Evaluation of TransFix/EDTA CSF Sample Storage Tubes compared to alternative preservation methods

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Abstract

Many central nervous system (CNS) diseases such as inflammatory neurologic diseases, leukaemia, or autoimmune diseases, are associated with the presence of white blood cells in the cerebral spinal fluid (CSF). The detection of leukocyte subsets in CSF is of vital importance for the accurate diagnosis and treatment of such diseases. However, low cellularity and cell viability of leukocytes causes challenges when analysing CSF with flow cytometry, and it is recommended that native CSF samples are analysed within 1 hour of lumbar puncture (Kraan *et. al.* 2008, De Graaf *et al.* 2011, Korfel *et al.* 2016). This eliminates the possibility of multicentre studies and centralised diagnostics (Sedek *et al.* 2020) resulting in heterogeneity in interpretation of the results of clinical trials and difficulty in the homogenous management of affected patients (Del Principe *et al.* 2020). Various methods of preservation/stabilisation of CSF samples have been attempted but there is little data comparing their performances in preserving lymphoid and myeloid cells in CSF for flow cytometry assessment. In this study, mock CSF (MAS-CSF spiked with PBMCs) was stabilised with TransFix/EDTA, basal RPMI and complete RPMI, and compared to untreated mock CSF to determine the most optimal stabilisation method. Samples were tested by flow cytometry at different time points over 3 days. Data showed that the TransFix treated samples provided consistent cell recovery for at least 72 hours after collection. Conversely, untreated, basal RPMI and complete RPMI samples exhibited a dramatic reduction in cell events compared to TransFix at all time points, including 0 hours (75.5, 83.0 and 47.8% reduction, respectively) and 0.5 hours (56.1, 73.3 and 35.3% reduction, respectively). These data indicate that TransFix/ETDA CSF Sample Storage Tubes provide clear benefits over the use of untreated or RPMI stabilised samples and should be used for all CSF samples destined for flow cytometric analysis.

Introduction

Cerebral Spinal Fluid analysis for diagnosis of Central Nervous System Diseases

White Blood Cells (WBC) are not normal constituents of CSF and many diseases are associated with WBC infiltration in the CNS, including inflammatory neurologic diseases, acute and chronic leukaemia, myelodysplastic syndromes, lymphomas, myeloma, autoimmune diseases, immunodeficiencies and solid tumours (Campos *et al.* 2017). The diagnosis of CNS diseases by the accurate identification of leukocyte populations in CSF is therefore extremely important. Upon diagnosis, important therapeutic decisions are required, including the administration of intrathecal chemotherapy. Rapid, accurate, and available diagnostic tools are required to start treatment early and confirm the clearance of lymphoma cells post-treatment (Korfel *et al.* 2016). Improved diagnostics may also identify patients at very low risk of CNS relapse and could be offered less toxic CNS therapy (Levinsen *et al.* 2016).

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The analysis of lymphoid or myeloid infiltrated CSF samples for disease diagnosis is difficult. Low cellularity is an issue, and malignant cells may be present in samples with a normal or low absolute cell count (<5/µl of CSF). Therefore, a highly sensitivity detection technique is required. Cell viability is also a problem in CSF specimens. It has been hypothesized that an increase in pH in CSF after removal from the body, occurring via diffusion of CO_2 out of the sample, and the hypertonicity of CSF causing water and solutes to move from the extracellular into the intracellular compartments, may contribute to cell death (Steele *et al.* 1986, Cunniffe *et al.* 1996). Chow and Schmidley showed leukocyte loss between 15% and 62% after 2 hours, and only 39%-60% of leukocytes remained detectable after 24 hours (Chow *et al.* 1984). De Graaf *et al.* found that approximately 50% of leucocytes and leukocyte subsets were lost when counted by flow cytometric and microscopic methods after 1 hour (De Graaf *et al.* 2011, Journal of Neurology).

It is widely accepted that to alleviate the cell viability issues, native CSF samples must be processed immediately for analysis (Korfel *et al.* 2016), although this can be challenging, as De Graaf *et al.* 2011 (Journal of Neurology) commented, "*CSF samples may remain unattended at room temperature for considerable and unknown amounts of time: at a busy emergency room, during prolonged transportation towards the laboratory, and on the laboratory bench pending analysis*". Prolonged storage of any biological specimen without the use of a fixative results in surface marker degradation, increase of cellular debris, loss of cell viability, increase in unspecific antibody binding, and cellular autofluorescence (Diaz *et al.* 2006 and Harrison *et al*. 2019). The particularly rapid deterioration of cells in CSF samples has hampered the opportunity of prospective, multicentric clinical trials (Quijano *et al*. 2009), and centralised diagnosis (Sedek *et al*. 2020) as these samples cannot be batched for analysis.

Preservation/Stabilisation of CSF Specimens for Flow Cytometry

Attempts have been made to 'preserve' the cells within the CSF samples using buffers, which may prevent cell death due to an increase in pH (Cunniffe *et al.* 1996). Steele *et al*. compared leukocytes spiked into buffered saline with those spiked into acellular CSF and found that lymphocytes and monocytes were maintained for 4 hours at room temperature in the buffered saline, whereas in the CSF counts had dropped to 66% and 61% of the original cell counts for lymphocytes and monocytes (Steele *et al.* 1986). Dux *et al*. showed that phosphate-buffered saline with 1% bovine serum albumin and 5% foetal calf serum ('FC buffer') preserved cells with 90% of lymphocytes surviving at 90 minutes, compared to 65% in the native CSF (Dux *et al.* 1994).

More recently, the cell culture medium Roswell Park Memorial Institute (RPMI) 1640 has been used as a stabilisation medium (De Jongste *et al.* 2013, De Graaf *et al.* 2011 Journal of Neurology)). The work by De Graaf *et al.* compared leukocyte counts and their major subsets in native CSF to CSF samples stabilised using RPMI-1640 which was supplemented with 25 mM HEPES, 1 mM L-Glutamine, 2% penicillin/streptomycin, 5% heat-inactivated foetal bovine serum (FBS), and 2,500 IU heparin. Half of the leukocytes and all their subsets were lost after 1 hour, measured by flow cytometry and microscopy. However, the samples stabilised with supplemented RPMI had no significant decrease after 5 hours, except for granulocytes at 1 hour. Further studies have shown that the foetal calf serum additive may not be necessary and that un-supplemented RPMI 1640 may be sufficient, as demonstrated in a study by Grieg *et al.* 2014.

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While CSF samples stabilised using RPMI are usable in flow cytometry, the shelf life of RPMI-1640 once opened is limited to 1 month. Furthermore, it is sold in volumes of 100 ml minimum and, since only ~2 ml is added to CSF samples, aseptic dispensing techniques are required. Moreover, the preservation of CSF by RPMI-1640 beyond 5 hours is unclear (De Graaf *et al.* J. Neurol 2011), restricting the analysis of the samples to a tight time window.

TransFix solution was originally developed for stabilising blood for external quality assurance programs by UK NEQAS. TransFix provides a buffer which may prevent the increase in pH after CSF leaves the body (Cunniffe *et al.* 1996). It also contains an aliphatic aldehyde which fixates cells by crosslinking amino acid residues. Heavy metal salts and other components further stabilise the cells and reduce excessive autofluorescence caused by aliphatic aldehydes (Barnett *et al.* 1995).

Since the commercialisation of TransFix for CSF stabilisation, the use of TransFix/EDTA CSF Sample Storage Tubes has been widely endorsed by the clinical community, providing increased sensitivity of detecting disease and the significant benefits of transporting stabilised CSF samples for centralised diagnostic analysis and multicentre clinical studies (Kraan *et al.* 2008, Quijano *et al.* 2009, De Graaf *et al.* 2011 (Cytometry), Baraniskin *et. al* 2012, Subira *et al.* 2012, van Dongen JJ *et al.* 2012, de Jonste *et. al.* 2014, Johansson *et al.* 2014, Correia *et al.* 2015, Subira *et al.* 2015, Korfel *et. al.* 2016, Levinsen *et al.* 2016, Penvaler *et al.* 2017, Campos *et al.* 2017, Bento *et al.* 2018, Szánthó *et. al.* 2019, Del Prinicipe *et al.* 2020, Domingues *et al.* 2020, Sedek *et al.* 2020, Thastrup *et al.* 2020).

However, there are limited head-to-head studies comparing TransFix/EDTA CSF Sample Storage Tubes with alternative CSF stabilisation/preservation methods. In this study, mock CSF samples were stabilised with either TransFix/EDTA, basal RPMI or complete RPMI. Lymphocytes subsets and monocytes were analysed at various time points over a 72-hour time frame by flow cytometry. Event data and partial percentages were analysed at each time point to determine the stability performance of these methods.

Methods

Mock CSF (Thermo Fisher Scientific MAS-CSF (CSF-MP) spiked with PBMCs) was stabilised with TransFix/EDTA, basal RPMI (Thermo Fisher Scientific 11835030) and complete RPMI (Thermo Fisher Scientific 11835030 supplemented with 10 % Foetal Bovine Serum) and compared to untreated mock CSF (control). Samples were tested in triplicate using the Beckman Coulter Navios flow cytometer along with the SST antibody panel from Cytognos (CYT-SST) at time points 0, 0.5. 1.5, 3, 24, 48 and 72 hours. The acquisition threshold on the flow cytometer for this protocol was set to 5 minutes. Each replicate was 100 µl of sample at approximately $1x10^6$ PBMCs/ml, so a total of approximately 100,000 PBMCs per replicate should be present in each tube.

Recovery of monocytes (CD45+CD14+), T cell subsets (CD45+CD3+CD4+/CD8+), B cells (CD45+CD3-CD19+CD20+,) and NK cells (CD45+CD3-CD56+) from the stabilised samples at each time point were compared to day 0 untreated control. Gating strategies were devised following recommendations for the SST antibody panel.

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Results

Figure 1 shows the recovery of lymphocyte subsets and monocytes at each time point. The event numbers for each cell subpopulation in the TransFix/EDTA-treated sample remained relatively consistent for at least 72 hours whereas the event numbers in all 3 other samples were low, particularly within the first hour. Cell events also decreased sharply in non-TransFix treated samples after 24 hours.

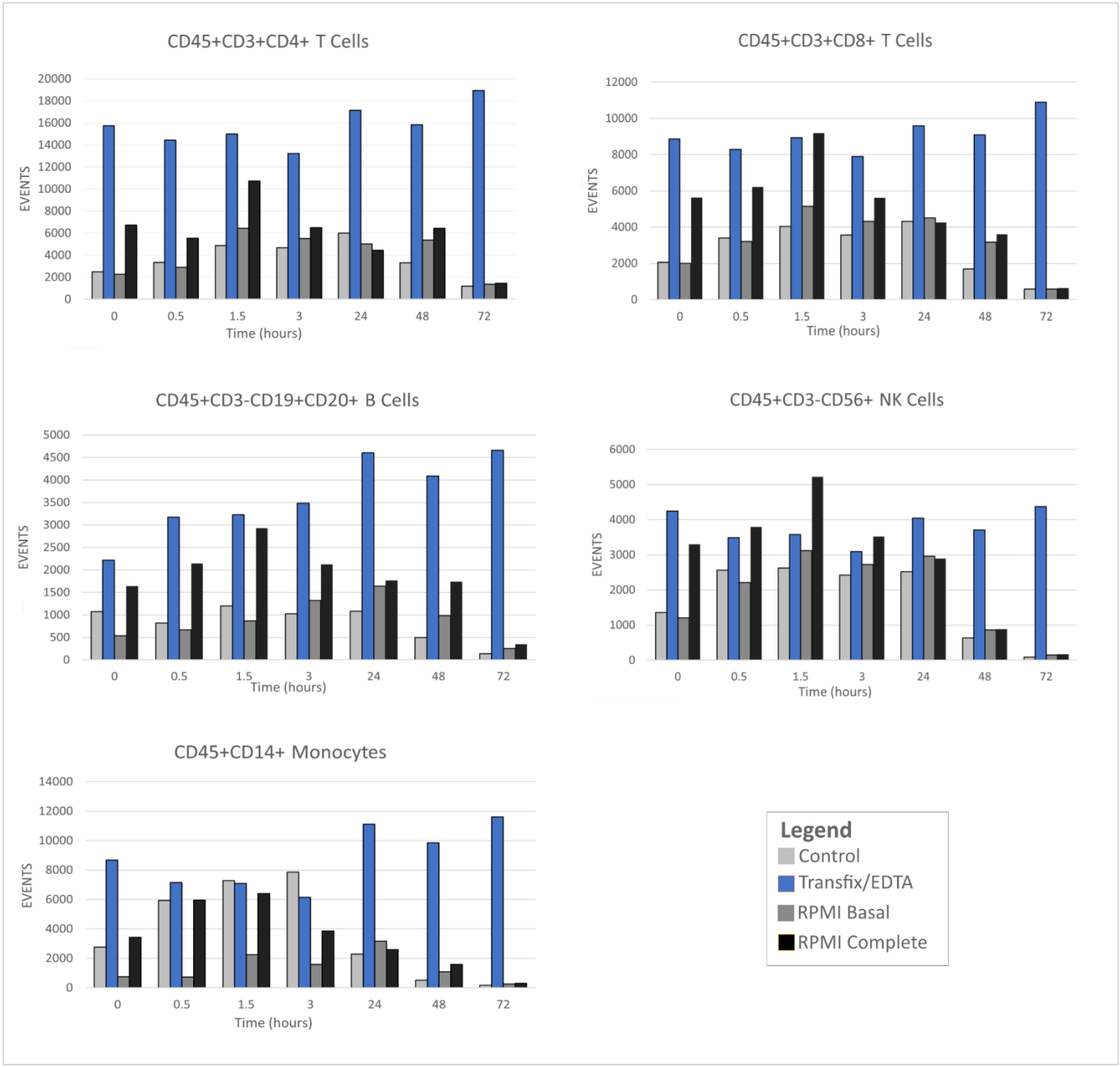

Figure 1. Average (n=3) number of classified events for leukocyte subpopulations of the TransFix/EDTA stabilised sample (blue) compared to the untreated sample (light grey) and samples treated with RPMI basal (dark grey), RPMI complete (black) over 3 days.

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Figure 2 shows the total classifiable cell events for each sample over the time frame. A higher number of events were recovered from the TransFix-treated sample at all time points. Conversely, untreated, basal RPMI and complete RPMI treated samples exhibited a dramatic reduction in cell counts compared to TransFix at all time points, including 0 hours (75.5, 83.0 and 47.8% reduction, respectively) and 0.5 hours (56.1, 73.3 and 35.3% reduction, respectively), see **Figure 3.**

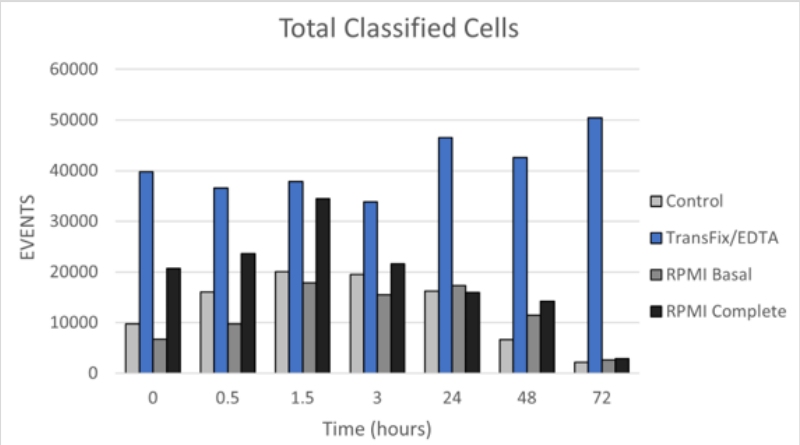

Figure 2. Total classified events for each sample type over 3 days. The data in this figure is the sum of the events from each test (n=3) for each subpopulation of cells.

Hours	Control	RPMI Basal	RPMI Complete
0	75.5%	83.0%	47.8%
0.5	56.1%	73.3%	35.3%
1.5	47.1%	52.9%	8.9%
3	42.3%	54.3%	36.2%
24	65.1%	62.8%	65.7%
48	84.4%	84.4%	66.5%
72	95.7%	94.8%	94.2%

Figure 3. Percentage reduction in cell events of untreated and RPMI treated samples compared to the TransFix treated sample.

The changes in the partial percentage of cell subpopulations over the course of three days are shown in **Figure 4**. The data shows that the partial percentages remained consistent over the time period for the TransFix-treated sample. The partial percentages of the untreated, basal RPMI and complete RPMI treated samples were variable.

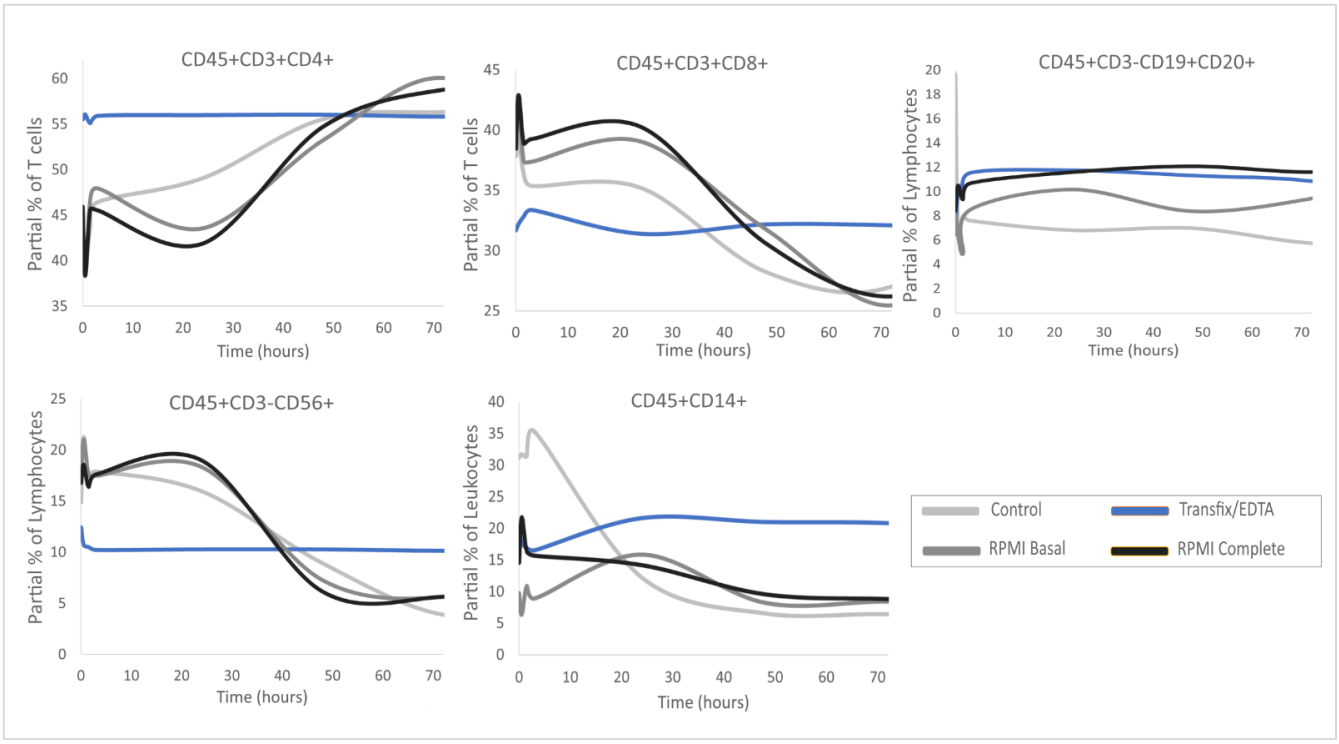

Figure 4. Average (n=3) partial percentages of leucocyte subpopulations of the TransFix/EDTA stabilised sample (blue) compared to the untreated sample (light grey) and samples treated with RPMI basal (dark grey), RPMI complete (black) over 3 days.

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Figure 5 shows example side scatter vs CD45+ dot plots for the untreated control, complete RPMI and TransFix-treated samples at 0, 24 and 72 hours (data for 0.5, 1.5, 3 and 48 hours not shown). The plots show that the density of events decreased with time in the control and RPMI-treated samples which is consistent with the findings in Figure 1 and 2. The density of the populations remained consistent for the TransFix treated sample and there was good separation of monocytes and lymphocyte populations.

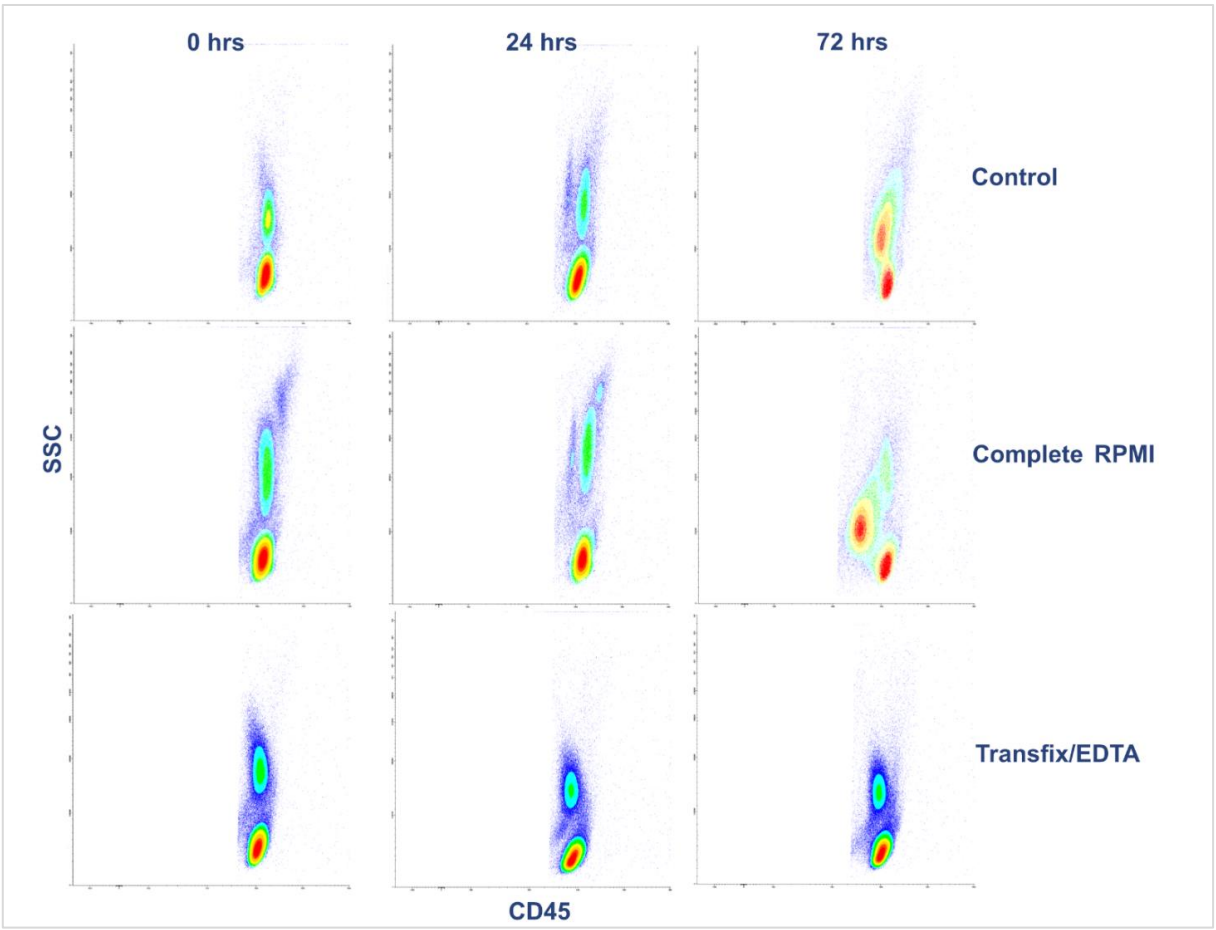

Figure 5. Side scatter vs CD45+ dot plots for the untreated (control), RPMI Complete and TransFix/EDTA treated samples. Basal RPMI is not shown as it's comparable to the control.

Discussion

The results clearly show the benefits of TransFix/EDTA treatment of mock CSF as indicated by the consistent recovery of total classifiable cell events and subpopulations for 72 hours (see Fig 1-4). Although the recovery of cells from the complete RPMI sample was better than the untreated and basal RPMI treated mock CSF samples, which supports the findings by De Graaf *et al.* J. Neurol 2011, the number of PBMCs recovered in the TransFix treated sample was consistently higher for CD4+ T cells and B cells at all time points. The untreated, basal RPMI and complete RPMI treated samples all appear to suffer immediate and substantial cell loss between 0-0.5 hours. It could be argued that using complete RPMI provided comparable results to TransFix at 1.5 hours for most cell types bar CD4+ T cells (see Fig 1). Complete RPMI is designed to support cells *in vitro* so an increase in recovered events after the initial shock is expected. However, this was not maintained and thus offers an extremely small analysis time-frame for analysing complete RPMI treated samples. It is important to note that while 100,000 PBMCs

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should be present in each sample, samples were not acquired in their entirety (acquisition time 5 min/replicate) and 2 washing steps were carried out during sample preparation. Therefore, full recovery would not be expected. Nevertheless, these data show how Transfix outperformed RPMI-treated and untreated samples throughout the course of the study and provides a reliable stabilisation method.

These findings are similar to published findings by De Jongste *et al.* 2014 who found that lymphocyte numbers in Transfix-stabilised CSF were higher than native CSF and comparable to complete RPMI after 30 minutes. However, our data indicates a similar finding at 1.5 hours as opposed to 30 minutes, where higher cell numbers were recovered in the TransFix treated sample. Moreover, De Jongste *et. al.* also showed that TransFix significantly enhanced the detection of leptomeningeal malignancies compared to both native CSF and CSF with serum-containing medium when samples were stored for 18 hours. Although our study has been performed using mock CSF, the MAS-CSF diluent is designed to be an artificial CSF control solution for CSF chemical analyses and thus we consider it a suitable surrogate for clinical CSF samples. Conducting such a study with multiple time points in quick succession in a clinical setting could be challenging to conduct logistically and ethically.

The dot plots in Figure 4 also confirm the ability of TransFix to maintain a clear separation between monocytes and lymphocytes over a 72 hour window. Despite some decrease in side scatter seen in the plots in Figure 4, the populations stabilised with TransFix were easy to gate and the fluorescence intensity of surface antigens was adequate. This data corroborates the findings of Del Principe *et al.* and De Jongste *et al.,* who showed that TransFix does not interfere with identification of cellular subsets or malignant cells. However, it is recommended that antibody conjugates are validated in association with TransFix/EDTA CSF Sample Storage Tubes prior to use.

Although it is widely accepted that native CSF samples should be processed within 1 hour of lumbar puncture (Kraan *et. al.* 2008, De Graaf *et al.* 2011, Korfel *et al.* 2016), we have shown in this study that immediate and significant cell loss may occur even if CSF samples are processed within the 1 hour purported 'acceptable' time frame. Moreover, even samples treated with complete RPMI provided poor results within 0-0.5 hours. This demonstrates the necessity of using TransFix/EDTA CSF Sample Storage Tubes for all CSF samples, regardless of potential analysis time frames. The addition of CSF samples to TransFix/EDTA CSF Sample Storage Tubes after lumbar puncture is a simple and quick process which can be performed by clinic staff. Conversely, transport of native CSF samples to the laboratory and flow cytometric processing within 1 hour is a complex process requiring skilled laboratory personnel. Moreover, TransFix/EDTA CSF Sample Storage Tubes provide the opportunity for the transportation of samples for centralised diagnosis or multicentre studies (Quijano *et al.* 2009, Levinsen *et al.* 2016, Thastrup *et al.* 2020, Sedek *et al.* 2020).

Conclusions

TransFix/EDTA CSF Sample Storage Tubes provide a superior standardised and flexible stabilisation method for end-users in the clinic compared to other preservation methods. The tubes are ready to use, have a long shelf life of 12 months, and stabilise CSF samples for up to 3 days. At the time of publication, TransFix/EDTA CSF Sample Storage Tubes are the only CE/IVD product available for the stabilisation of leukocytes in CSF.

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