ+ T cells
preGC B cells
CS memory B cells
MFI
hours
days
control
TransFix RT
TransFix 4°C
Cyto-Chex
Time
C
Transfix RT
Transfix 4°C
Cyto-Chex RT
PC3
PC1
T cells
Baso
Eo
Mono
Neutro
pDCs
CD16+ Mono
NK cells
B cells
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48h
72h
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7 days
10 days
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Cyto-Chex RT
fold change
hours
days
Time
B cells
NK cells
Neutrophils
CD16+ Monocytes
T cells
Eosinophils
Monocytes
Plasmacytoid DC
Basophils

basophils, neutrophils and eosinophils at multiple time points deviated more than 1SD from their reference population (**Figure 4C**). In TransFix-treated samples stored at 4°C, myeloid cells were heavily affected from the start. Already at 24h time point neutrophils, CD16+ monocytes and NK cells deviated >1SD from baseline samples. Deviations in eosinophils, monocytes and basophils showed a gradual increase in time. Evaluation of Cyto-Chex treated samples revealed a hampered identification of pDCs, neutrophils, basophils, CD16+ monocytes and eosinophils at most time points. Thus, whole blood fixation seemed to have the biggest detrimental impact on cells of myeloid origin.

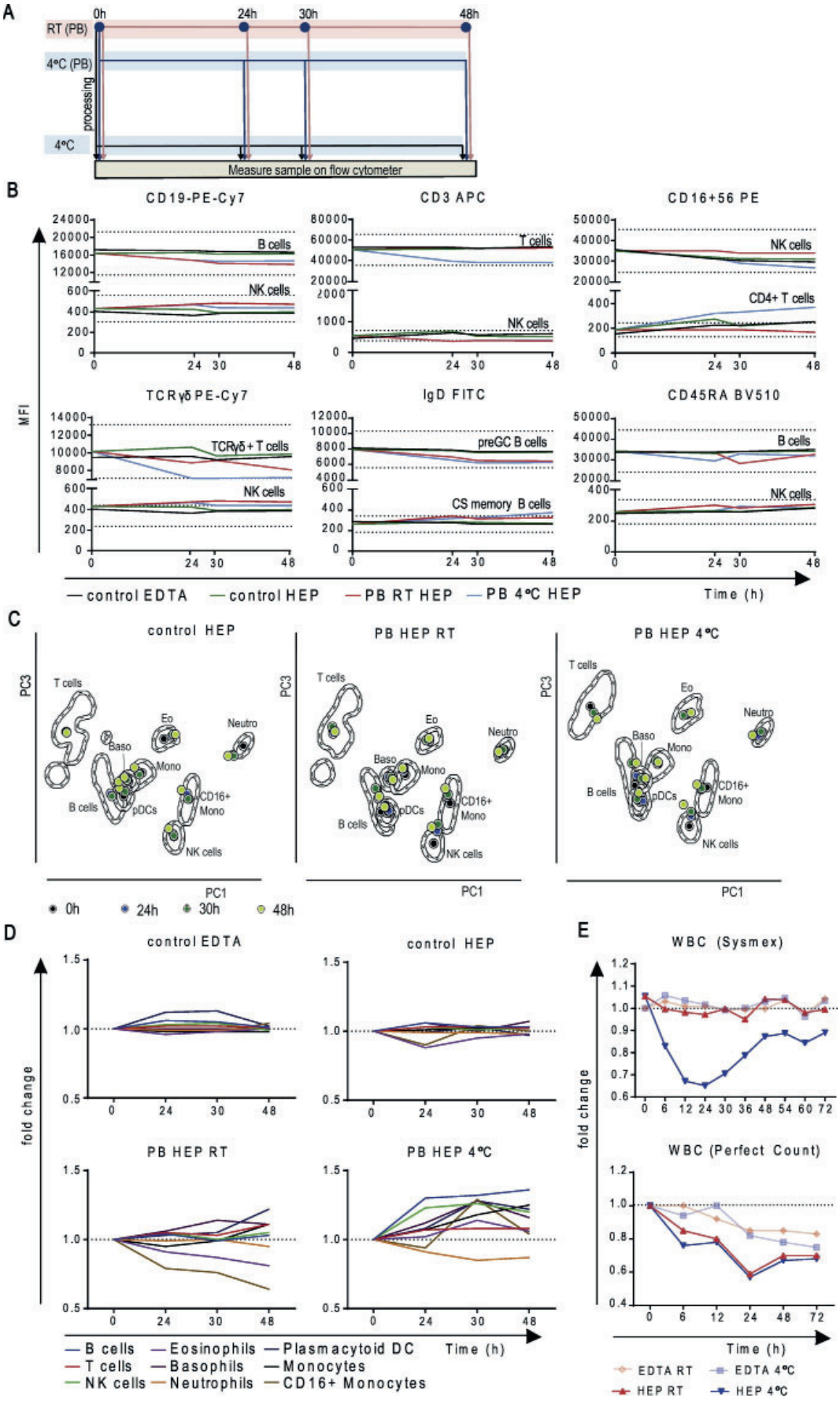
Although at most of the time points populations of cells in Cyto-Chex and TransFix samples cluster together in the multidimensional space, the initial deviation from baseline samples renders them unsuitable for comparison with baseline samples and use for detailed immunophenotyping.

Both Cyto-Chex and TransFix affected the relative distribution of multiple cell populations. Lymphocytes and monocytes were overrepresented at the expense of granulocytes, similar to what was observed in blood samples stored at 4°C (**Supplemental Table 2, Figure 4D**). Decrease in neutrophil numbers was especially prominent in samples treated with TransFix. After these initial changes, the distribution of cell populations remained relatively stable over time. Overall, samples stored at RT performed slightly better than samples stored at 4°C.

Good performance of PIDOT in Na-Heparin blood

Heparin is often the anticoagulant of choice when immunophenotyping needs to be combined with functional assays. To compare EDTA and Na-Heparin blood samples, Na-Heparin and EDTA anticoagulated blood was stained directly and stored at 4°C for acquisition at 0h, 24h, 30h and 48h. Additionally, samples in Na-Heparin tubes were stored at RT or at 4°C for delayed staining and acquisition at 24h, 30h and 48h (**Figure 5A**).

Figure 5 (next page). Longitudinal analysis of blood samples collected into Na-Heparin tubes. Peripheral blood samples were collected into Na-Heparin tubes, stained and acquired directly or stored at 4°C for delayed acquisition. Additional samples were collected into Na-Heparin tubes and stored either at RT or at 4 °C for delayed staining. All results were compared to freshly stained K3EDTA samples of the same donors, n ≥ 3. In 1 donor, the PIDOT in dried format was used, in all others, the liquid format was used for staining. (A) Flow chart showing different storage conditions tested in this experiment (B) Mean fluorescence intensity (MFI) of selected cell surface markers on positive and negative reference populations. Each point indicates a mean value from ≥3 individual experiments. Dashed lines depict a 30% deviation from the MFI value at baseline (fresh sample without an additional fixative). (C) Individual populations from different time points separated by an automated population separator (APS) and visualized in 2D projections of the first and third principal components. Each dot represents a median value and each dashed line represent 1 standard deviation line of the indicated population at t = 0 h. Data of one representative donor is shown. Obtained data were analyzed with Infinicyt™ Software. (D) Changes in the frequency of individual cell populations over time relative to baseline. (E) Changes in leukocyte count over time relative to baseline as measured by two independent methods.


A
0h
24h
30h
48h
RT (PB)
4°C (PB)
processing
4°C
Measure sample on flow cytometer
B
CD19-PE-Cy7
CD3 APC
CD16+56 PE
B cells
NK cells
T cells
CD4+ T cells
MFI
TCRγδ PE-Cy7
IgD FITC
CD45RA BV510
TCRγδ+ T cells
preGC B cells
CS memory B cells
control EDTA
control HEP
PB RT HEP
PB 4°C HEP
Time (h)
C
control HEP
PB HEP RT
PB HEP 4°C
PC3
PC1
T cells
Eo
Neutro
Baso
Mono
B cells
pDCs
CD16+ Mono
NK cells
0h
24h
30h
48h
D
control EDTA
control HEP
PB HEP RT
PB HEP 4°C
fold change
B cells
T cells
NK cells
Eosinophils
Basophils
Neutrophils
Plasmacytoid DC
Monocytes
CD16+ Monocytes
Time (h)
E
WBC (Sysmex)
WBC (Perfect Count)
fold change
EDTA RT
EDTA 4°C
HEP RT
HEP 4°C
Time (h)

Stained and stored EDTA and Na-Heparin samples performed equally well with regards to marker stability and cell population distribution (**Figure 5B-D, Supplemental Table 3, Supplemental Table 4**). None of the markers in samples collected in Na-Heparin tubes, besides CD16+56 PE showed a >10% decrease in MFI signal, and the increase in background staining was limited (>30% for only CD16+CD56 PE and CD27 BV421). None of the analyzed cell populations were clearly over- or underestimated over time. When looking at a representative donor in the APS plot, baseline samples showed a smaller standard deviation in directly stained Na-Heparin samples compared to directly stained EDTA samples. For neutrophils and CD16+ monocytes, >1SD difference was seen after 24h and 48h, respectively. Nevertheless, stored samples closely represented baseline samples (**Figure 5C**).

Samples stored at RT in Na-Heparin tubes prior to staining showed stable expression of most of the evaluated cell surface markers (none showed decreased MFI values of >30%) and outperformed samples stored at RT in EDTA tubes (**Supplemental Table 1**). Sample storage at 4°C had a more detrimental effect on marker expression in both EDTA and Na-Heparin. While CD3 APC and CD27 BV421 performed better in Na-Heparin tubes, CD8 FITC was more stable in EDTA.

Na-Heparin samples stored at RT showed virtually no deviations >1SD in the multidimensional space up to 48h of PB storage. Na-Heparin samples stored at 4°C showed deviations >1SD from 30h onwards for NK cells, B cells and neutrophils (**Figure 5C**).

The relative distribution of distinct cell populations was more stable when samples were stored at RT, and Na-Heparin tubes were associated with more stable results than EDTA tubes (**Figure 5D**). Up to 24h no major deviations were observed in Na-Heparin samples stored at RT, while at later time points lymphocytes (mainly T cells), monocytes and basophils were slightly overrepresented at the expense of eosinophils, CD16+ monocytes and neutrophils. These changes were more prominent in Na-Heparin blood stored at 4°C, where almost all cell populations deviated from baseline by >10% already at 30h. Again, lymphocytes (mainly B cells), monocytes and basophils were overrepresented at the expense of neutrophils.

In contrast to the expression of cell surface markers and the distribution of cell populations, absolute leukocyte numbers were less stable in samples stored in Na-Heparin than in EDTA (**Figure 5E**). After 24h, in samples measured by Perfect-Count Microspheres™, absolute cell counts decreased by ~40% in Na-Heparin and by ~20% in EDTA. Remarkably, all samples were stable over time when measured on Sysmex XP-300 except for Na-Heparin samples stored at 4°C. Na-Heparin samples stored at 4°C showed a strong initial drop in absolute cell counts in all donors (>30% after 12h), followed by gradual recovery from 30h onwards.

Bulk lysis protocol should be adjusted for limited sample volume
Bulk lysis SOP is optimized for 1.5-2ml of blood, which results in more than the 2.5 x 106 cells required for PIDOT. As different lysis protocols may impact sample properties like scatter and marker expression, it is recommended to use the same protocol for all samples that are compared to one another, especially when automated analysis is used. In case too little sample volume is available for bulk lysis, the protocol may need to be adjusted. To evaluate the procedure for limited blood volumes, 100µl and 200µl of blood were lysed either with 50ml of NH_4Cl (bulk lysis SOP) or 3ml of NH_4Cl (blood/NH_4Cl ratio similar to official EuroFlow SOP; **Supplemental Table 5**) and stained with PIDOT. If a reduced NH_4Cl volume was used, lysis was performed directly in the staining tube to minimize cell loss during transfer of sample between tubes.

If the original blood/NH_4Cl ratio was maintained, the distribution of cell populations in small volume samples was more comparable to the distribution of such cell populations in samples processed according to the SOP. In small volume samples lysed with 50ml of NH_4Cl most B-cell subsets were underestimated by >20%, mainly in favor of granulocytes and monocytes. In all tested conditions, the fluctuations of plasma cell percentages were large (>50%), most likely due to the small population size at the detection limit of the method.

Prolonged storage of diagnostic samples affects data analysis
To evaluate the impact of sample storage in diagnostic settings, 88 EDTA blood samples from PID-suspected individuals were stored for up to 8 days at RT and analyzed with PIDOT. As all samples were sent for routine diagnostics, no baseline measurements were available. For analysis purposes samples were anonymized and divided into five categories based on storage duration: 0-12h, 12-24h, 24-48h, 48-72h and >72h.

As in the whole blood storage experiments with healthy controls, most markers showed >10% decrease in MFI on the positive reference population already 12-24h after collection (**Supplemental table 6, Figure 6**). In accordance with the previous findings in PB stored at RT, the most dramatic decrease in signal was observed for TCRγδ PE-Cy7 and CD27 BV421, which showed >30% and 28% decrease in MFI in samples acquired >48h after drawing, respectively. Furthermore, control and diagnostic samples shared some common features regarding relative changes in population distribution over time, i.e. a gradual loss of eosinophils and NK cells (**Figure 6, Supplemental table 7**). Although in both control and diagnostic samples most populations could be identified in aged blood, the situation in PID-suspected individuals was complicated by the unknown diagnosis. Therefore, the absence or an aberrant phenotype of a given population could be

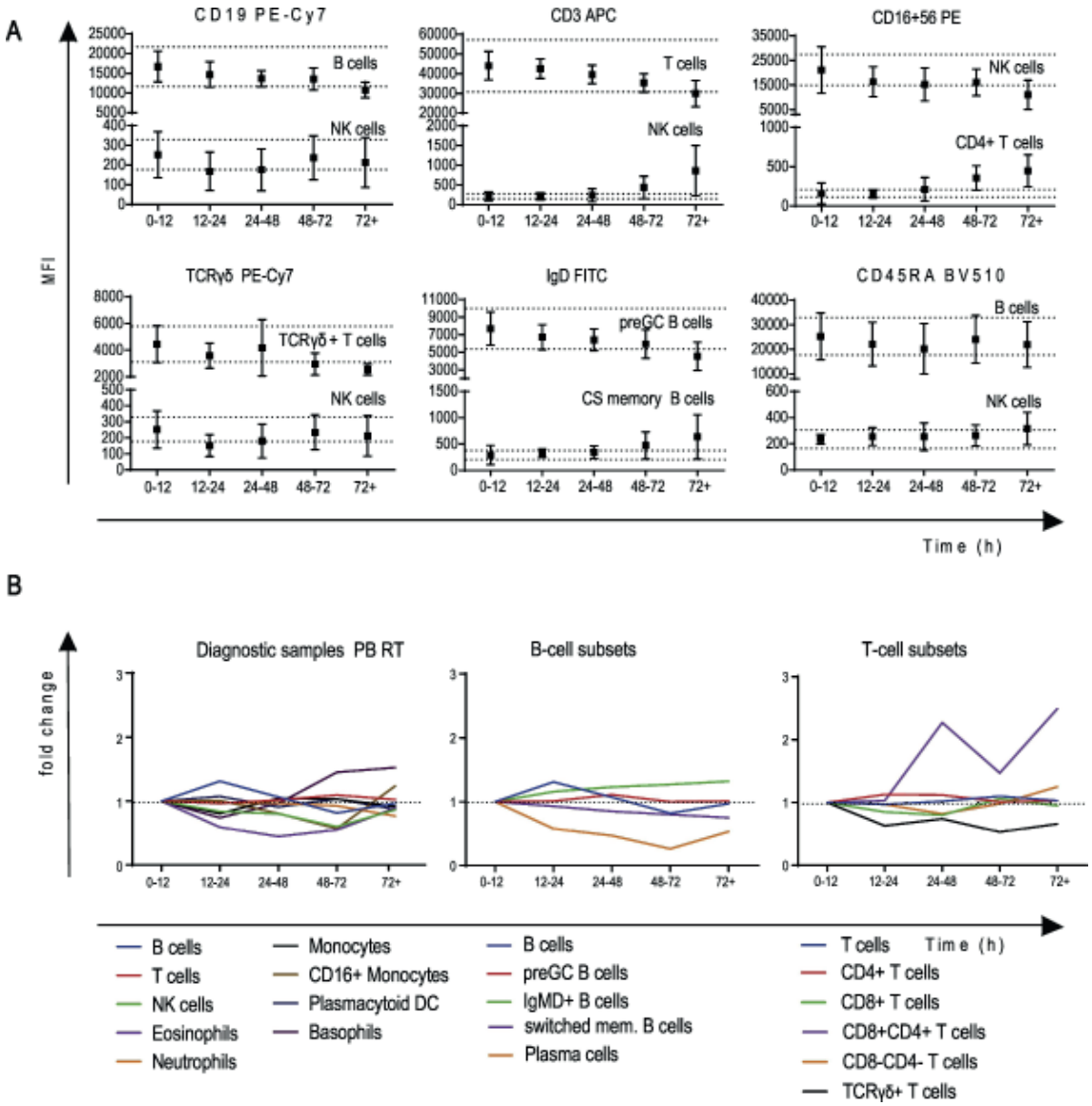


Figure 6. Impact of sample storage on diagnostic samples collected in K3EDTA tubes. Peripheral blood samples were collected into K3EDTA tubes and stored at RT until staining (n = 88). Mean age and range per group: 0–12 h (20.1; 1–67.4), 12–24 h (23.0; 0.4–71.3), 24–48 h (36.5; 0.4–78.8), 48–72 h (39.9; 5.9–68.7), 72 h + (28.6; 1.2–76.8) (A) Mean fluorescence intensity (MFI) of selected cell surface markers on positive and negative reference populations. Each point indicates a mean value of all diagnostic samples collected within that timeframe (n ≥ 11). Dashed lines depict a 30% deviation from the MFI value from the samples acquired 0–12 h after collection. (B) Changes in the frequency of individual cell populations over time relative to the samples acquired 0–12 h after collection.

attributed either to prolonged sample storage or to the underlying disease.

Discussion

Complex sample logistics in multicenter clinical studies require careful alignment of working procedures among participating laboratories. Extra challenges are caused by limited sample volume (e.g. in case of infants or young children) and the need of sample transportation to another (e.g. reference) laboratory.

Here we evaluated several critical steps in the process of obtaining high quality flow cytometry data from peripheral blood samples. We showed how the choice of anticoagulant influences the stability of cell surface markers and cell populations, and tested a range of possibilities to preserve whole blood and stained samples for delayed acquisition. These results were translated to clinical settings by retrospective analysis of routine diagnostic samples. Finally, we recognized the need for adjusting working procedures in case of insufficient sample volumes. Enumeration of white blood cells is one of the most frequently requested routine diagnostic tests. Here we evaluated two alternative strategies to determine absolute cell counts: flow cytometry-based Perfect-Count Microsphere™ measurements and the Sysmex XP-300 hematological analyzer. Overall, results obtained with Sysmex XP-300 in aged blood better reflected results from fresh samples. Analysis with Perfect-Count Microspheres™ was preceded by sample treatment for 10 min with NH_4Cl. This lysis step can have a detrimental effect on the – already vulnerable- aging cells which then become classified as debris or dead cells rather than viable cells; alternatively speed of analysis and electronic-digital signal processing, together with different rates of doublet formation might contribute to explain such differences. Furthermore, the potential inclusion or exclusion of apoptotic cells by different counting methods could result in different blood cell counts. Irrespective of the blood storage temperature, measurement of Na-Heparin samples resulted in lower absolute cell counts than those obtained for EDTA anticoagulated samples. Therefore, whenever longitudinal analyses are required, or subtle changes in cell numbers are expected, it is crucial to always use the same blood collection tubes, preferably EDTA tubes if samples will be processed immediately (<24-30h).

Reliable analysis of absolute cell counts by Sysmex XP-300 was not possible in Na-Heparin samples stored at 4°C. For these samples leukocyte numbers showed an apparently rapid initial decrease followed by a slow gradual recovery after 24h. These changes were positively correlated with the platelet counts (Spearman correlation $P<0.0001$, correlation coefficient of 0.65). A likely explanation is that in Na-Heparin samples stored at 4°C platelets become activated and start to form aggregates [31, 32] including platelet-leukocyte aggregates. This formation and disintegration may explain fluctuations in the absolute leucocyte count.

Total white blood cell numbers can be reliably determined in a limited volume of blood. However, if infrequent cell populations need to be quantified, analysis might reach the detection limit. Here, we stained 2.5 x 106 cells (resulting in acquisition of ~1-1.5 x 106 cells) and observed that small populations like plasma cells, eosinophils and specific T-cell subsets showed a greater variation than larger cell populations, implying that the enumeration of less represented cell populations has to be more critically assessed. Increasing the number of cells evaluated will most likely improve accurate quantitation of such rare cells. As stated by Oldaker et al., and proven by Theunissen et al., and Flores Montero et al., detection of low represented abnormal cell populations is possible as long as an adequate number of events is acquired [26, 33, 34].

In fact, the limit of detection of one or more cell populations becomes an important problem when limited blood volumes are available. Here we found that the distribution of populations analyzed in the small volume samples better reflects the distribution of populations in large volume samples when the volumes of reagent are adjusted to maintain the same blood/reagents volume ratio. Fewer transfer steps between tubes in the protocol with adjusted blood/reagents volume may further influence better preservation of initial population distribution. Consequently, reagent volumes and protocols need to be adapted to sample volumes. In any case, the total number of acquired white blood cells should always be acknowledged to indicate the reliability of the data.

Populations of cells are defined based on the presence or absence of specific markers and their expression levels (especially of importance if these are modulated during cell activation and/or maturation)[35-38]. Therefore, each marker should not only provide a proper discrimination between strong and dim positive cells and between positive and negative cells, but it should also provide an adequate resolution to detect more subtle changes. Here we confirmed and extended previous observations [12-17, 22-25] by showing that the type of anticoagulant, storage time, storage conditions and treatment all had an impact on marker expression levels and background staining. Similar findings on the impact of anticoagulant on sample stability have recently been published by Kárai et al. in the context of bone marrow and PB processing [39].

None of the two tested anticoagulants (Na-Heparin, EDTA) had a clear advantage over the other with regard to stability of marker expression levels. However, individual markers performed better in one of the conditions. Therefore, if MFIs are to be compared, it is crucial to be consistent with the choice of anticoagulant.

For most markers the resolution of signal decreased over time. The decrease in signal and increase in background staining was more prominent in stored whole blood than in stored processed and stained samples. This is likely because the signal intensity can be influenced by unspecific binding of mAbs to dying cells and shedding or internalization of markers due to e.g. apoptosis or (in)activation [38, 40]. Furthermore, sample (including whole blood) fixation (Solution A from F&P, Cyto-Chex, TransFix) had a negative impact on marker expression. In these circumstances the most prominent loss of signal occurred directly after addition of a fixative, possibly due to conformational changes of the antibody targeted epitopes or impact of the fixative on the fluorochromes [41].

Certain fluorochromes, e.g. some of the tandem-dyes, are known to be less stable [42]. Despite a broad range of antibodies assessed, we have not observed antibodies conjugated with any specific dye to be more affected. At the same time, we found that antibodies with lower affinity for the antigen, such as TCRγδ, were especially sensitive to prolonged sample storage. This is in line with what has been reported by Davis et al., who showed that CD4 PE conjugates from different clones showed great variability in both avidity and antibody stability [43] and by

Van der Velden et al., who showed that TCRγδ PE-Cy7 showed greater variability when used in different formats (freeze-dried or liquid antibodies) [28].

In whole blood samples the largest differences in cell distribution were caused by progressive cell death (mainly granulocytes), which appeared to be associated with both changes in the cell phenotype and an increase in doublets and debris. Although whole blood fixation had a detrimental effect on the relative distribution of distinct cell populations directly after treatment, it stabilized the relative distribution afterwards, making this condition more beneficial at the later time points. Thus, if blood cannot be processed within 24-30h, and the distribution of cell populations is of primary interest, whole blood fixation might be an option. However, we would not recommend it for general immunophenotyping. Of note, it has been reported that PB fixation may preserve leukocyte counts compared to untreated PB [44, 45]. Again, here we show that additional fixation of processed samples provides better results than fixation of whole blood samples.

Processing of diagnostic samples can be delayed by transportation as well as by personnel and equipment availability. This can have several consequences for the outcome of flow cytometric analysis. First, we showed in healthy controls that, depending on the storage conditions, absolute cell counts can decrease by up to 40% already 24h after collection. Since absolute cell count is one of the diagnostic criteria for immunodeficiencies, cell loss in aged samples can affect proper diagnosis. Secondly, several immunodeficiencies are characterized by the absence or reduced number of cell populations or their aberrant phenotype [27, 46]. A recent study by van der Burg et al. showed reduced numbers of memory B cells in confirmed SCID patients. The markers used in PIDOT to separate memory B cells from pre-germinal center cells are mainly CD27 and IgM. Thus, the observed decrease in CD27 BV421 and increased background in the PerCP-Cy5.5 channel (which harbors both IgM and CD4) can eventually hamper discrimination of memory B cells. Analogical problem can arise in identification of TCRγδ T cells, as TCRγδ PE-Cy7 was one of the most storage-affected markers, and lack of these cells can be associated with immune deficiency. Thus, prolonged storage (>24h) of diagnostic samples should be avoided and, if not possible, they should be evaluated critically, keeping in mind that changes may be attributed to sample aging. In this study we used a <30% deviation from baseline MFI as a measure of marker stability. In many cases, samples could be reliably analyzed by experienced personnel despite greater signal changes. This was at least in part based on the software used for data analysis (Infinicyt™ Software v2.0, Cytognos), which allows for simultaneous multidimensional gating rather than 2-dimensional consecutive gating. In this multidimensional approach changes in expression of cellular markers over time were absorbed by other markers in such a way that population separation was still possible. Recently, a database has been released for automated analysis of PIDOT (Infinicyt™ Software 2.0, Cytognos). Although during the construction of a database high quality samples are required [47], it would be of great interest to evaluate how aged and differently treated samples can be run through such a database.

Concluding remarks

This intricate set of data presented here was based on the analysis of a single antibody combination: PIDOT. Therefore, it cannot provide universal guidelines. Still, certain conclusions can be drawn about what can and should not be done in the pre-analytical phase of multicenter clinical trials:

- Delay from blood collection to blood processing should be minimized, especially in a diagnostic setting.
- PB samples stored for <24h at RT seem suitable for reliable immunophenotyping, although losses in absolute cell numbers should be taken into account.
- PB samples stored for 24-48h at RT may be used for immunophenotyping, but results will be less reliable. Again, losses in absolute cell numbers should be taken into account.
- In whole PB samples, most populations are more stable when stored in Na-Heparin than in EDTA tubes at RT.
- However, storage of processed and stained samples is usually more beneficial than storage of whole blood.
- B and T cells are the most stable populations, followed by NK cells, whereas myeloid cells and monocytes are more sensitive to storage. Thus, based on cell type of interest, different delays are acceptable.
- The impact of anticoagulant and potential fixation steps on the expression of individual cell markers varied significantly; thus, it is crucial to be consistent in the use of anticoagulant, storage time and temperature.
- Any deviations from standard operating procedures should be recorded.
- When determining absolute counts, minimize storage or aim for consistent delays. Be consistent with the method used. In our experience an automated hematological analyzer is more stable than flow cytometric determination of absolute counts.

Specific recommendations for individual types of cells are summarized in **Supplemental Figure 1**.

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Declaration of interests

The authors declare no conflict of interest apart from J.J.M. van Dongen and A. Orfao, who are chairman and co-chairman of the EuroFlow scientific consortium, which receives royalties from licensed patents; this income is solely used for continuation of the collaboration within the EuroFlow consortium. In addition, J.J.M. van Dongen and A. Orfao have an Educational Service Agreement with BD Biosciences; income from this agreement goes to LUMC and USAL respectively.

Supplemental Figures and Tables can be found in the online article:

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