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RESEARCH ARTICLE

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A field method for cryopreservation of whole blood from a finger prick for later analysis with flow cytometry

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Abstract

Objectives: Flow cytometry is a powerful tool for investigating immune function, allowing for the quantification of leukocytes by subtype. Yet it has not been used extensively for field work due to perishable reagents and the need for immediate analysis of samples. To make flow cytometry more accessible, we devise and evaluate a field protocol for freezing capillary blood.

Materials and Methods: We collected finger prick blood samples from 110 volunteers, age 18 to 42. Blood samples were analyzed immediately for 18 cell surface markers. Aliquots of whole blood were frozen in the vapor phase of a liquid nitrogen tank with 10% dimethyl sulfoxide in medium. Samples were analyzed on a Guava EasyCyte HT flow cytometer after 2, 4, or 14 weeks.

Results: Major lymphocyte fractions in frozen samples were correlated with fresh values (T-cells: r = 0.82; Natural Killer [NK] cells: r = 0.64; CD4: r = 0.67; CD8: r = 0.82; Naïve CD4: r = 0.73, Naïve CD8: r = 0.71; B-cells: r = 0.73; all $p < 0.001$), and mean values were similar to those from fresh samples. However, correlations for smaller subsets of CD4 and B cells were generally poor. Some differences resulted from changes in non-specific binding for some antibody-conjugate pairs. Cryopreservation also resulted in a reduction in granulocytes more than lymphocytes.

Discussion: Our results suggest that antibody/fluorochrome combinations should be validated before use on frozen samples, and that functional changes in cells may affect some cell markers. However, this simple freezing protocol utilizing finger pricks, whole blood, and a liquid nitrogen shipping tank is viable for obtaining samples for flow cytometry under field conditions.

KEYWORDS
cryopreservation, field methods, flow cytometry, immune function

1 | INTRODUCTION

Flow cytometry is a tool widely used in laboratory and clinical settings for counting and phenotyping white blood cells. When employed in evolutionary and anthropological studies, it is a powerful tool for examining environmental, social, and genetic impacts on immune function; for example, flow cytometry has been to assess immunological differences between agriculturalists and hunter gathers (Harrison et al., 2019), to examine the immune profiles of horticulturalists exposed to high levels of parasites and pathogens (Blackwell et al., 2016), tradeoffs between these immune shifts and growth in children (Garcia et al., 2020), and to examine the effects of social rank manipulation on the immunity of captive macaques (Snyder-Mackler et al., 2016).

However, flow cytometry still receives relatively limited use in human biology, field primatology, and related disciplines. In large part,

this may be due to the difficulty of using flow cytometry in field settings. Flow cytometry works by labeling cells with antibodies against cell-surface markers of interest, for example, the T cell co-receptor CD3, which is present on the surface of all T cells but absent from other cells. Antibodies against markers of interest are conjugated to fluorescent dyes which produce a color when stimulated with a laser. To count cells with colored labels, cells are diluted and passed through a thin tube, such that they pass by detectors one at a time, allowing the color labels on each cell to be measured individually.

In practice, flow cytometry is relatively straight-forward, but has many requirements that make it difficult to use in field settings. The first is the flow cytometer itself. Currently small, relatively light-weight flow cytometers are available, such as the Accuri C6 (BD Biosciences) and the Guava easyCyte line of cytometers (formerly from Millipore, but currently sold by Luminex). These are relatively light (~30 lbs for the Accuri), but must be packaged and moved carefully, making transport to remote field sites possible, but cumbersome and somewhat risky. One of us (ADB) had the experience of transporting an Accuri C6 to the Tsimane Health and Life History Project (THLHP) lab in San Borja, Boliva, a trip that required trusting airlines with an expensive piece of equipment, negotiating with customs to avoid paying import fees, and finally a harrowing taxi ride down Yungas Road (a.k.a. "Death Road") with the Accuri loosely strapped to the roof. Although this trip was a success, a further risk of this type of transport is that any of these actions could have knocked the lasers out of alignment, requiring professional servicing.

Once the flow cytometer is in the field, two other barriers create difficulties. The first is the need for a clean workspace with a reliable power source. The second is the need for reagents and a refrigerator in which to keep them. The THLHP solved these problems by maintaining a laboratory in San Borja, Bolivia, and transporting participants to the laboratory site for visits with project physicians (Gurven et al., 2017). For many field projects, this kind of infrastructure is not available.

An alternative to analyzing blood samples immediately after collection is to freeze them for later analysis, which negates the need to own or transport a flow cytometer since samples can be run on a flow cytometer located at the home laboratory or a core facility. However, the freezing of blood samples presents at least two complications for flow cytometry. Firstly, freezing can cause cells to lyse. If all cells have an equal propensity to lyse when frozen, then freezing will affect absolute cell counts, but not relative cell fractions. However, because different leukocytes are different sizes and may differ in resilience, there is a possibility for freezing to alter fractional leukocyte percentages. Secondly, freezing represents a stressor for immune cells, both due to the freezing itself as well as to the exposure to cryopreservatives and novel cell media. Immune cells may respond to this stressor as they would to any other, by altering functional responses, including changing the concentration of cell surface proteins that would otherwise be used as markers. This may be particularly true for cell surface proteins known to be labile, and which are used as markers of cellular activation.

There is nothing particularly novel about the cryopreservation of white blood cells for later flow cytometry. Numerous papers validating the use of freezing have been published, showing that freezing does not alter the major mononuclear cell proportions (helper T cells, cytotoxic T cells, B cells, natural killer cells, and monocytes) (Costantini et al., 2003; Fiebig et al., 1997; Hayes et al., 2002; Hviid et al., 1993; Ichino & Ishikawa, 1985; Jones et al., 1986; Kreher et al., 2003; Sleasman & Leon, 1997; Tollerud et al., 1991; Weinberg et al., 2009). However, freezing may alter some subsets, such a regulatory T cells (Elkord, 2009; but see Van Hemelen et al., 2010), and alters the ability to use some markers, such as CD62L (Costantini et al., 2003; Weinberg et al., 2009). Moreover, a number of studies report that freezing alters cellular function and response to stimulation (Best et al., 2007; Costantini et al., 2003; Ichino & Ishikawa, 1985; Kreher et al., 2003; Owen et al., 2007; Weinberg et al., 2000) though correlations between fresh and frozen responses are frequently maintained (Disis et al., 2006; Kreher et al., 2003; Weinberg et al., 2000).

In the field, freezing presents additional complications not present in a laboratory setting. To freeze cells without lysing, a cryoprotectant (typically 10% dimethyl sulfoxide; DMSO) that limits ice crystal formation is usually added to the samples. More important than this, however, is the rate at which the temperature is lowered. In general, a slower rate of freezing results in less ice crystal formation. The two most common methods for obtaining a slow rate of freezing are to use a programmable freezer, which lowers the temperature at a programmed rate (Hayes et al., 2002; Ichino & Ishikawa, 1985; Sleasman & Leon, 1997; Van Hemelen et al., 2010), or to use a freezing container (such as a Nalgene Mr. Frosty) which provides a controlled amount of insulation and when placed in a −80°C freezer produces a relatively fixed freezing rate (Best et al., 2007; Costantini et al., 2003; Disis et al., 2006; Kreher et al., 2003; Lavoie & Grasman, 2005; Stevens et al., 2007; Weinberg et al., 2000, 2009).

Most of these papers also introduce an additional step by isolating and freezing only peripheral blood mononuclear cells (PBMCs). The isolation of PBMCs removes granulocytes and red blood cells which may contribute debris to flow cytometric analysis and require the collection of additional events (particles detected by flow cytometry) to acquire the desired number of lymphocytes. The isolation of PBMCs is not particularly complicated, but does require the presence of Ficoll reagents, access to a centrifuge, and pipetting instruments. In a field setting the isolation of PBMCs also adds additional opportunities for contamination and error due to the lack of a controlled environment.

The use of controlled freezing and isolation of PBMCs are common practice in cryopreservation for flow cytometry, yet several papers suggest that simpler protocols may work nearly as well. First, as an alternative to isolating PBMCs, several studies have utilized whole blood (Cheng et al., 2001; Fiebig et al., 1997; Hayes et al., 2002; Pinto et al., 2005; Stevens et al., 2007). These studies all show that lymphocytes in frozen whole blood retain good viability and high correlations with analyses on fresh blood. Second, in the absence of electricity, the next best option for freezing is often to use

a liquid nitrogen sample tank. Liquid nitrogen may be difficult to acquire in some field settings, yet when used in an appropriate tank can keep samples frozen at between −140°C in the vapor phase and −196°C in the liquid phase for several months without a refill. Because of the lower temperature, liquid nitrogen presents a challenge for slowly freezing cells. Samples dropped directly into the liquid phase are likely to experience near universal lysing (as our own experiments with this approach confirm). However, in a liquid nitrogen freezer, the liquid usually fills only a few inches at the bottom of the tank. Above this lies the vapor phase (cold air, nitrogen, water vapor), which is warmer and less conductive. The handful of studies that utilize liquid nitrogen tanks freeze samples in the vapor phase (Jones et al., 1986). Hviid et al (Hviid et al., 1993) go so far as to engineer a device to slowly lower samples down into this phase at a controlled rate, since the vapor phase is typically warmer toward the top and cooler at the bottom.

Here, we take advantage of these two simplifications to devise and validate a protocol for the collection and cryopreservation of whole blood in a field setting. To further simplify the protocol, we use blood collected from a finger prick, rather than a venous draw. Typically flow cytometry is performed on venous blood, yet in truth this usually produces far more sample than is needed, in many circumstances is less comfortable for participants, and requires the collaboration of a trained phlebotomist. Major lymphocyte fractions in capillary blood are highly correlated with fractions in venous blood (Sitoe et al., 2011; Srisala et al., 2019). By optimizing our protocol for small volumes, we also make storage and transport easier. In lieu of a device for lowering samples into vapor, we use a vapor shipping tank for freezing and a liquid nitrogen tank for storage. The vapor shipper is a liquid nitrogen tank which has an absorbent material as a liner. The tank is filled with liquid nitrogen which absorbs into the material. Any excess liquid nitrogen is then poured off, leaving a tank that is purely vapor phase. In our initial experiments we found that the vapor shipper provided more consistent results than utilizing the vapor phase in a liquid nitrogen tank. An additional benefit of the vapor shipper is that it can be shipped or taken on an airplane once samples are collected, given the absence of any liquid nitrogen which could spill.

We validate this procedure by comparing flow cytometry results for 18 different antibodies on paired fresh and frozen samples from 110 participants. Our objective here is to both validate the field protocol and examine which surface markers are robust against cryopreservation, and which might be altered by the process. In general, we find that our procedure works well for common surface markers, while some other markers may be more problematic.

2 | METHODS

2.1 | Participants

Participants were 121 undergraduate student volunteers who were each paid $10 for their participation. Of these, we obtained sufficient blood from the finger prick to analyze both a fresh and a frozen sample for 110. For this sample, age was 18.1–42.3 years (mean 22.2). 72.5% of the participants were female. The study was approved by the Institutional Review Board at the University of California, Santa Barbara and all participants provided written informed consent for the study.

2.2 | Blood collection

Participants visited the UCSB Ecoimmunology Laboratory in the morning (Mean 9:34 am, range 8:00 am–11:08 am). A finger prick was used to collect ∼500 μl capillary blood in a heparin coated capillary blood tube. Total leukocytes and a two-part differential (lymphocytes/monocytes and granulocytes) were measured immediately with a QBC Autoread Plus (QBC Diagnostics Inc.). Samples were prepared for cryopreservation or analysis within 3 hours of collection (mean 1.07 h). For many flow cytometric analyses whole blood is considered stable for 24 h or more (Davis et al., 2013; Diks et al., 2019), however, we verified that time to analysis was not associated with significant alterations in cell type percentages (see analysis code).

2.3 | Cryopreservation

Prior to collecting samples, we prepared a liquid nitrogen shipping tank by filling it with liquid nitrogen, waiting for the liquid nitrogen to absorb into the shipping sponge, and then draining any remaining liquid. We also prepared 100 μl aliquots of a mixture of 20% DMSO and 80% filter sterilized RPMI 1640 medium and kept these stored in the liquid nitrogen tank.

Prior to collecting blood, we thawed DMSO/RPMI aliquots and allowed them to warm to just below room temperature. For freezing, we mixed 100 μl of blood with 100 μl DMSO/RPMI. Blood and medium were mixed by tapping or very gentle vertexing.

Within 5 min we placed the mixture in a cane in the drained shipping tank, such that the sample was held in the vapor phase. After 6–24 h we transferred the sample from the shipping tank to the liquid phase in another liquid nitrogen tank for long term storage.

2.4 | Thawing

Prior to analysis, frozen samples were removed from the liquid nitrogen tank and placed in a 37°C water bath to thaw. Samples were checked every few minutes to see if they were still frozen and moved to the next step as soon as they were thawed. As soon as the sample thawed, we added ∼1 ml of RPMI which had been warmed to 37°C to each tube. Samples were then centrifuged at 400×*g* for 7 min. We aspirated the supernatant, then resuspended the cells in 100 μl of RPMI (matching the original blood volume). The cells were then left to rest for ∼30 min in the 37°C water bath before assaying.

2.5 | Flow cytometry

Flow cytometry staining antibodies were pre-mixed in staining sets (Table 1) with 0.5 μl of antibody per sample to be measured and enough Flow Cytometry Staining Buffer (FCSB, eBiosciences, Inc.) to reach a final volume of 10 μl per sample. Ten microliters of fresh blood or 20 μl of frozen cells in RPMI were mixed with the 10 μl antibody mix in a 96-well conical tray. Cell/antibody mixes were incubated for 30 minutes at 4°C, then mixed with 200 μl Fix/Lyse Solution (eBioscience, Inc.) and incubated for an addition 30 min at 4°C. Following incubation, cells were pelleted at 200 x g, the supernatant was aspirated, and the cells were resuspended in 150 μl FCSB for data collection on a Guava EasyCyte 6HT 2 L (MilliporeSigma) flow cytometer using InCyte 2.7 software. For cell counts, lymphocyte and granulocyte gates were first established using forward and side scatter. Cells within the lymphocyte gate were then gated further based on florescence.

Cell viability after freezing and thawing was assayed using Guava ViaCount Reagent (MilliporeSigma). Following the manufacturer's directions, 10 μl of resuspended cells were mixed with 190 μl of ViaCount reagent. Cells were gated in the ViaCount Software to obtain a viable cell proportion. For a second subset, we used Fixable Viability Dye eFluor 660 (eBioscience, Inc.) combined with antibodies listed in Table 1 to assess viability within subsets of leukocytes.

2.6 | Analysis

Lymphocyte subset percentages from each batch were exported from InCyte as csv files and combined in R 4.0.3 (https://cran.r-project.org/). Mixed models were fit with `lmer` in the `nlme` package (Pinheiro et al., 2016), with variance components estimated using `get_variance` from the `insight` package (Lüdecke et al., 2019). Pearson's correlations and Bland–Altman plots (Altman & Bland, 1983; Vesna, 2009) were used to evaluate correspondence between fresh and frozen samples. The flow cytometry data file and analysis scripts at the time of publication are available at https://doi.org/10.5281/zenodo.4421667 and on Github at https://github.com/adblackwell/flowcytometryfreezing.

3 | RESULTS

3.1 | Sample

Of the 121 participants initially recruited, we obtained sufficient blood sample from the finger prick to complete flow cytometry for both a fresh and at least one frozen sample for 110. Of these, 71 were frozen and analyzed after approximately 2 weeks (mean 14 days, range 12–15 days). From this set, 39 had a second frozen sample which was analyzed 4 weeks after collection (mean 28 days, range 21–29 days). A separate set of 39 samples were analyzed only at baseline and after 14 weeks in liquid nitrogen (mean 99 days, range 85–109 days).

3.2 | Identification of lymphocyte subsets with flow cytometry

Because our assay used whole blood rather than isolated PBMCs, we first assessed the preservation of the major granulocyte and lymphocyte subsets (Figure 1). Overall, freezing modestly altered the forward and side scatter characteristics of the recovered cells, particularly for granulocytes (Figure 1a). However, the granulocyte and lymphocyte clusters were still easily identified and gated. In cryopreserved samples, granulocytes were significantly reduced relative to lymphocytes (Figure 1b,c), with lymphocytes representing an average of 37% of cells in the fresh samples (compared to 34% from the QBC) but representing 52% in the frozen samples. On an individual level, lymphocyte percentages estimated by flow cytometry were significantly

TABLE 1 Antibodies used for flow cytometry

	Color channel			
Set name	**Green; FITC**	**Yellow; PE**	**Red; PerCP-Cy5.5**	**Red2; APC/eFlour**
1. Basic	CD3 (OKT3)[a]	CD16 (CB16)[b] CD56 (MY31)[b]	CD45 (HI30)[a]	CD19 (HIB19)[a]
2. T-cell	CD3 (OKT3)[a]	CD4 (RPA-T4)[a]	CD45 (HI30)[a]	CD8 (RPA-T8)[a]
3. Naïve CD8	CD45RA (HI100)[a]	CD57 (TB01)[b]	CD8 (RPA-T8)[a]	CD28 (CD28.2)[a]
4. Naïve CD4	CD45RA (HI100)[a]	CD57 (TB01)[b]	CD4 (RPA-T4)[a]	CD28 (CD28.2)[a]
5. Th1/2/17	CD196 (CCR6)[b]	CD183 (CXCR3)[b]	CD4 (RPA-T4)[a]	CD294[c] (CRTH2)[b]
6. Treg	CD45RA (HI100)[a]	CD25 (BC96)[a]	CD4 (RPA-T4)[a]	CD127 (R34-34)[a]
7. NK/NKT	CD3 (OKT3)[a]	CD56 (MY31)[b]	CD8 (RPA-T8)[a]	CD16 (CB16)[b]
8. B cell	CD27 (O323)[a]	IgD (IA6-2)[b]	CD19 (HIB19)[a]	CD20 (2H7)[a]

Note: Antibody clone is shown in parentheses.
[a]Tonbo Biosciences, Inc.
[b]eBiosciences, Inc.
[c]eFluor conjugate instead of APC.

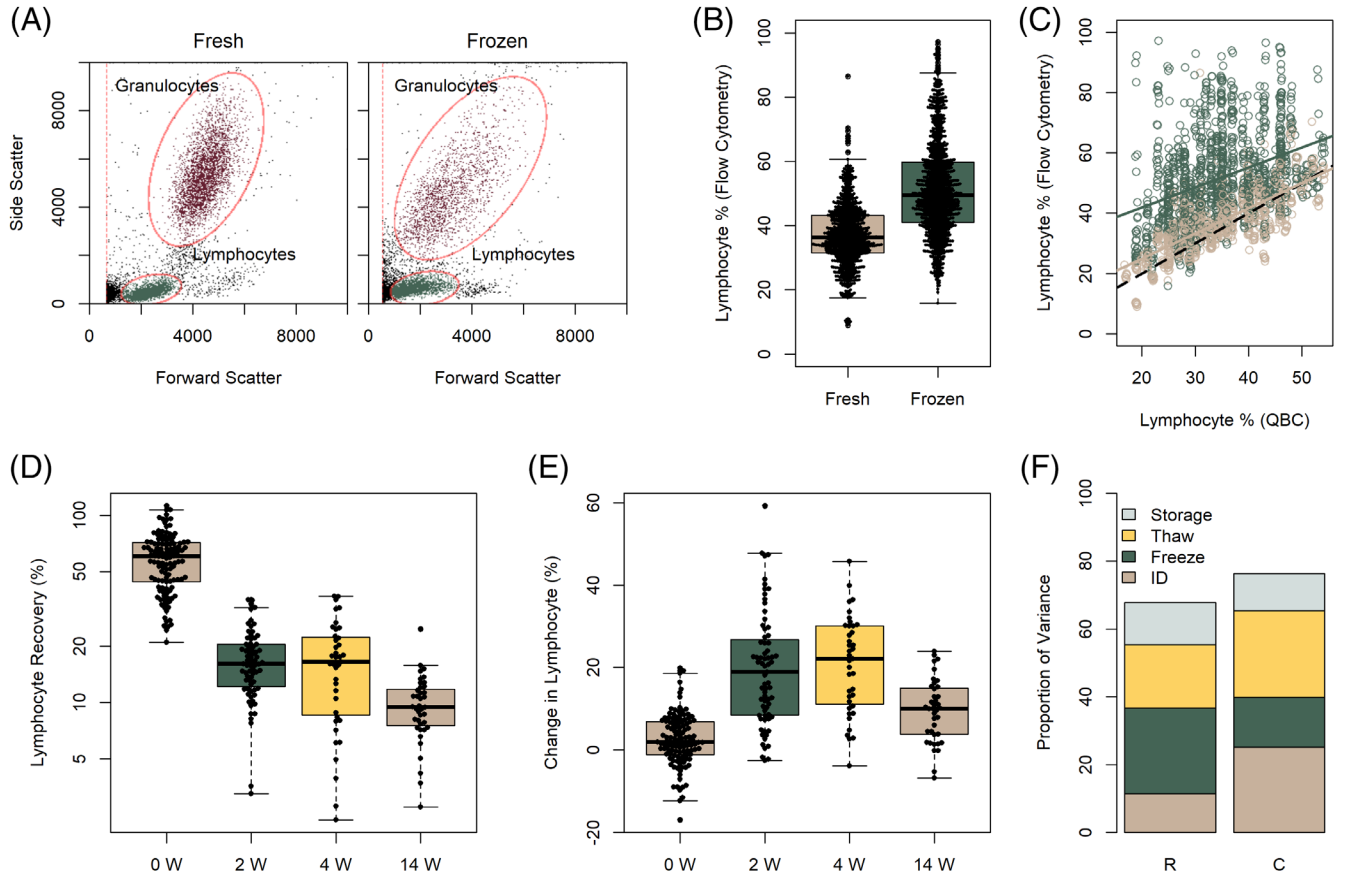

FIGURE 1 Recovery of lymphocytes and granulocytes in frozen samples. (a) Location of the granulocyte and lymphocyte gates in a sample while fresh and after cryopreservation. (b) Proportion of cells in the lymphocyte gate in fresh and frozen samples. (c) Lymphocyte percentage as measured with the QBC versus in fresh (beige) and frozen (green) samples. Dashed line shows the expected 1:1, tan line the fit for fresh samples, green line for frozen. (d) Estimated lymphocyte concentration after correcting for dilutions as a proportion of the QBC concentration. Frozen samples correct for multiple dilution, wash, and resuspension steps. (e) Change in lymphocyte percent of total lymphocytes and granulocytes as measured by flow cytometry, relative to QBC measurements. (f) Variance components from mixed models for lymphocyte recovery (R) and change in lymphocyte percent (C). Colors: Beige = subject ID, green = freezing batch, yellow = thawing/analysis batch, and pale blue = time in storage

correlated with QBC estimates (r = 0.74, CI = 0.65;0.82, df = 108, p < 0.001), though were moderately higher by flow cytometry (Paired t-test mean difference 2.4%, CI 1.3–3.6%, t = 4.16, p < 0.001). Frozen lymphocyte percentages were also correlated with original QBC measures, but much less so (r = 0.44, CI = 0.28;0.58, df = 108, p < 0.001), and flow cytometry estimates were considerably higher (Paired t-test mean difference 15.6%, CI 13.5–17.8%, t = 14.46, p < 0.001).

We define lymphocyte recovery as estimated concentration of lymphocytes in the sample as measured by flow cytometry, after correcting for dilutions, divided by the original concentration of lymphocytes as measured by QBC. In fresh samples, lymphocyte recovery was 59 ± 20%. In frozen samples it was 15 ± 8%. Although low lymphocyte recovery and a loss of granulocytes were both characteristics of frozen samples, within the frozen samples these were not correlated with one another (r = −0.09, CI: −0.07;0.25, df = 147, p = 0.27).

Samples were collected and frozen in 21 batches and thawed and analyzed in 31 batches. To investigate the contributions of freezing conditions, thawing conditions, and time in storage on lymphocyte recovery and percentage we used mix models with "time frozen" as a linear term and random effects for subject, freezing batch, and thawing and analysis batch (Figure 1f). The largest proportion of variance in lymphocyte recovery was explained by freezing batch (25.2%), followed by thawing batch (18.7%). This was followed by weeks in storage (12.4%), which was associated with reduced lymphocyte recovery (β = −0.58 ± 0.27 points/week, p = 0.05), and finally subject (11.4%; correlation between 2-week and 4-week recovery r = 0.36, CI: 0.05;0.61, df = 37, p = 0.02). Change in lymphocyte percent was explained more by thawing batch (25.6%), subject (25.1%; 2-week and 4-week r = 0.55, CI: 0.28;0.73, df = 37, p < 0.001), and freezing batch (14.7%). Lymphocyte percent also declined with more weeks frozen (β = −0.83 ± 0.41 points/week, p = 0.05, 10.9% of variance).

3.3 | Coefficients of variation in repeat samples

Cells were stained with eight different antibody combinations (Table 1). Before examining changes between fresh and frozen samples we assessed consistency in repeat measures on fresh samples,

taking advantage of the fact that we used the same antibody multiple times. These coefficients of variation (CVs) are useful for setting expectations for agreement between fresh and frozen replicates. Table 2 shows the CVs for markers that were repeated. For fresh samples, most markers had reasonable CVs (<12%). CD19 had a moderately higher CV for fresh samples (13.8%) but was measured using two different antibody-conjugate sets (see Table 1 and Table 3). CD28 had a reasonably high CV (16.1%). The worst performance was from CD57 (29.0%), however, given a low proportion of CD57+ cells (8.1%; Table 3) this represents a smaller absolute change in value.

For frozen cells, CVs were generally higher. Two are worth noting as particularly high: the CV for CD19 was 56.0%, while the CV for CD57 was 45.5%. For CD19 this was due to poor performance from one of the two antibody-conjugates used. We discuss this in the next section.

3.4 | Performance of individual antibodies in the lymphocyte gate

Table 3 shows the mean percentage of the lymphocyte gate positive for each antibody-conjugate pair in fresh and frozen samples, as well as the change as a percentage of the fresh value. For most antibodies, percentages were similar for fresh and frozen cells (Figure 2), though almost all showed significant differences in paired t-tests. In part this was likely due to the inclusion of non-lymphocytes in the lymphocyte gate (perhaps dead granulocytes) as indicated by the drop in CD45 percent. Correcting for CD45 percent moved several means closer to their fresh values (Figure 2). However, there were still a handful of antibody-conjugate pairs that yielded poor results on frozen samples. In particular, CD19-PerCP-Cy5.5 appeared to have significant issues with non-specific binding in the frozen samples, as there were many positive cells in the granulocyte as well as lymphocyte gate, and a much higher percentage estimated in the lymphocyte gate. This was despite CD19-APC performing quite well using the same antibody clone but a different conjugate. CD4 also had some non-specific binding with the PerCP-Cy5.5 conjugate, although we were mostly able to correct for this by adjusting the gating. CD56-PE, CD20-APC, CD27-FITC, and CD127-APC yielded lower results on frozen cells, while CD25-PE was higher.

For antibodies labeling major lymphocyte subsets there were good correlations between percentages measured in fresh and frozen samples (Table 4). This included CD3-FITC, CD4-PE, CD8, CD19-APC, CD20-APC, and CD45RA-FITC. Antibodies with poor correlations included the previously mentioned CD4-PerCP-Cy5.5, CD19-PerCP-Cy5.5, CD16-APC, CD25-PE, CD27-FITC, CD28-APC, CD56-PE, CD57-PE, CD127-APC, and IgD-PE. Notably there was a poor correlation with CD45-PerCP-Cy5.5, however this is likely due to there being little variation between individuals in CD45+ percent, leaving most variation due to debris and dying cells in the lymphocyte gate after thawing. Interestingly, CD8-PerCP-Cy5.5 also performed much more poorly than CD8-APC, despite yielding similar mean percent positives. As with mean values, many correlations were improved by adjusting for the CD45+ percent of the lymphocyte gate.

It is worth noting that correlations for many of the problematic PerCP-Cy5.5 antibodies were improved in the samples thawed after 14 weeks. These were also the samples that had the smallest change in lymphocyte percent compared to fresh (Figure 1e), suggesting, perhaps, that these samples were less affected by freezing and thawing.

3.5 | Lymphocyte subsets identified by multiple antibodies

We next identified lymphocyte subsets using combinations of multiple markers (Table 5). The use of multiple markers yielded a high consistency between fresh and frozen samples for B cells, CD4 T cells, CD8 T cells, natural killer cells, and naïve subsets of CD4 and CD8 cells. At an individual level, correlations between fresh and frozen measurements were high for these subsets (Table 6, Figure 3). Bland–Altman

TABLE 2 Mean coefficients of variation and intraclass correlations for markers measured more than once on the same sample

			ICC	
	Mean fresh CV	Mean frozen CV	Fresh	Frozen
Lymphocytes	5.1%	11.0%	0.92	0.68
CD45	2.8%	3.1%	0.47	0.75
CD3	3.7%	4.0%	0.78	0.77
CD4	11.7%	18.0%	0.95	0.68
CD8	11.7%	18.0%	0.80	0.32
CD19	14.2%	56.0%	0.59	0.27
CD28	16.3%	17.2%	0.79	−0.15
CD45RA	6.6%	7.2%	0.47	0.23
CD57	28.6%	45.5%	0.70	0.69

Note: Coefficient of variation (CV) was calculated for each individual and condition and then averaged across individuals. The intraclass correlation (ICC) reports the correlation within individual, relative to variation between individuals. CD19 was measured with an APC conjugate in one replicate and PerCP-Cy5.5 in the other. CD4 includes only the PerCP-Cy5.5 conjugates. See Table 1 for further details.

TABLE 3 Mean percent lymphocytes positive for antibody tags in fresh and frozen samples

	Non-adjusted			CD45 adjusted		
	Fresh mean %	Frozen mean %	Δ (95% CI)	Fresh mean %	Frozen mean %	Δ (95% CI)
CD3-FITC	69.7	66.2	−3.5 (−4.9; −2.1)	75.0	74.2	−0.7 (−1.4; 0.3)
CD4-PE	40.4	36.9	−3.5 (−4.8; −2.1)	43.2	41.3	−1.9 (−3; −0.7)
CD4-PerCP-Cy5.5	33.6	38.7	5.1 (2.9; 7.3)	36.1	43.3	7.2 (5.1; 9.4)
CD8-APC	23.1	23.8	0.7 (−0.1; 1.7)	24.8	26.6	1.8 (1.2; 2.8)
CD8-PerCP-Cy5.5	22.4	24.0	1.6 (0.4; 2.8)	24.1	26.8	2.7 (1.5; 4)
CD16-APC	9.6	9.4	−0.2 (−1.6; 1.2)	10.3	10.7	0.4 (−1.1; 2)
CD19-APC	10.3	10.4	0.2 (−0.4; 0.7)	11.0	11.7	0.7 (0.1; 1.3)
CD19-PerCP-Cy5.5	10.2	26.7	16.5 (13; 20)	11.1	29.8	18.7 (15; 22.7)
CD20-APC	11.8	9.1	−2.7 (−3.3; −2.2)	12.7	10.1	−2.6 (−3.2; −1.9)
CD25-PE	18.7	25.7	7.0 (1.4; 12.6)	19.8	28.9	9.1 (3.3; 15.1)
CD27-FITC	55.6	38.8	−16.8 (−20.1; −13.4)	60.1	43.4	−16.6 (−19.9; −13)
CD28-APC	48.2	43.2	−5.0 (−7.5; −2.6)	51.9	48.4	−3.5 (−5.8; −1.1)
CD45-PerCP-Cy5.5	93.2	89.2	−4.0 (−5.9; −2.2)			
CD45RA-FITC	61.8	51.9	−9.9 (−11.5; −8.2)	66.3	58.3	−8.1 (−10; −6.4)
CD56-PE	17.9	10.3	−7.6 (−11.3; −4)	18.8	11.5	−7.3 (−11.2; −3.5)
CD57-PE	8.1	9.7	1.6 (0; 3.1)	8.7	10.9	2.2 (0.4; 3.6)
CD127-APC	61.4	28.9	−32.5 (−37.5; −27.6)	66.5	31.9	−34.6 (−39.7; −28.9)
CD183-PE	22.7	37.1	14.5 (11.6; 17.3)	24.4	41.7	17.3 (14.1; 20.4)
CD196-FITC	7.4	2.7	−4.7 (−7.2; −2.3)	8.2	3.1	−5.0 (−7.8; −2.2)
CD294-APC	2.5	9.1	6.6 (3.8; 9.5)	2.6	10.5	7.9 (4.5; 11)
IgD-PE	5.3	6.4	1.1 (0.1; 1.9)	5.7	7.3	1.6 (0.5; 2.5)

Note: Δ is the change in mean value. 95% CI is the CI for the mean difference from a paired *t*-test.

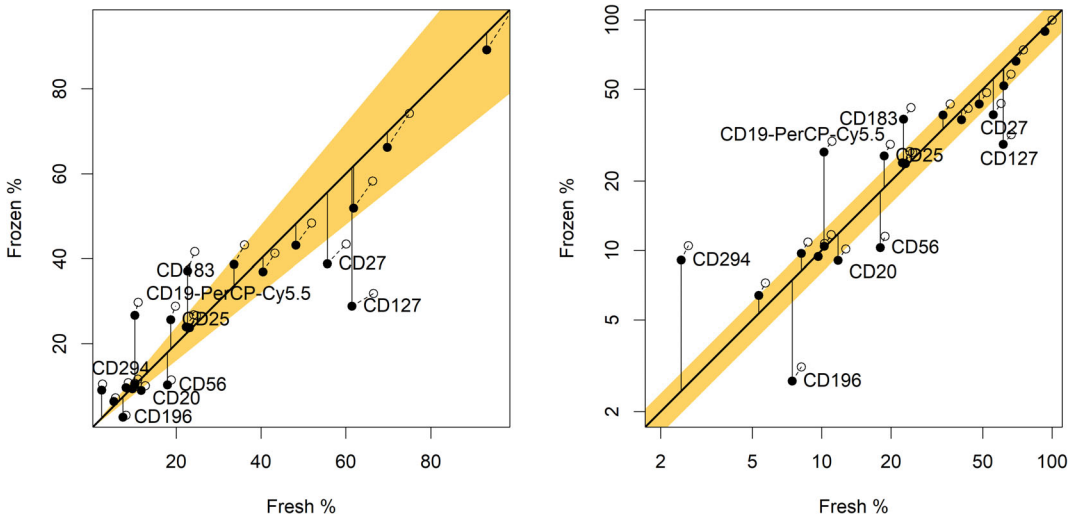

FIGURE 2 Mean percentage of cells in the lymphocyte gate tagged with each antibody in fresh and frozen samples. A linear scale is used on the left and a log scale on the right. Shading indicates a 20% change from fresh to frozen, relative to the original value. Antibodies outside of this range are labeled. Values are equal to those in Table 2

plots (Figure 4) suggested that for most subsets differences were <10% and were not systematically biased in one direction. An exception to this was for naïve cells, which had greater error and were reduced in frozen samples. CD4 cell percentages also showed slightly higher error than other subsets. We also obtained reliable correlations for counts by multiplying percentages obtained from flow cytometry times the lymphocyte/monocyte count taken with the QBC at sample collection (Figure 5). The identification of senescent cells (CD57

TABLE 4 Pearson's correlations between percentages measured in fresh and frozen samples

	Un-adjusted				Adjusted for CD45+			
	Overall[a] (*n* = 110)	2 W (*n* = 71)	4 W (*n* = 39)	14 W (*n* = 39)	Overall (*n* = 110)	2 W (*n* = 71)	4 W (*n* = 39)	14 W (*n* = 39)
CD3-FITC	0.59	0.63	0.49	0.62	0.78	0.79	0.67	0.80
CD4-PE	0.59	0.47	0.38	0.86	0.66	0.53	0.47	0.88
CD4-PerCP-Cy5.5	0.16^{ns}	0.11^{ns}	0.43	0.71	0.23	0.18^{ns}	0.43	0.72
CD8-APC	0.66	0.66	0.57	0.68	0.75	0.77	0.66	0.73
CD8-PerCP-Cy5.5	0.45	0.52	0.55	0.56	0.48	0.45	0.56	0.72
CD16-APC	0.16	0.47	0.12^{ns}	−0.39^{ns}	0.19	0.54	0.07^{ns}	−0.37^{ns}
CD19-APC	0.71	0.66	0.63	0.76	0.71	0.72	0.65	0.66
CD19-PerCP-Cy5.5	−0.09^{ns}	0.01^{ns}	−0.10^{ns}	0.58	−0.06^{ns}	0.06^{ns}	−0.05^{ns}	0.51
CD20-APC	0.68	0.69	0.52	0.75	0.68	0.73	0.55	0.68
CD25-PE	−0.38^{ns}	0.07^{ns}	0.26^{ns}	0.72	−0.29^{ns}	0.11^{ns}	0.30	0.72
CD27-FITC	0.19	−0.01^{ns}	0.36	0.38	0.24	0.00^{ns}	0.40	0.46
CD28-APC	0.19	0.18^{ns}	0.42	0.44	0.35	0.24	0.45	0.61
CD45-PerCP-Cy5.5	0.14^{ns}	−0.04^{ns}	0.04^{ns}	0.49				
CD45RA-FITC	0.62	0.53	0.40	0.76	0.59	0.49	0.41	0.66
CD56-PE	−0.20^{ns}	−0.33^{ns}	0.00^{ns}	0.16^{ns}	−0.21^{ns}	−0.32^{ns}	0.02^{ns}	0.04^{ns}
CD57-PE	0.24	0.10^{ns}	0.10^{ns}	0.47	0.28	0.13^{ns}	0.11^{ns}	0.56
CD127-APC	0.07^{ns}	0.05^{ns}	−0.08^{ns}	−0.61^{ns}	0.16^{ns}	0.11^{ns}	−0.05^{ns}	−0.42^{ns}
CD183-PE	0.07^{ns}	0.11^{ns}	0.30	−0.12^{ns}	0.01^{ns}	0.14^{ns}	0.33	−0.33^{ns}
CD196-FITC	−0.12^{ns}	−0.13^{ns}	−0.03^{ns}	−0.01^{ns}	−0.12^{ns}	−0.14^{ns}	−0.04^{ns}	0.04^{ns}
CD294-APC	−0.05^{ns}	0.01^{ns}	0.54	−0.21^{ns}	−0.07^{ns}	0.01^{ns}	0.51	−0.25^{ns}
IgD-PE	0.20	−0.11^{ns}	0.32	0.23^{ns}	0.19	−0.05^{ns}	0.32	0.11^{ns}
Mean *r*	*0.23*	*0.22*	*0.30*	*0.37*	*0.28*	*0.27*	*0.34*	*0.38*

Note: ns (superscript) indicates $p > 0.05$ in a one-sided test against the null that $r \leq 0$. Percentages were adjusted by dividing by the CD45+ percent within the lymphocyte gate.

[a]For overall statistics, duplicated frozen measurements for the same individual at weeks 2 and 4 (*n* = 39) were averaged together before calculating the correlation.

+CD28−) was noisier; even in CD8 cells, where the correlation was reasonable ($r = 0.61$), the proportion of senescent cells was higher in frozen samples (12.4 vs. 7.7%). Correlations for CD56 were reliable, despite poor results for the CD56-PE antibody alone, largely due to the exclusion of CD3 and CD19 positive cells which likely eliminated much of the nonspecific binding. Correlations for further subsets of CD4 T cells and B cells were poor. This is largely because the antibodies used to identify further T cell subsets (CD25, CD127, CD183, CD196, and CD294) were unreliable in frozen samples. For B cells the CD19-PerCP-Cy5.5 antibody was also unreliable, as were CD27 and IgD.

To determine whether differences between fresh and frozen samples were due to freezing conditions, thawing/analysis conditions, or neither, we examined the proportion of the variance in frozen minus fresh value explained by these two batches (Table 6). For major subsets, freezing batch explained more variance than thawing batch. However, for T-cell and B-cell subsets thawing and analysis batch explained more of the discrepancy.

3.6 | Stability in storage

In general, there was no systematic evidence for poorer results after longer storage in liquid nitrogen. The samples stored for 14 weeks had lower lymphocyte recovery (Figure 1d) but had less change in lymphocyte percentage that the samples analyzed at 2 and 4 weeks (Figure 1e). There were changes in mean values with freezing, but few clear trends related to time in storage (Figure 6). For many markers, correlations between fresh and frozen values were higher in the samples analyzed at 14 weeks (Tables 4 and 6).

TABLE 5 Mean percent lymphocyte subsets in fresh and frozen samples

Percent of	Cell type	Markers	Fresh mean %	Frozen mean %	Δ (95% CI)[a]
CD45	B cells	CD45+CD19+	11.1	11.6	0.5 (0; 1.1)
	T cells	CD45+CD3+	74.5	74.3	−0.1 (−0.9; 0.7)
	T CD4	CD45+CD3+CD4+CD8−	41.7	39.4	−2.3 (−3.5; −1.1)
	T CD8	CD45+CD3+CD8+CD4−	22.4	25.4	3.0 (2.4; 3.7)
	Natural Killer	CD45+CD3−CD19−CD16/56+	9.9	12.0	2.0 (1.1; 2.8)
Helper T	Naive	CD4+CD45RA+CD57−	53.6	50.2	−3.4 (−5.0; −1.8)
	CD45RA	CD4+CD45RA+	57.0	51.4	−5.6 (−7.5; −3.7)
	Senescent	CD4+CD57+CD28−	1.7	2.0	0.3 (−0.3; 0.8)
	Th17	CD4+CD196+	4.4	2.8	−1.6 (−3.4; 0.2)
	Th1	CD4+CD183+	28.2	44.3	16.1 (12.8; 19.4)
	Th2	CD4+CD294+	2.8	17.3	14.5 (9; 20)
	Treg	CD4+CD25+CD127−	3.0	6.5	3.5 (1.6; 5.4)
Killer T	Naïve	CD8+CD45RA+CD57−	62.8	54.8	−8.0 (−9.9; −6.1)
	CD45RA	CD8+CD45RA+	72.6	65.4	−7.4 (−9.1; −5.7)
	Senescent	CD8+CD57+CD28−	7.7	12.4	4.8 (3.3; 6.2)
B cells	B Memory	CD19+CD27+IgD−CD20+	9.5	3.6	−5.8 (−7.2; −4.4)
	B Naive	CD19+CD27−IgD+	20.8	28.7	7.9 (3.1; 12.7)
	B Non-switched	CD19+CD27+IgD+	3.0	1.9	−1.1 (−1.7; −0.5)
	B Plasma	CD19+CD27+IgD−CD20−	4.7	22.6	17.6 (14.4; 20.9)

[a]Mean difference and 95% CI for the mean difference from a paired samples *t*-test.

TABLE 6 Pearson's correlations between percentages obtained for fresh and frozen samples

Percent of	Cell type	Overall[a] (*n* = 110)	2 W (*n* = 71)	4 W (*n* = 39)	14 W (*n* = 39)	Freezing batch[b]	Thawing batch[b]
CD45	B cells	0.73	0.75	0.65	0.68	20.0%	27.7%
	T cells	0.82	0.86	0.53	0.87	25.0%	6.6%
	T CD4	0.67	0.50	0.46	0.90	42.2%	1.7%
	T CD8	0.82	0.79	0.79	0.86	28.8%	7.0%
	Natural Killer	0.64	0.75	0.29	0.55	34.9%	10.9%
Helper T	Naive	0.73	0.71	0.33	0.87	30.3%	28.7%
	CD45RA	0.62	0.67	0.39	0.75	21.7%	41.5%
	Senescent	0.37	0.57	0.70	0.20^{ns}	15.9%	0.0%
	Th17	-0.04^{ns}	-0.14^{ns}	-0.01^{ns}	-0.08^{ns}	22.3%	24.2%
	Th1	0.07^{ns}	0.13^{ns}	0.19^{ns}	0.00^{ns}	6.7%	37.3%
	Th2	-0.06^{ns}	-0.04^{ns}	0.43	-0.06^{ns}	16.2%	56.9%
	Treg	0.22	0.35	0.42	0.01^{ns}	3.8%	51.5%
Killer T	Naïve	0.71	0.58	0.75	0.74	10.4%	7.4%
	CD45RA	0.65	0.52	0.51	0.77	20.7%	27.2%
	Senescent	0.61	0.46	0.76	0.73	0.0%	42.9%
B cells	B Memory	0.22	0.12^{ns}	0.07^{ns}	0.33	54.4%	0.0%
	B Naive	0.12^{ns}	0.00^{ns}	0.05^{ns}	0.44	0.0%	42.2%
	B Non-switched	-0.09^{ns}	-0.24^{ns}	-0.08^{ns}	0.20^{ns}	5.8%	24.8%
	B Plasma	0.29	-0.04^{ns}	0.09^{ns}	0.05^{ns}	7.5%	61.6%

Note: ns (superscript) indicates $p > 0.05$ in a one-sided test against the null that $r \leq 0$. Percentages were adjusted by dividing by the CD45+ percent within the lymphocyte gate.

[a]For overall statistics, duplicated frozen measurements for the same individual at weeks 2 and 4 ($n = 39$) were averaged together before calculating the correlation.

[b]The proportion of the difference between fresh and frozen explained by freezing batch and thawing batch, estimated by random effects.

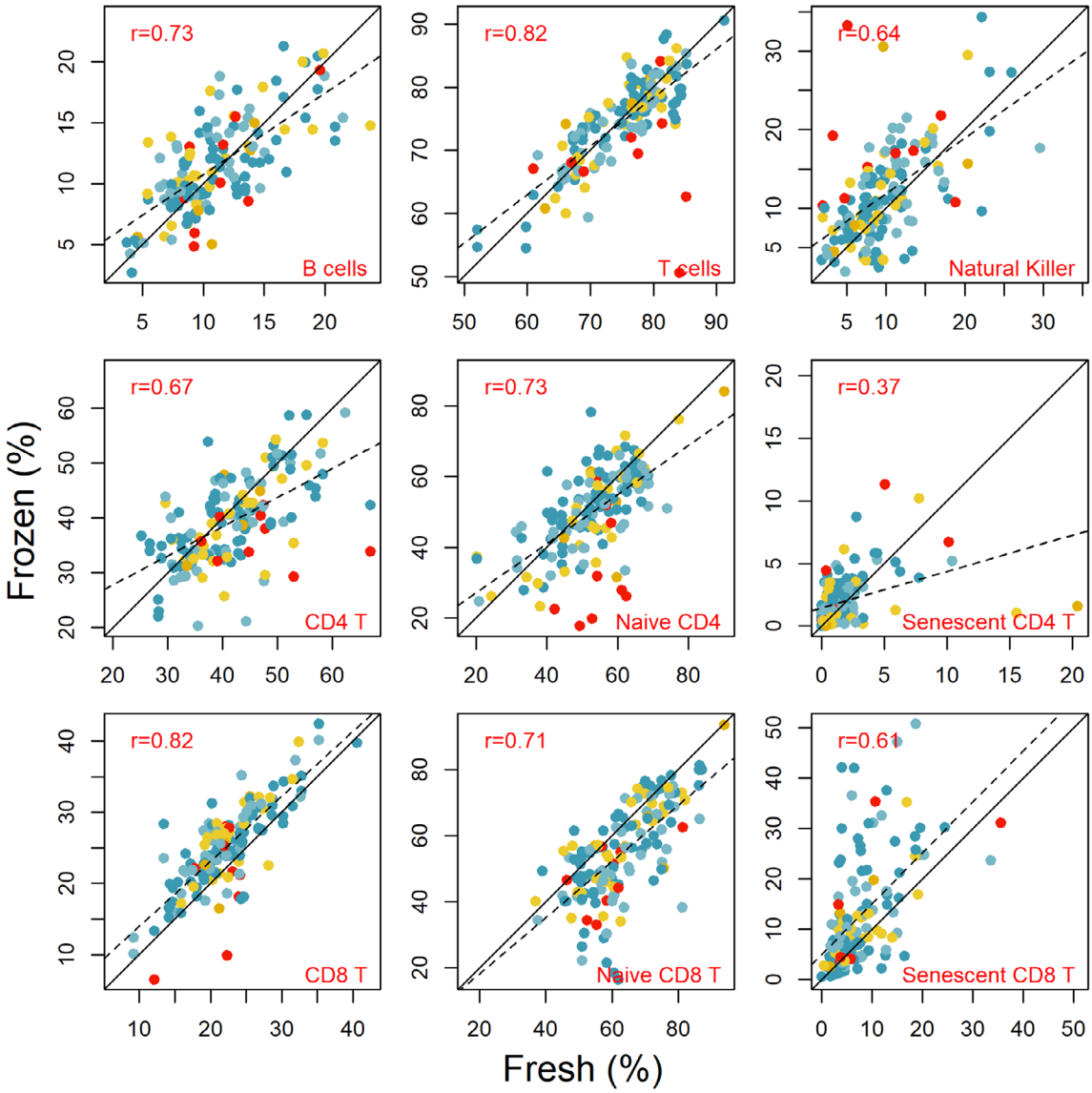

FIGURE 3 Percent of CD45+ cells or percent of respective T cell subset (CD4 or CD8 for naive and senescent cells) in matched fresh and frozen samples. Black line is the 1:1 slope. Dashed line is the best linear fit line. Colors indicate the lymphocyte recovery of the sample, with red = recovery <5%; yellow = recovery 5–10%; and blue = >10%

3.7 | Viability of frozen cells

After assaying the first sets of samples we decided to add a viability assay to assess cell viability in thawed samples. We used two different viability assays. Guava ViaCount Reagent uses two DNA-binding dyes. One labels nucleated cells to eliminate debris, and the other labels dead or dying cells. Using Viacount, we evaluated the viability of 34 samples after 2 weeks, 23 samples after 4 weeks, and 39 samples after 14 weeks. Viability of fresh samples was not assayed, as initial testing of fresh blood samples revealed viability of 99.5% with little variability. Mean viability of frozen samples by ViaCount was 25.3 ± 10.5%, with no significant differences by weeks frozen. Viability was weakly correlated with lymphocyte recovery (r = 0.18, p = 0.08) and the lymphocyte percent (r = 0.32, p = 0.001).

To assess the viability of separate subsets of leukocytes, we used Fixable Viability Dye eFluor 660 along with combinations of antibodies for an additional subset of samples which were frozen for 2 or 4 weeks (n = 16). The eFluor 660 viability dye yielded much higher overall viability estimates (Mean 70 ± 17%) than the ViaCount assay, though these were different samples so may not be directly comparable. Viability was highly correlated with lymphocyte recovery (r = 0.78, p < 0.001), and weakly and non-significantly with lymphocyte percentage (0.30, p = 0.25). Viability was much higher within the lymphocyte gate (85 ± 12%) than the granulocyte gate (32 ± 20%; paired t = −8.85, p < 0.001). Viabilities for CD45, CD3, CD4, CD8, CD4+CD45RA+, CD8+CD45RA+, and CD16/CD56+ were high though slightly lower than for lymphocytes overall (means ranged from 72–82 ± 11–19%). There were no significant differences in viability by lymphocyte type.

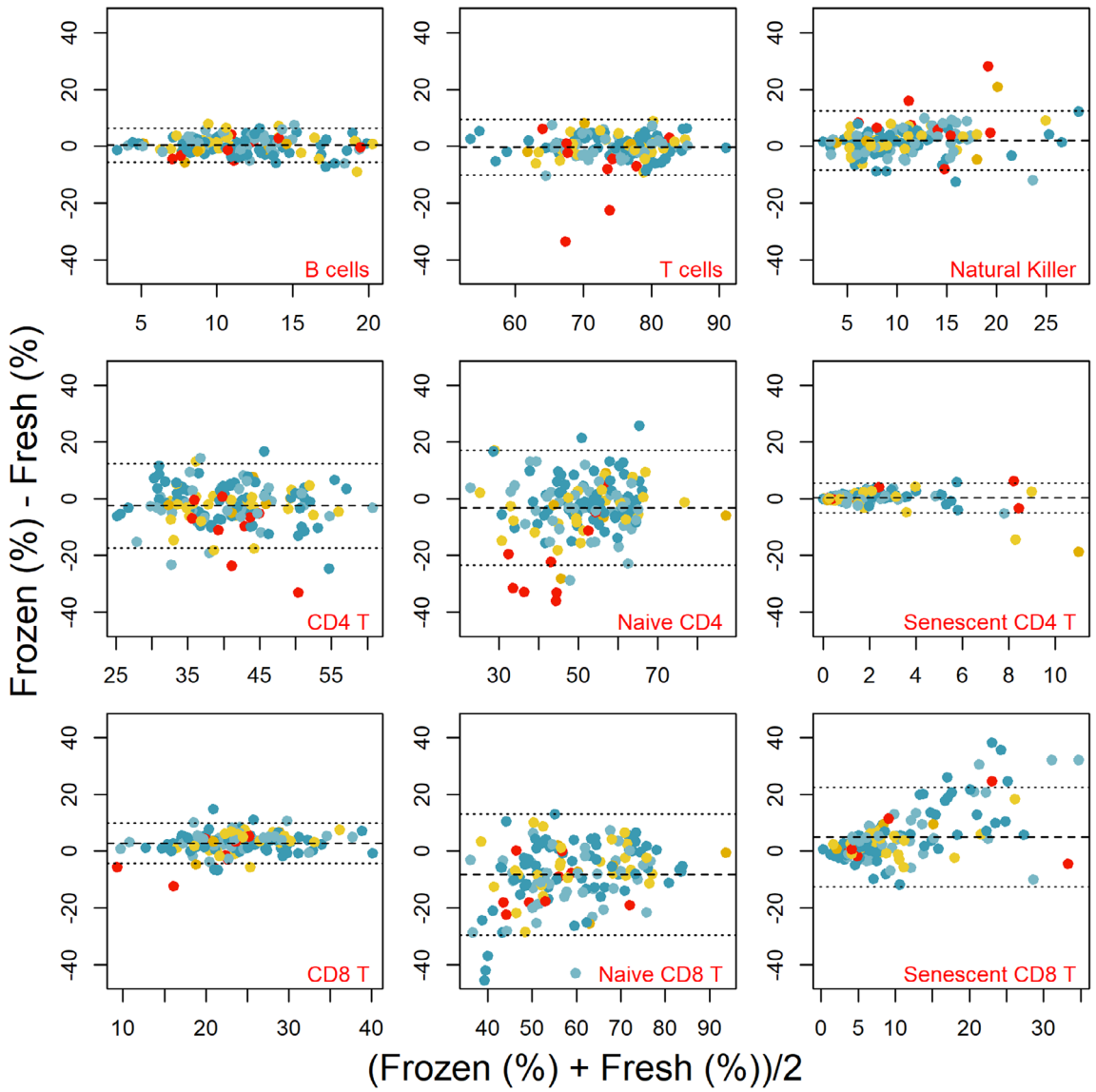

FIGURE 4 Bland–Altman plot showing the relationship between the average measured value for the two conditions (fresh and frozen) and the difference between these values. Values used are percent of CD45+ cells or percent of respective T cell subset (CD4 or CD8 for naive and senescent cells). Long dashed line shows the mean value for the difference and short dashed lines ± 2 SD for the difference

4 | DISCUSSION

Flow cytometry can be a powerful tool for studying immune function and responses, but there are several barriers to its use in field settings. Here, we have evaluated the performance of a simplified procedure for cryopreserving samples in the field using a liquid nitrogen shipper tank. Our protocol has several advantages over other cryopreservation protocols. First, it avoids the use of an electric freezer or other complicated equipment. Second, it avoids extra steps, such as the isolation of PBMCs, by using whole blood. Third, we avoid the use of fetal calf serum (FCS) or other reagents that are frequently used as medium for freezing PBMCs by freezing whole blood mixed with RPMI-1640. FCS requires refrigeration, whereas RPMI-1640 is available in a powder form which can be reconstituted in the field.

For the identification of major lymphocyte subsets using multiple markers, this procedure works well. Mean percentages were very similar to those obtained on fresh samples (Table 5). More importantly for studies of individual differences, correlations were high (Table 6). Changes in mean values are comparable in magnitude to those reported in other studies using more controlled methods (i.e., Costantini et al., 2003; Hviid et al., 1993; Tollerud et al., 1991; Weinberg et al., 2009). Note that many of these studies report differences as non-significant, whereas most of the changes we have observed are "significant" in the statistical sense. However, many of these studies have reported on much smaller sample sizes than we have used here, limiting power to detect significant changes of comparable magnitude.

Few studies have reported correlations for individual samples. Weinberg et al. (2009) reports correlations with r = 0.86 for CD4+

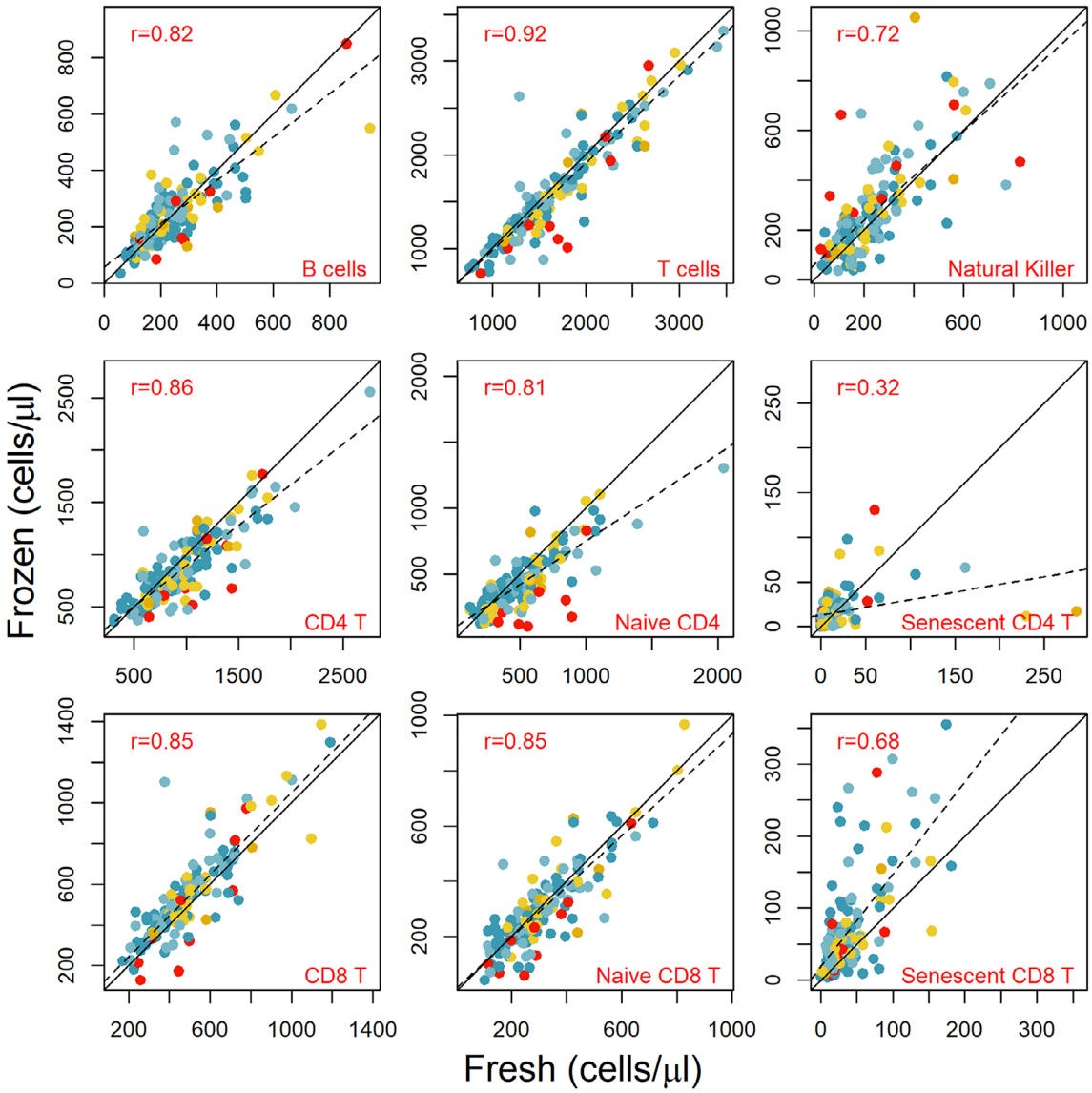

FIGURE 5 Estimated cell counts in matched fresh and frozen samples. Counts were calculated by multiplying percentages obtained from flow cytometry times the lymphocyte/monocyte count from the QBC at baseline. Black line is the 1:1 slope. Dashed line is the best linear fit line. Colors indicate the lymphocyte recovery of the sample, with red = recovery <5%; yellow = recovery 5–10%; and blue = > 10%

cells, and r = 0.82 for CD8 cells, in the ballpark of what we report here, while Fiebig et al. (1997) reports correlations with r = 0.99 for both CD4+ and CD8+ cells, suggesting that these might be improved further. However, CVs for many repeats without freezing for our study were in the range of 5–15% (Table 2). The International Council for Standardization of Haematology (ICSH) and International Clinical Cytometry Society (ICCS) recommend target CVs <10% for common subsets and <20% for rare cells (Wood et al., 2013). The values we have obtained are close to these targets and are comparable to past studies, which have also obtained CVs in the range of 5–15% for common subsets, and higher CVs (~20–25%) for less common subsets (Hasan et al., 2015; Kalina, 2020). A CV of 15% would be associated with a correlation of $r \sim 0.90$, meaning that without cryopreservation this is the best we should expect from our current assay methods. That correlations between fresh and frozen percentages are only a bit below this suggests that cryopreservation introduces only a moderate amount of error.

This study involved a relatively homogenous population of participants, particularly in terms of age. When variation in the sample is minimal, differences will be largely due to error, rather than between individual differences. Studies involving a wider range of natural variation might expect better correlations than we report here. Conversely, future studies with little between individual variation may have a harder time reliably reporting individual values with these methods.

The fairly large batch effects observed in lymphocyte recovery suggest that small variations in freezing conditions were influential on some outcomes. Freezing batch also explained more of the difference in measurement in major lymphocyte subsets than did thawing and analysis batch. This may have related to the amount of liquid nitrogen still present in the shipping container at the time of freezing, which

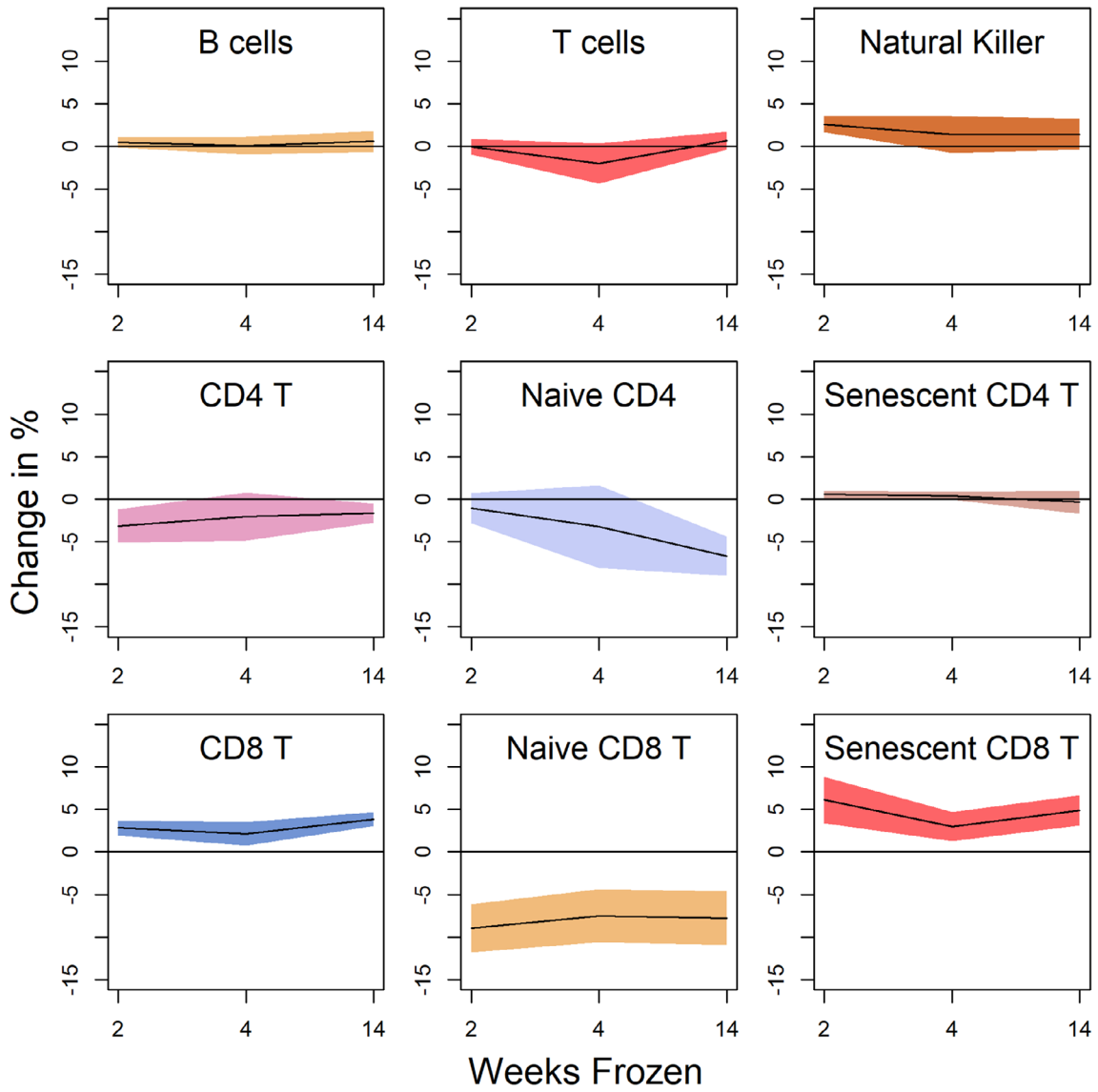

FIGURE 6 Change in mean measured percentage at 2, 4, and 14 weeks, relative to measurements in fresh samples

may have not been perfectly standardized and thus may have affected the temperature of the vapor phase. The same author was primarily responsible for the flow cytometry (RLK), with assistance from the other authors, and all gating analysis was conducted on all samples at the end of collection, so technician differences are unlikely to explain batch effects. Thawing/analysis batch did explain some differences in T-cell and B-cell subsets, however, so technical error may have affected counting of these smaller subsets.

Our study used whole blood rather than isolated PBMCs in order to simplify sample collection. Isolating PBMCs is useful when the cell populations of interest are rare, because rare cells can be counted without counting as many other cells that may not be of interest. Isolating PBMCs also eliminates granulocytes, which, as our results suggest, are sometimes not well preserved by freezing and can contribute debris. However, the isolation of PBMCs can also introduce bias in some cell populations, such as CD8 cells, such that many studies consider the use of whole blood to be more accurate (Appay et al., 2006; Ashmore et al., 1989; De Paoli et al., 1984; Renzi & Ginns, 1987; Romeu et al., 1992). The choice of whether to isolate PBMCs should therefore depend on research goals and practical concerns.

Similarly, the use of capillary blood from a finger prick makes collection easier and reduces the sample collected and transported. For the most part measurements in capillary blood correlate strongly with measurements in venous blood, but caution should be used when comparing data from both sources, since small differences have been noted (Sitoe et al., 2011; Srisala et al., 2019).

4.1 | Less than ideal

Despite good correlations for major lymphocyte subsets, we report much poorer performance for many markers delineating further subsets. There are at least three reasons poor correlations might arise: (a) changes in cell composition due to differential resilience to

cryopreservation, (b) changes in cell function and surface marker expression, (c) changes in antibody binding. This final reason could likely result from changes in protein conformation, interference from cellular debris or antigen, which might block binding or cause non-specific binding.

A number of papers have reported changes in cellular function or responsiveness following cryopreservation in terms of proliferation or cytokine secretion (Best et al., 2007; Costantini et al., 2003; Disis et al., 2006; Weinberg et al., 2000, 2009). Much of this may be due to changes in cell viability (Weinberg et al., 2009), but not all responses were equally affected in these studies, suggesting some differential changes in viability.

Papers have also reported changes in cell type or cell marker expression, however there is sufficient variation in protocol and marker choice across studies that it is difficult to determine whether there are reliable patterns. One study reported changes in regulatory T-cells (Elkord, 2009), though this was later challenged (Van Hemelen et al., 2010). Two studies reported reductions in naïve cells as labeled by CD45RA, a form of CD45 found on naïve cells, and CD62L, a cell adhesion molecule related to localization of naïve and central memory T cells to lymphoid organs (Costantini et al., 2003; Weinberg et al., 2009). These changes were largely due to changes in CD62L, rather than CD45RA. Costantini et al. (2003) also reported an increase in CD57, a marker we use to characterize senescent cells and exclude naïve cells (though also present on natural killer cells). In our study, we found that CD45RA expression decreased and CD57 expression increased, consistent with a maturation of cells away from a naïve phenotype toward a senescent phenotype. These changes are consistent with what we might expect to observe with cryopreservation, since they relate to cell life stage or activation state, rather than cellular identity. However, we caution that we cannot exclude changes in antibody binding rather than cellular phenotype as the explanation for these changes.

We are not aware of any studies explicitly noting problems with antibodies in cryopreserved samples. This may be due to the use of isolated PBMCs, instead of whole blood, which likely eliminates some debris from red blood cells and granulocytes. Alternately, authors may have chosen to report procedures that worked, rather than the iterations that were attempted but failed. Here we have chosen to report the parts of our procedure that worked as well as those that did not to highlight issues researchers developing their own protocols might face. In our results, it is clear that antibody binding conjugates were responsible for some poor correlations. Notably, CD4 and CD19 antibodies with a PerCP-Cy5.5 conjugate produced poor results in frozen samples relative to the same antibodies with PE or APC conjugates. Our CD56-PE antibody also performed poorly in frozen cells. For some of the other markers, particularly those that are cytokine receptors (i.e., CD127, CD25) it is less clear whether poor correlations were due to antibody binding or due to actual functional changes in the cells. Either way, caution is warranted.

4.2 | Recommendations for implementation

Our results suggest that a simplified field procedure for freezing whole blood can be useful for preserving cells to identify major lymphocyte subsets. Despite some changes in mean percent, correlations were also good for naïve, and to a lesser extent, senescent subsets. However our results suggest that even for these subsets certain steps should be taken that include (a) pre-screening antibodies to ensure they work well with the experimental conditions and (b) using multiple markers to isolate the cell population of interest, for example labeling with CD45 or CD3 to screen out additional debris or granulocytes that can end up in the lymphocyte gate. In contrast to results with lymphocytes, frozen samples are not reliable for counting granulocytes, and consequently, may not be reliable for counting absolute lymphocyte counts, rather than percentages. Using a point of care device such as the QBC Autoread Plus to obtain a two-part differential at the time of collection is a simple way to negate this limitation. Finally, our results suggest caution in using frozen samples to analyze further lymphocyte subsets. Markers related to lymphocyte activation or maturation may be affected by freezing, particularly when freezing conditions are less closely controlled, as would occur in a field setting.

4.3 | Opportunity for further refinement

The protocol used here has been heavily simplified for field conditions, but we would like to suggest two further modifications that might be investigated in the future. The first is the possibility of fixing cells prior to cryopreservation. Pinto et al. (2005) fixed isolated PBMCs with Optilyse or 2% formaldehyde and then froze cells directly in the fixative. Flow cytometry results obtained from fixed and frozen cells were comparable to fresh. At the time of writing, formaldehyde diluted to less than 10% is not considered hazardous by airlines in the United States (though regulations may change), so formaldehyde might be transportable to field sites. Further steps might be necessary when working with whole blood instead of PBMCs (i.e. lysis of fixed red blood cells (Chow et al., 2005)). Additionally, to the best of our knowledge, no study has directly compared fixation to DMSO cryopreservation. However, fixation might be worth investigating further as a field protocol.

Here we mixed whole blood with RPMI and DMSO prior to cryopreservation, in order to dilute the blood and limit clumping and to approximate many of the PBMCS protocols which use FCS or something similar. However, other studies with whole blood mix the blood directly with DMSO, suggesting the RPMI may not have been necessary (Cheng et al., 2001; Hayes et al., 2002; Stevens et al., 2007). This might be a further simplification worth investigating.

Finally, our results do suggest that these methods might be used for cell sorting and functional assays. Our viability analyses suggest 25–70% of cells remained viable after freezing, with no significant differences in viability by lymphocyte subtype. However, caution is

warranted since changes in markers such as CD45RA and CD57 due to cryopreservation may reflect changes in the functional capacity of some cells. Further optimizations to the protocol might be warranted if cells are to be used in functional assays, for example by using different medium additives after thawing (Disis et al., 2006).

5 | CONCLUSION

Flow cytometry is generally not a very field friendly technology. However, the cryopreservation of samples for later analysis can help make it part of field studies by allowing for transport and analysis in a central location. Samples frozen in liquid nitrogen can be stored for several months without electricity. When care is taken, major lymphocyte percentages determined from whole blood frozen in the vapor phase of a liquid nitrogen shipper are highly correlated with results from fresh samples. Importantly, antibody conjugates may differ in their efficacy at labeling previously frozen samples, and some markers may change due to alterations in the expression of surface proteins on previously frozen cells. As such, pilot work should be done prior to field data collection, in order to determine which sets of antibodies and conjugates optimally capture the desired cell subsets. Further, caution should be employed when using frozen samples for finer grained cytometric analysis. Despite these potential complications, flow cytometry is currently the best option for immunophenotyping and investigating the cellular components of immunity in the field.

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AUTHOR CONTRIBUTIONS

Aaron Blackwell: Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; resources; supervision; visualization; writing-original draft. **Angela Garcia:** Conceptualization; investigation; methodology; project administration; supervision; writing-review and editing. **Ryan Keivanfar:** Formal analysis; investigation; methodology; writing-review and editing. **Sarah Bay:** Investigation; methodology; writing-review and editing.

CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

DATA AVAILABILITY STATEMENT

The flow cytometry data file and analysis scripts at the time of publication are available at https://doi.org/10.5281/zenodo.4421667 and on Github at https://github.com/adblackwell/flowcytometryfreezing.

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