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How fixation affects the results of lymph node immunophenotyping by flow cytometry

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Abstract

Aim: Flow cytometric diagnosis of lymphoma and leukemia is of high clinical and research importance. However, performing flow cytometry analysis on the day of biopsy might be of challenge due to several reasons, including late sample delivery, problems of preparing the reliable panel for immunophenotyping based on other diagnostic studies, etc. This problem could be partially solved if cell suspension could be fixed and stained on another day or after several days after standard FFPE (formalin-fixed and paraffin-embedded) procedure.

Material and methods: Addressing this issue, we compared staining of live lymphocytes in suspension obtained from lymph node biopsies and same specimens fixed using 2-4%-paraformaldehyde, 1-3%-glyoxal, and 0.1-1% glutaraldehyde with subsequent immunostaining on the next day or later.

Results: Staining after fixation could be partially representative only after paraformaldehyde fixation for 20 min and subsequent storage of cell suspension in phosphate-buffer saline within not more than 3 days. Probes stained after fixation always shows lower stain index compared to staining of live cells.

Conclusion: Staining after fixation cannot be used for determining of the percentage of CD45-positive cells and for testing B-cell lymphomas since antigens against light chains of IgG cannot be properly detected in fixed specimens.

Key words: flow cytometry, immunophenotyping, cell suspension, lymph node, sample fixation, B cells, T cells, lymphoma

Introduction

Nowadays, flow cytometry (FC) is one of the major clinical tools in immunophenotyping patients with hematological and lymphoproliferative disorders. Meanwhile, performing flow cytometry analysis on clinical samples upon receiving them might be a challenge for some laboratories. Moreover, researchers or doctors might decide on performing additional studies based on the result of morphological or immunohistochemical studies later, which also requires preservation of biological specimens.

A comprehensive review on the problems appearing throughout the process of preparing a single cell suspension from solid tissues for prolonged storage prior to flow cytometry analysis was recently published [1].

Chemical fixation is a routinely used procedure aimed at maintaining cell or tissue architecture and antigenicity [2]. There is a wide range of fixators, mixtures of different fixators, and procedures, where each laboratory adapts their own protocol for achieving the optimal staining results [2,3]. The choice of the fixative depends on its maximum fixation ability without adverse effect on antigenicity thus providing long-term storage and reliable results of immunophenotyping post fixation. Apart from losing antigenicity, fixation can cause autofluorescence and nonspecific fluorescence [4,5]. In flow cytometry, researchers generally suggest staining live cells first and then preserve them by fixation known as post fixation procedure [4-6]. A variety of studies has been performed to preserve mainly liquid forms of human samples (whole blood, bone marrow and cerebrospinal fluid) using different fixatives and methods [4-7]. However, no data is available on chemical preservation of cells isolated from lymphatic nodes.

Formaldehyde or paraformaldehyde (PFA) solutions are common fixatives routinely used in flow cytometry and in diagnostic pathology [8]. It has been suggested that short fixation of cells from solid tissues

might prevent their degradation, while not affecting light scatter characteristics [9]. Alcohol fixation is another type of fixators successfully used for intracellular staining in flow cytometry [10], but it results in cell clumping and loss of light scatter thus it is completely unacceptable for cell fixation and storage [11]. Less popular among fixators is glyoxal-based solutions, which is nowadays positioned as alternative fixator to paraformaldehyde in immunostaining [12,13]. Authors emphasize glyoxal's advantages including safeness and easiness in handling, better preservation of cell fine structure and more intense immunostaining without antigen retrieval. However, no information was published on preservation of surface antigens in cell suspension using glyoxal solution. Since glyoxal was introduced as a fixative more recently than formalin and show at the microscopic level better tissue preservation [12,14], we decided that it is important to test this fixative along with formalin and glutaraldehyde. In this study, we approbated two different concentrations of glyoxal – 1% and 3% for fixation of cells in suspension. To the best of our knowledge, no data has been previously published on assessing the effect of fixation with glyoxal on cells isolated from solid tissues.

Neither, we found any papers related to such multi-parametric analysis of the effect of fixation imposed on the main lymphocytic antigens. Developing easy, safe, and fast method of chemical fixation allowing prolonged storage without deterioration of FC results might be very useful for clinical laboratories and field research. In this study, we suggest fixation of unlabeled cells first following with antibodies staining a few days later. This will benefit both clinicians and researchers by extending the range of panels used and experiments conducted on a single sample.

Aim of the study

In the current study, we used live cells in suspension obtained from the lymph node biopsy for immediate multicolor immunostaining and then stained and analyzed PFA, glyoxal, and glutaraldehyde fixed samples to compare the staining and expression of lymphocyte specific antigens post fixation. The research objectives were the following:

Firstly, to approbate widely used (PFA, glutaraldehyde) and recently suggested (glyoxal) chemical fixators aiming to preserve lymphoid cell morphology (reflected as light scatter properties) and antigenicity (antibody staining) in suspension stored for at least three-days at +4ºC.

Secondly, to test different concentrations of PFA (2-4%) and glyoxal (1-3%) identifying the optimal working concentrations.

Thirdly, to compare the percentage of positive cells, mean fluorescence intensity, and stain index between live and fixed cells, as a measure to analyze the effect of fixation posed in cell surface antigen expression.

Material and methods

Sample collection

In this study, lymph node biopsies with suspected lymphoma were provided by the National Research Oncology and Transplantology Center and delivered to the laboratory for the analysis within an hour. Adult patients with clinically evident lymphoma with excision biopsy recommended at the National Research Oncology and Transplantology center were included in this study upon informed consent. Sample group contained 29 patients, among which there were 15 women and 14 men, median age 49.

Approval №9 dated 21.06.2021 was obtained from the Ethical Committee of National Research Oncology and Transplantology Center. Written informed consent was obtained from the patients. Flow cytometric analysis was performed on the same day of biopsy on live cells in suspension isolated from solid tissue.

Cell suspension preparation for immunostaining

One third of biopsy was disaggregated by either or both mechanical and manual methods providing sufficient cell recovery to perform flow cytometric analysis. The manual tissue disaggregation method was adapted by our laboratory with some minor changes [15]. The specimen was hold in place with forceps and multiply perforated using a 10-ml syringe with a 21-gauge needle. Approximately 10 ml of phosphate buffered saline (PBS) containing 0.1% sodium azide was repeatedly injected into the tissue until complete tissue dissociation. For better cell preservation no vortexing of biopsy tissue used at any time of obtaining cell suspension. Finally, cell suspension was filtered through a 40-μm cell strainer to get rid of any connective tissue that can occasionally appear. After centrifugation (500 g for 5 min) cell pellets were resuspended in sufficient amount of RPMI-1640 medium and collected into one tube, labelled MD (manual disaggregation).

Cells were treated with erythrocyte lysing solution containing ammonium chloride for 10 min, then washed twice with cold PBS (5 min). Mechanical disaggregation was used when poor cell yield came from manual disaggregation, or when (i) biopsy was very small; (ii) contained mainly connective tissue, and (iii) had a rigid structure. The number of cells were then counted using hemocytometer and cell viability accessed through staining with Trypan blue. Cell suspension has been then split into two parts: one stained immediately with fluorophore-conjugated antibodies, and another fixed using PFA, glyoxal, or glutaraldehyde and stored at 4°C for 1-5 days until further staining and analysis.

Flow cytometric analysis of live/unfixed cells

Panels for analysis were composed depending on patient's initial diagnosis and accounting for fluorophores compatibility. Immunostaining of cells included the following monoclonal antibodies from BD (Becton Dickinson, Franklin Lakes, NJ, USA), BioLegend (San Diego, California, USA) and Abcam (Cambridge, United Kingdom) companies against: common leukocyte antigen (CD45-APC-Cy7 clone 2D1), B-cell antigens (CD19-FITC clone HIB19, CD19-BV711 clone SJ25C1, CD20-BV421 clone 2H7, CD22-BV510 clone HIB22, CD23-APC clone EBVCS-5, Ig light chain κ-FITC clone TB28-2, Ig light chain λ-PE clone MHL-38, and CD10-BV421 clone HI10a), and T-cell antigens (CD3-PE-Cy7 clone SK7, CD5-PE clone UCHT2, CD5-BV510 clone HIT2, CD4-FITC clone OKT4, CD8-Alexa Fluor 647 clone SK1).

The same antibodies were used through the whole study. Two extended 8-color panels were used for all patients, namely general lymphoid panel and B-cell panel. General panel (including anti-CD45, anti-CD19, anti-CD3, anti-CD5, anti-CD10, anti-CD38, anti-CD4, and anti-CD8) was used to detect any most common aberrations in surface antigen expression among B- and T-lymphocytes in lymphomas. B-cell panel (including anti-CD45, anti-CD19, anti-CD20, anti-CD22, anti-CD23, as well as anti-kappa and anti-lambda light chains of immunoglobulins) was used to identify light chain restriction and changes in B-cell antigens expression if any. FC studies might require the inclusion of additional markers (extended

T-cell panel, intracellular staining panel, etc.) [16], which is out of scope of this study.

Cells before staining were washed three times with PBS and then the cell number was adjusted to roughly $1*10^6$ cells/100 µl of PBS containing 1% of BSA and incubated on ice for 30 min. For each panel we mixed the corresponding antibodies first and then added 100 µl of washed cell suspension. Staining was performed on ice for 20 minutes. After incubation, cells were rinsed in PBS and resuspended in 500 µl of fresh PBS ready for flow cytometric analysis.

Stained cells were analyzed using FACS Aria II cell sorter with FACS Diva software version 8.0 equipped with 405 nm (violet), 488 nm (blue), 561 nm (yellow-green), and 638 nm (red) lasers using the following channel/bandpass filter combinations: BV421 (450/50), BV510 (530/30), BV711 (730/45), FITC (530/30), PE (586/15), PE-Cy7 (780/60), Alexa Fluor 647 (660/20), APC-Cy7 (780/60). (Becton Dickinson, Franklin Lakes, NJ, USA). For each sample analyzed, 100 000 events were collected. The acquired data were analyzed using FlowJo software v10.2. Gating was carried out as following: A. Forward (FSC-Area) versus side scatter (SSC-Area); B. FSC-Height/FSC-Width (to exclude doublets); and C. SSC-Area/CD45 (to gate leukocytes).

Fixators

We used freshly (the same day) prepared 4% and 2% w/v paraformaldehyde (PFA), a 3% and 1% v/v glyoxal solutions, and 0.1% and 1% glutaraldehyde solutions. PFA solution was prepared by dissolving 1 g of powder PFA, 96% (Alfa Aesar, A11313) in 25 ml of PBS. Paraformaldehyde was slowly dissolved in PBS on a heated magnetic stirrer, then solution was cooled down, filtered, and stored at +4°C until use. Similarly, a 2% PFA solution was prepared by dissolving 0.5 g of stock powdered PFA in 25 ml PBS. Glyoxal solution was made by mixing the following components: 2.835 ml ddH_2O, 0.789 ml ethanol (96%), 0.313 ml glyoxal (40% stock solution, Sigma Aldrich), and 0.03 ml acetic acid. The pH of the solution was adjusted between 4 and 5 by adding dropwise about 0.12 ml of 1M NaOH. The solution was also stored at +4°C until use. A 1% glyoxal solution was prepared by diluting initially prepared 3% glyoxal with water. Glutaraldehyde in two concentrations (0.1% and 1%) was prepared by diluting the corresponding amount of stock glutaraldehyde (25% stock, AppliChem, A5252) in phosphate-buffer saline.

Cell fixation and flow cytometric analysis

The second tube of suspension cells was equally divided into tubes and washed once with PBS before fixation. The supernatant was then discarded and pellets were resuspended in the corresponding fixative. Primarily we employed 4% (final conc.) PFA, without washing and leaving tubes at +4°C for prolonged storage (several months). Prior to staining 200 µl of paraformaldehyde stored cells was washed twice with PBS (5 min, 300 g) and stained following the protocol as for live cells.

The second method was cell fixation in 4% and 2% PFA in PBS for 20 mins on ice. In this case, fixed cells were washed twice with PBS (400g, 5 min), the resuspended in sufficient amount of PBS, and left at +4°C.

The fixation with 3% and 1% v/v glyoxal solutions was performed on ice for 10 min, followed the double wash in PBS and leaving samples in PBS at +4°C.

The fixation with 0.1% and 1% glutaraldehyde solution was performed on ice for 20 min, followed the double wash in PBS and leaving samples in PBS at +4°C.

For staining fixed cells in suspension on day 1-5, 200 µl of suspension was washed once with PBS, and then stained with the cocktail of antibodies.

Data processing and analysis

The analysis of fixed cells by staining with antibodies was performed usually within 5 days after fixation; some samples were analyzed one week and one month after fixation (data not shown). Staining effectiveness of fixed specimens was assessed through the calculations of the stain index (SI), as well as comparison of the percentage of positive cells and MFI (median fluorescence intensity) of a given population between live/unfixed and fixed cells. For quantitative analysis, the stain index was calculated using the following equation: SI = (Median positive - Median negative)/ (2×rSD negative) as described elsewhere [17-20].

All bar graphs represented mean values, and all error bars denote the standard error of the mean. All statistical analysis was performed in GraphPad Prism 8.0 software (GraphPad, San Diego, CA, USA). Differences between means were compared using paired t-test, where the significance was identified by P-value: $p<0.05$.

Results

In the present study, we assessed the extent to which fixation before immunostaining affects the surface antigen expression of lymphocytes from lymph node biopsies by flow cytometry. The effect of different fixatives was analyzed through comparison of stain index (SI), MFI and percentages of positive cells between live and fixed specimens. The results show that cell fixation prior to immunostaining and subsequent cell storage results in

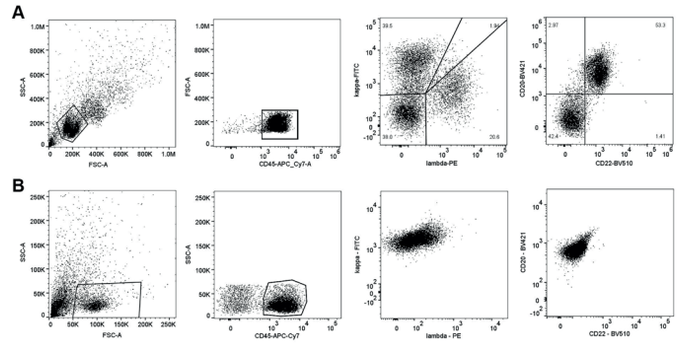

Figure 1 - (A) light scatter characteristics and results of immunophenotyping of live cells; (B) light scatter characteristics and results of immunephenotyping of cells stored in 4% PFA for a month.

a decrease in SI incompatible with the one demonstrated by live cells, yet to the different extent.

Prolonged storage (more than 24 h) of suspended cells from lymph nodes in 4% PFA did not provide satisfactory staining results. Fixation resulted in the loss of the majority of surface antigens (Figure 1). Leaving samples for one hour or overnight in PBS to get rid of excess PFA did not make any improvements in antigen expression. Moreover, staining with two or more fluorophore-conjugated antibodies resulted in high autofluorescence without possibility to differentiate between populations.

0.1% and 1% glutaraldehyde fixative resulted in changes in the light scatter and showed worse surface antigens preservation – lower SI (in the case of CD45) or sometimes inability to

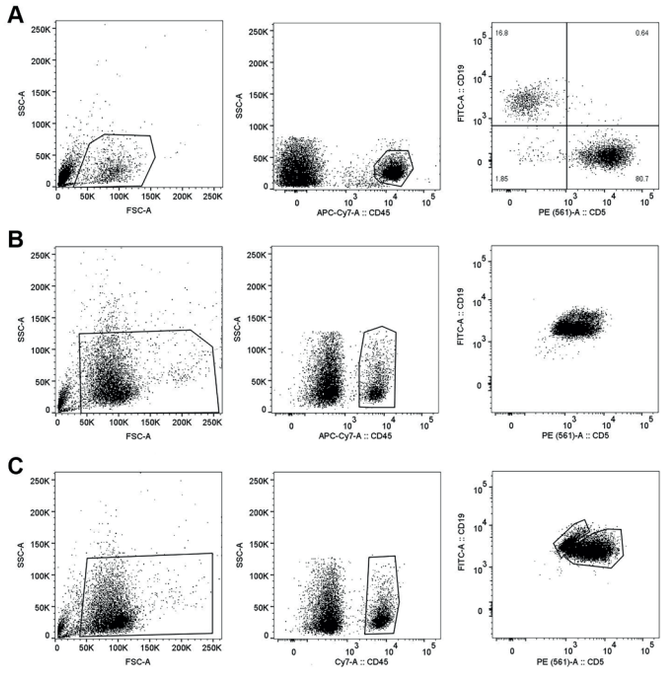

Figure 2 - The result of glutaraldehyde fixation. The upper line (A) shows light scatter characteristics and results of CD45, CD5 and CD19 immunostaining of live cells, the middle line (B) - after 1% glutaraldehyde fixation, and the bottom line (C) - after 0.1% glutaraldehyde fixation.

differentiate between positive and negative cell populations (CD19 and CD5) (Figure 2).

Thus, these two types of fixation procedures (leaving samples in 4% PFA, as well as glutaraldehyde at two concentrations) were excluded from the further study. We next used freshly prepared 2% and 4% PFA for cell fixation on ice for 20 minutes, washed off the fixative by PBS two-three times (5 min, 500 g) and left samples in PBS at 4ºC from 1 to 5 days before analysis. Similarly, we fixed samples with 1% and 3% glyoxal solution on ice for 10 minutes, washed off the fixative three times with PBS, and stored cells in PBS at 4ºC from 1 to 5 days.

The effect of fixation on light scatter parameters

Fixation of suspended lymphocytes caused significant changes in light scatter properties, resulting presumably in cell shrinkage and causing subsequent reduction of SSC and FSC values making gating more difficult.

Apparently, there is a loss of cell debris possessing the lowest light scattering values, where we can observe a single population of events with decreased SSC and FSC (Figure 3).

The decrease was apparent especially for SSC-A signal reflecting cell complexity and granularity. However, FSC-A was affected commensurately decreasing the cell size, which might be important in lymphoma evaluation and detection of large cells in case of large B-cell lymphomas. In fixed cells, the exclusion of cell debris is almost impossible, due to a single cell population observed in light scatter.

The least effect on light scatter properties of fixed cells was observed in samples left in 4% PFA without washing (Figure 3). However, this treatment dramatically decreased antigenicity of cells affecting staining with antibodies.

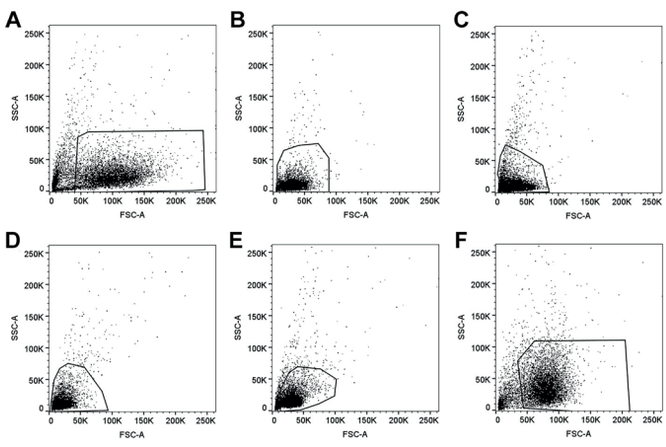

Figure 3 - Changes in light scatter properties of cell suspensions after different fixations. (A) – live cells; (B) – cells fixed with 4%-PFA, stored in PBS; (C) – cells fixed with 2%-PFA, stored in PBS; (D) – cells fixed with 1%-glyoxal, stored in PBS; (E) – cells fixed in 3%-glyoxal, stored in PBS; (F) – cell left in 4%-PFA for long-term storage.

Leukocyte common antigen (CD45)

The light scatter properties of cells derived from a lymph node biopsy is different from whole blood or PBMC and is often not as obvious for identifying lymphocytes. Thus, we employed the SSC-A/CD45 plot to isolate the lymphocyte population. The subsequent analysis of B- and T-cells was performed within the CD45 positive population only, and it was important to access the effect of fixation on this particular antigen (Figure 4).

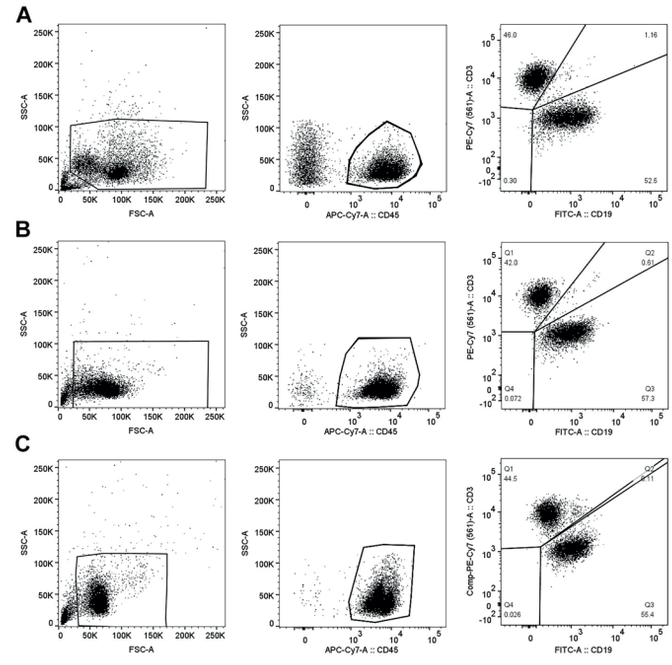

Figure 4 - Fixation with PFA and glyoxal show similar changes in antigen representation: (A) – light scatter and immunostaining (CD45/CD3/CD19) of live cells; (B) – cells, fixed in 4%-PFA, and stored in PBS before staining; (C) – cells, fixed in 3%-glyoxal, and stored in PBS before staining.

The percentage of CD45 positive cells as well as the SI values after fixation differed from live cells significantly for all treatments apart from the fixation with 3% glyoxal. Fixation resulted in an apparent increase in the percentage of CD45+ cells and decrease in SI. However, fixation did not pose any significant effect on MFI of CD45 positive cell population. On average, MFI was lower in fixed cells when compared to live cells (Figure 5). Generally, the side scatter characteristics of the lymphocytes gated through high CD45 expression and low side scatter were not markedly affected by fixation of cells in suspension.

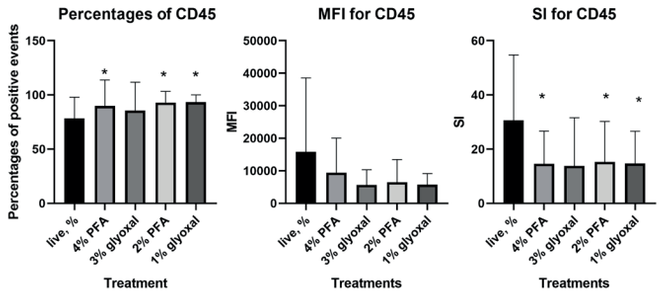

Figure 5 - The effect of different fixatives on percentage, MFI, and SI of CD45 positive cells. * - the difference from live cell staining is significant, p≤0.05.

B-cells specific antigens

The majority of lymphoma has B-cell origin, thus, it was important to analyze the effect of fixation on antigenicity of B-lymphocytes. In this study, we have assessed the effect of fixators on CD19 and CD20 antigens, as well as important in lymphoma evaluation analysis of kappa and lambda light chains of immunoglobulins.

The percentage of gated CD19-positive lymphocytes was not significantly altered with any of the variant procedures apart from 4% PFA (p=0.044). However, all types of fixation negatively affected SI and MFI of anti-CD19 stained cells (Figure 6).

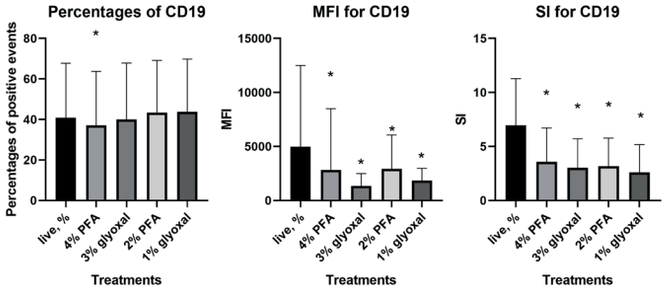

Figure 6 - The effect of different fixatives on percentage, MFI, and SI of CD19 positive cells. * - the difference from live cell staining is significant, p≤0.05.

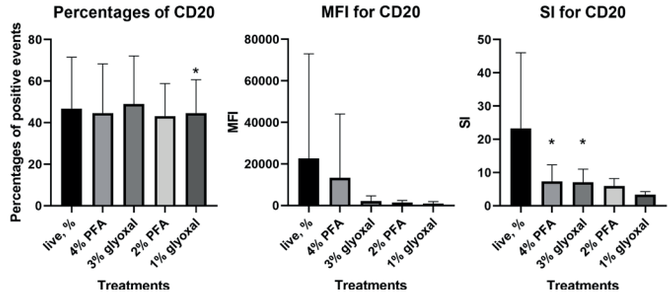

Figure 7 - The effect of different fixatives on percentage, MFI, and SI of CD20 positive cells, * - the difference from live cell staining is significant, p≤0.05.

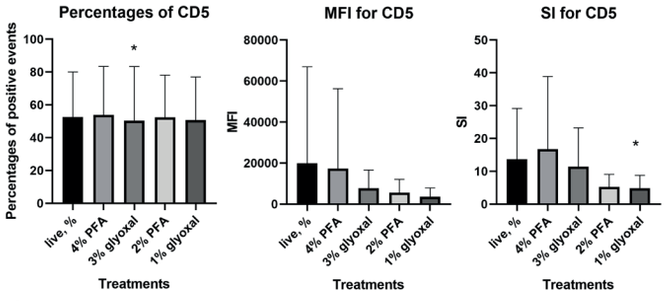

Figure 8 - The effect of different fixatives on percentage, MFI, and SI of CD5 positive cells, * - the difference from live cell staining is significant, p≤0.05.

B-cell specific CD19 antigen was better preserved in cells fixed in 4% PFA, washed and then stored in PBS at 4°C, when compared to continuous cell storage in the same fixative (data not shown). This was true for cells labelled with anti-CD19-FITC on either the next day post-fixation or 1 week after. Cells fixed with 4% PFA and stored in PBS for 1 month showed ten times decrease in SI values when compared to live cells. The result of fixation with 3% glyoxal was worse at preserving CD19 antigens over time when compared to 4% PFA.

Fixation caused changes in CD20 representation to a lesser extent when compared to other antigens. Nevertheless, we observed a decrease in SI values and in MFI, while the percentage of CD20 expressing cells in all samples was almost unaffected by fixation (Figure 7). It is important to mention that gating populations in fixed samples based on FSC/SSC is challenging and might be biased.

Evaluation of the expression of CD5 is important in lymphomas as it is a marker of normal T-cells and abnormal B-cells from CLL and mantle cell lymphoma. Based on the results obtained we can conclude that CD5 antigen is one of the least affected antigens, where no significant difference was found between live and fixed cells in any of the assessed parameters (Figure 8). However, similarly fixation caused gradual decrease in SI values for CD5 staining over time.

T-cells specific antigens

Flow cytometry plays an important role in evaluation of T-cell lymphoma. Thus, we assessed the expression of the main T-lymphocytic antigens (CD3, CD4 and CD8) by cells after fixation. There is only minor effect of fixation on antigenicity of T-cells, both the percentage of positively gated and MFI (Figure 9). Similarly as for all cell types analyzed in this study fixation negatively affected SI values of major T-cell antigens. CD3 was affected to a lesser extent (p=0.3), and CD4 and CD8 to a greater.

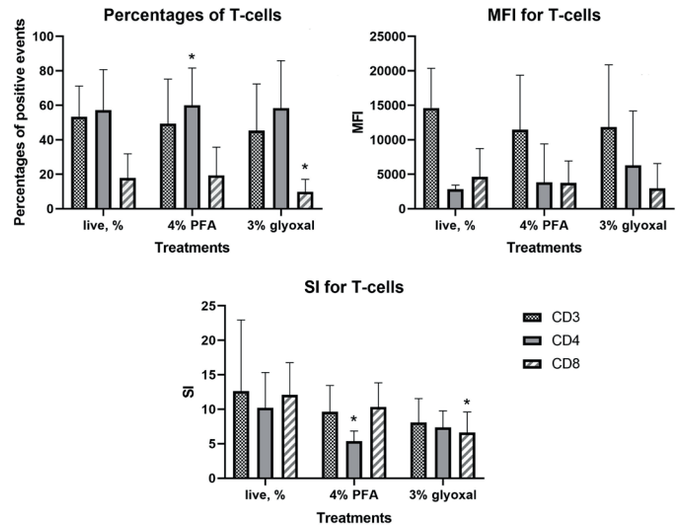

Figure 9 - The effect of different fixatives on percentage of positive cells, MFI, and SI for main T-cell antigens, * - the difference from live cell staining is significant, p≤0.05.

Discussion

We describe a dramatic effect of fixation on light scatter properties of suspended lymphocytes. Forward scatter/Side scatter-based dead cell discrimination is one of the possible and primary gating strategies applied in flow cytometry [21]. Light scattering of all cells after fixation became similar making it harder to discriminate lymphocytes from cell debris. The data

on the effect of fixation on light scatter properties of cells is in agreement with previously reported experiments [22,23]. Generally, fixation significantly affected cell distribution on SSC-A/FSC-A graph as well as the antibody binding efficiency for the majority of surface antigens [22,23]. Although, the data is variable, and fixation using 4% PFA before lysing red blood cells, might preserve whole blood light scattering profile and antigenicity of some leukocyte surface markers [24].

Despite the fact, that fixation is designed to stabilize biological samples close to the pattern demonstrated by live cells, and making further observations reliable, treating with the fixators cause cells to aggregate and shrink. Our results show that, fixation always leads to a substantial decrease in FSC-A values responsible for cell size. Cell morphology analyzed by atomic force microscopy and scanning ion conductance microscopy proofs that PFA (1-4%) fixation drastically change cell morphology through protein crosslinking when compared to live cells [25]. Recently glyoxal was described to be more efficient than PFA in preserving morphology of the attached cells and in cross-linking proteins [12]. However, we observed significant changes in light scatter properties of suspended cells subjected to glyoxal fixation.

Post-fixation procedure, where you first stain cell with fluorophores-conjugated antibodies and then fix them, is preferable by researchers as cell architecture change drastically during fixation, which might subsequently lead to false results [11,26]. PFA, when used prior staining, tend to interfere in the process of antibody-antigen complex formation by crosslinking with epitopes or their structural parts [26]. In previous work, stained lymphocytes fixed using 1% paraformaldehyde for 30 min following washing three times with HPSS and storing cells at 4°C retained its fluorescence properties for at least 2 weeks prior multicolor flow cytometry analysis [12]. Another study showed the possibility of one-week storage of cells fixed with 0.5-1% PFA and 0.05-0.1% glutaraldehyde prior to immunostaining of mouse T-lymphocytes [27]. On the contrary, fixation using 4% PFA is more effective for up to 10 days storage of mononuclear cells comparing to 1% PFA that only preserved cells for up to 3 days [28].

Cross-linkage formed during fixations with aldehydes partially explains loss of antigenicity or masking of surface epitopes, and subsequently decrease in the percentages of positively stained cells post-fixation [29]. However, our data suggests that alterations of cell surface caused by the action of fixators are very serious leading to the denaturation or even loss of surface epitopes available to antibodies.

Based on the previous research [6,30], the optimal concentration of PFA still needs to be identified, since generally used 4% PFA might not be the best working concentration for suspension cell fixation leading to permanent changes of surface antigens expression. However, in our studies reducing PFA concentration to 2% caused similar or even stronger reduction in SI values for some antigens. Overall, we did not find any substantial difference between fixation with 4% PFA or 2% PFA, and the same was true for two concentrations of glyoxal, 3% and 1%. Thus, in this study we mainly used 4% PFA and 3% glyoxal as fixators.

The fixation of leukocytes involving 2% PFA prior immunostaining, and without sample storage, considerably affects the consequent gating of T-lymphocytes, monocytes and basophils. This might lead to result falsifications and drawing a wrong conclusions, which is unacceptable for clinical samples [5]. It was also true for T-cell antigens CD4 and CD8 in our experiment, where fixation with both 4% PFA and 3% glyoxal resulted in a significant decrease in SI and the percentage of positive cells when compared to unfixed cells.

It turned out that washing cells after fixation and storing fixed cells in buffer is beneficial in both procedures of pre- and post-fixation [31]. Time of fixation might vary but generally, it does not exceed one hour [11,26]. In our experiments, the best results were achieved when fixation was performed for 20 min (with PFA) and 10 min (with glyoxal) on ice.

The feasibility of using glutaraldehyde even at low concentrations for the fixation of red blood cells is in doubt as it provides high autofluorescence and significantly reducing cell surface antigenicity [32]. Glutaraldehyde fixation at concentrations of 0.1% and 1% prior cell staining with antibodies did not show prominent results in antigen representation. Post staining fixation with glutaraldehyde resulted in high autofluorescence background, in accord with previous observations [33]. The same authors found that 1% PFA is the most appropriate fixator for stained cells, which can be stored for at least 1-week prior to analysis without significant changes in fluorescence. Another study performed on blood cell leukocytes (PBMC) and whole blood demonstrated the feasibility of fixation prior staining, however it resulted in a decrease in MFI (mean fluorescence intensities) for some antigens with little effect on percentages of positive cell [23].

We have noticed a decrease in positive cells number especially after fixation with glyoxal, where no cells were detected after 1-week storage at +4ºC. It has been found that long storage of fixed cells at +4ºC results in dramatic loss of positive cells when compared to storage in liquid nitrogen [34]. Prolonged storage of fixed cells by our method is in doubt as immunophenotyping can only be performed within 5 days without significantly compromising results. However, the analysis of some antigens might be of challenge. The fixation deteriorates immunostaining results by eradicating small aberrant populations and large cells [5,35]. The percentage of positive cells might increase or decrease depending on the clone and conjugate of the antibody used, which is not reflecting the real immune cell profile [5].

The expression of the common leukocyte antigen CD45 was stable after fixation of peripheral blood [4] or cryopreservation of suspension cells obtained from nodal biopsies [36], which has been also shown in our experiments. One study has shown long-term preservation of B-cell antigens when fixed chemically using a mixture of 2% formalin, enzyme inhibitors, glycine and gelatin [31]. This might suggest the usage of a mixture of fixators and other compounds for better antigen preservation. While, our experiments demonstrated dramatic changes in both SI and MFI of CD19 and CD20 antigens triggered by all types of fixation. This was supported by other study, where pre-fixation with PFA caused the reduction in staining intensity of CD19 in whole blood [24].

One of the problems arises with the fixation is a loss of negative populations and increased autofluorescence, which by use of the aldehyde fixators cannot be completely eliminated. Whole blood immunophenotyping is preferable with the use of fresh samples with no fixation of leukocytes, which reflects the true figure of cellular phenotype distribution [37]. Moreover, MFI tended to decrease by 10-30% for major leukocytes antigens after fixation with 1% paraformaldehyde [35]. Similarly, the study conducted on peripheral blood immunostaining demonstrated the worst preservation of MFI when blood samples were pre-fixed with both 1% and 4% PFA [30]. Our results on fixed lymphocytes isolated from fresh lymph node biopsies are in accord with these data.

Conclusion

The fixation of cells with 2% or 4% PFA for 20 min and then washing off the fixative and storing sample in PBS for up to 3 days partially retains cells antigenicity, although might cause the percentages changes while MFI and SI usually decreases. Other aldehyde fixatives give inappropriate results. The fixation with 4% PFA significantly increase the percentage of CD45 stained cells up to 95% or more. It also affects light scatter properties of cells. Fixation makes impossible to analyse large cells, which are important for detection of large cells lymphomas. Usually, the fixation causes slight increase in the number of CD19 positive B-cells, and slight reduction in the percentage of CD3 and CD5 positive T-cells. Some other B-cell antigens (CD10, CD22, CD23, and CD38) are strongly affected by fixation. Storing for more than 3 days results in the formation of the apparent CD19/CD5 double positive population, as well as the loss of CD10 and CD23 antigens, which is very important for lymphoma immunophenotyping. We recommend analyze fixed cells no later than three days after fixation.

Most papers describing chemical fixation of lymphoid cells in suspension were published in the last century, and used staining with only one-two antibodies available at that time. While in this study, we used antibody panels of eight antibodies when compared staining of live and fixed cells. This definitely complies with modern FC capabilities [16].

We approbated different concentrations of PFA (2%-4%) and glyoxal (1%-3%) aiming to retain compatible to live staining results for the most used lymphocytic antigens (CD45, CD19, CD20, CD3, CD5, CD4, CD8, kappa, lambda).

Even the best fixation affects representability of the staining to a lesser extend for antibodies CD45, CD19, CD20, CD3, and CD5, and to a greater extend for antibodies CD10, CD22, CD23, CD38, as well as antibodies against kappa and lambda light chains of immunoglobulins.

We tested the major clones of antibodies approved for lymphoma diagnostics. However, new monoclonal antibodies developed recently might give better results. Lymphomas are very heterogenic group of diseases, which falls into tens of separate clinical entities. Thus, comparison of staining live and post fixation samples might require more precise and individual assessment of each clinical case.

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