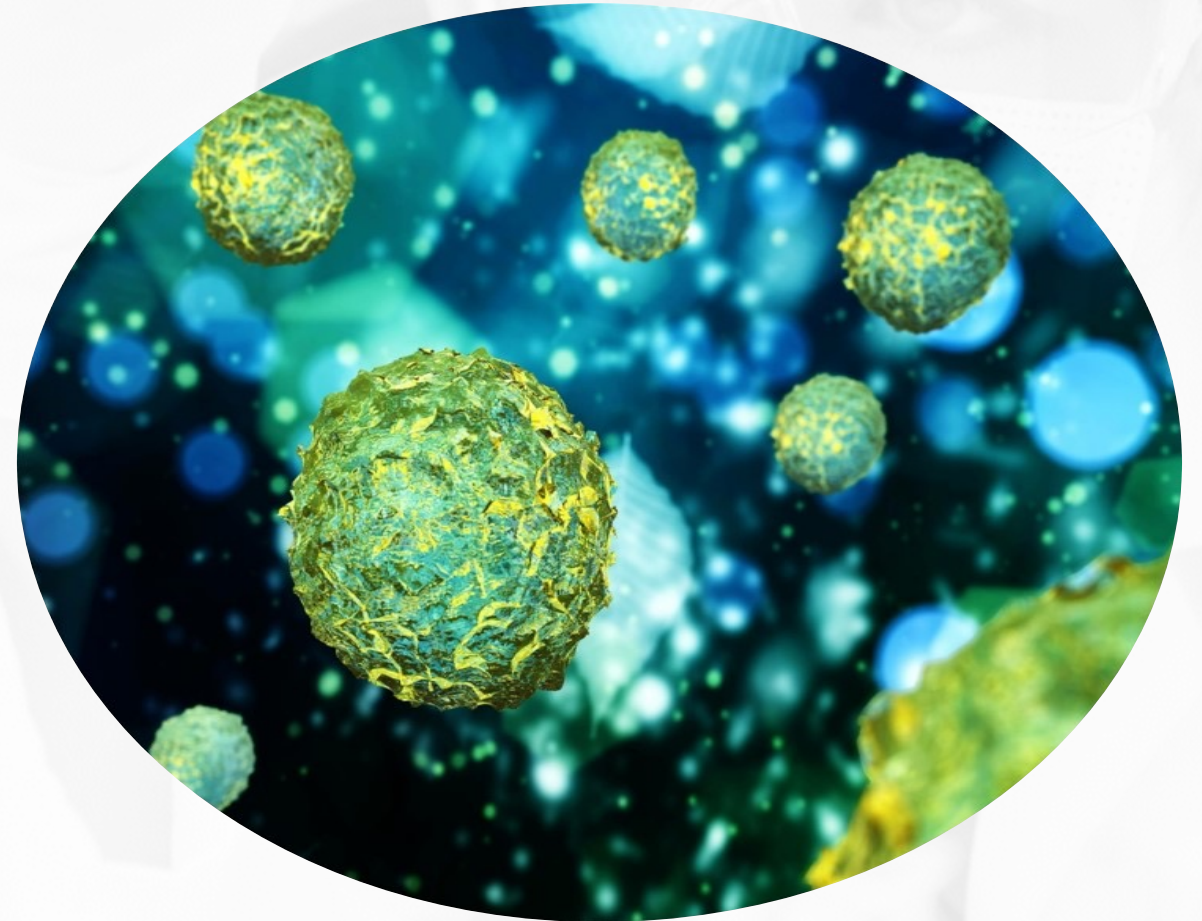


Sample Stability Assessments in Flow Cytometry Assays: Immunophenotyping Case Study and Critical Considerations

Petia Doytcheva, PhD

Lead Scientist Molecular and Cell Biology

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16th EBF Open Symposium



Flow Cytometry in Bioanalysis

✓ PD biomarkers

- Immunophenotyping (relative or absolute concentrations of various cell types)
- Rare cell enumeration (e.g. detection of circulating tumour cells)
- Target engagement (receptor occupancy & receptor internalization)
- Intracellular and cell surface markers (e.g. expression or inhibition of downstream targets)
- Functional cell assays (e.g. induction of apoptosis)

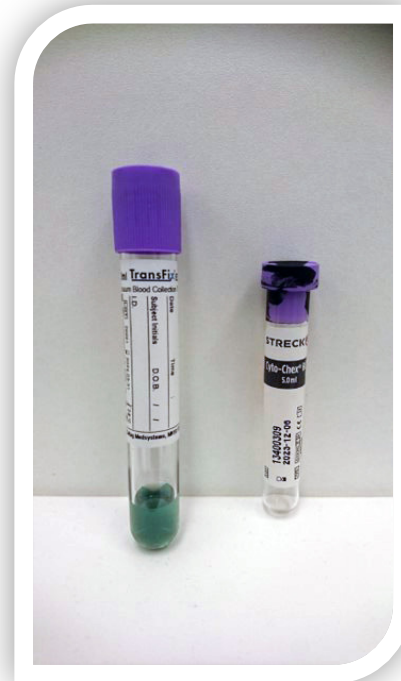
✓ PK and ADA analysis (e.g. CAR-T cell therapy)

✓ Power of single cell analysis and high dimensional multiplexing combined

✗ Sample stability of cells and cell markers is a major limitation (<24-48h)

Stability of Flow Cytometry Samples

- ✔ TransFix (formaldehyde-based) and Cyto-Chex (proprietary stabilizer) blood collection tubes
 - Direct draw blood collection and stabilization tubes
 - Maintain lymphocytes morphology and surface antigen expression for up to 14 days at RT (Cyto-Chex) or 5°C (TransFix)
 - Direct transport at RT (Cyto-Chex) or 5°C (TransFix)
 - Both products approved for IVD immunophenotyping of HIV patients

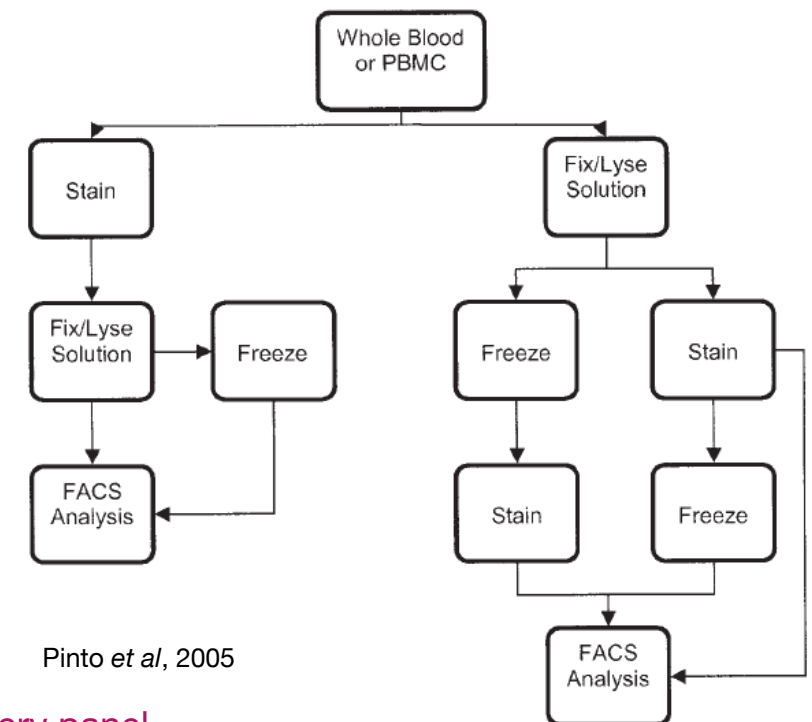


- ✔ 10% DMSO stabilization for storage at -80 °C
 - Comparable to frozen PBMCs

- ⊗ Different results in different cells types
- ⊗ Difference in sample handling after thawing/staining → avoid washing
- ⊗ Different markers could be differentially affected, stability needs to be assessed for every panel

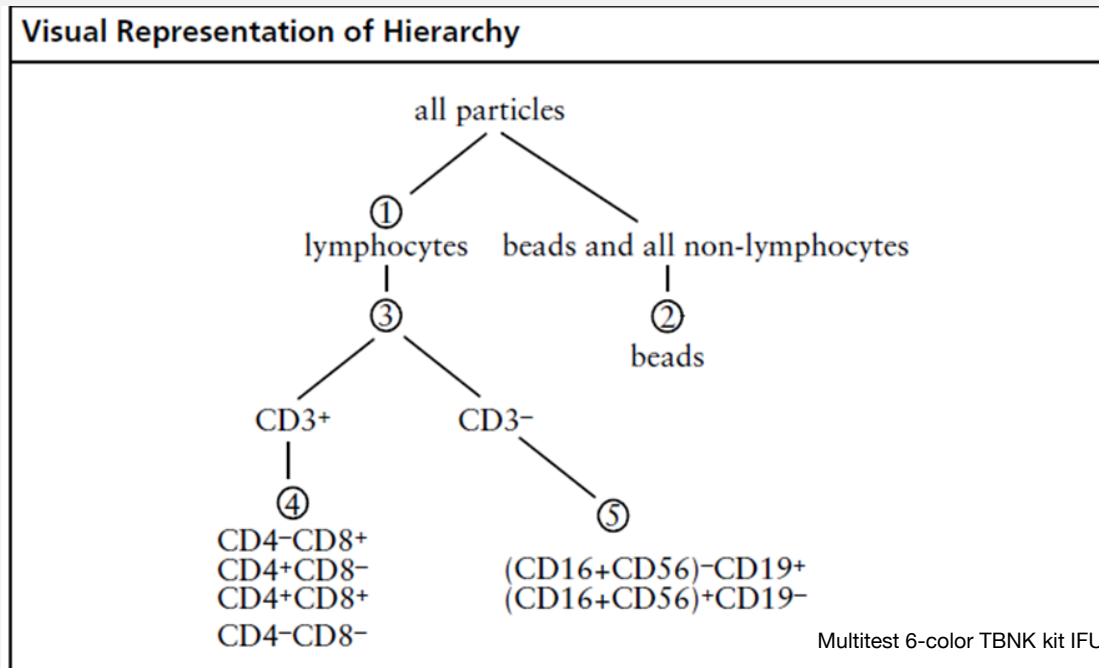
- ✔ Formaldehyde fixation for storage at -80 °C

- Lyse and fix samples prior to freezing
- Pinto *et al*, 2005: variations of fix/lyse-freeze-stain procedure are suitable for flow cytometric analysis



T, B, and NK Immunophenotyping Case Study

- Commercial BD Multitest 6-color TBNK kit with BD Trucount Tubes



Quantified cell types:

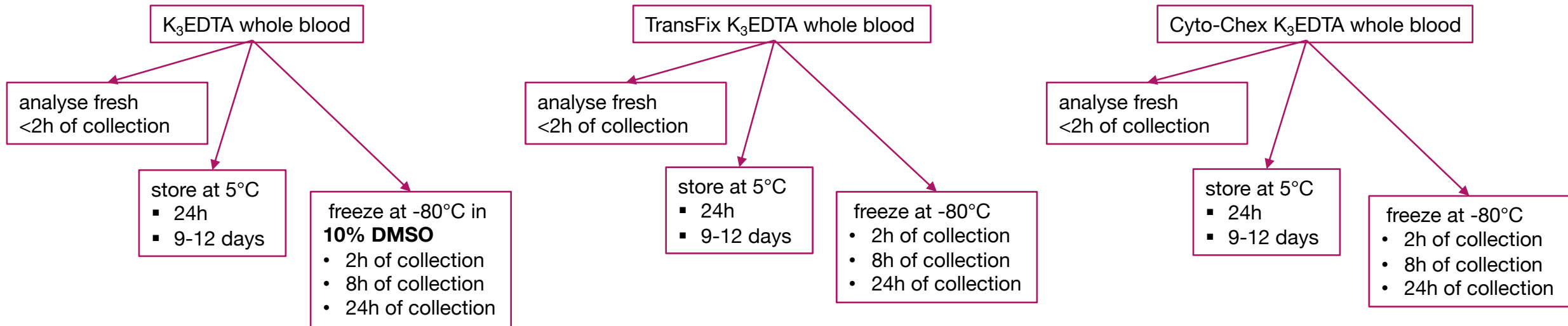
- total T cells = CD3⁺
- T helper cells = CD3⁺CD4⁺CD8⁻
- cytotoxic T cells = CD3⁺CD4⁻CD8⁺
- B cells = CD3⁻CD19⁺CD16⁻CD56⁻
- natural killer (NK) cells = CD3⁻CD19⁻(CD16/CD56)⁺

Control samples:

- BD Multi-Check Control = mimicking a healthy sample
- BD Multi-Check CD4 Low Control = mimicking an HIV sample

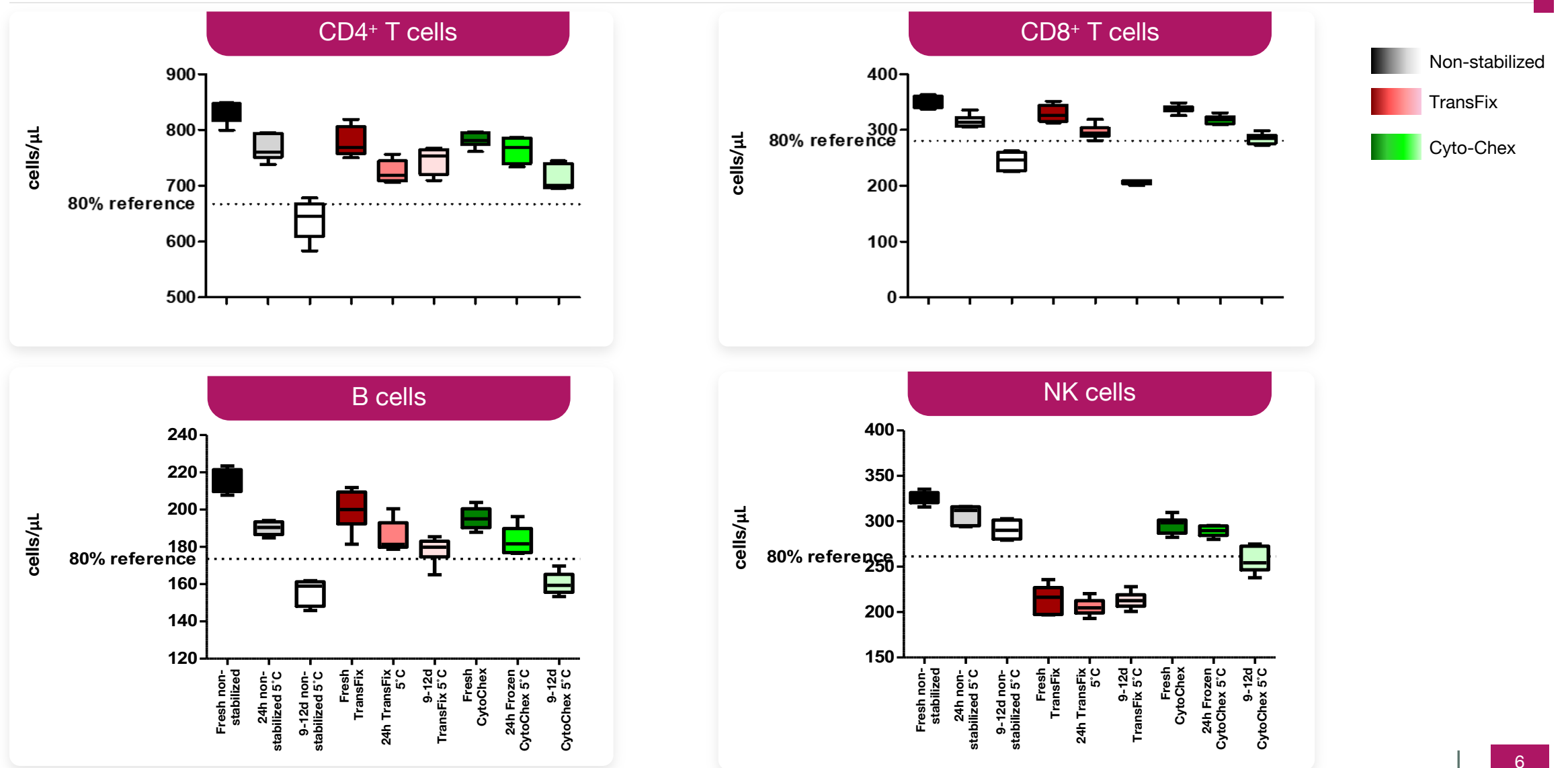
T, B, and NK Immunophenotyping Case Study

✔ Compare the following storage conditions in 5 healthy donors



✔ 80% of the results per cell type should be within $\pm 20\%$ change of the baseline (freshly analyzed non-stabilized blood)

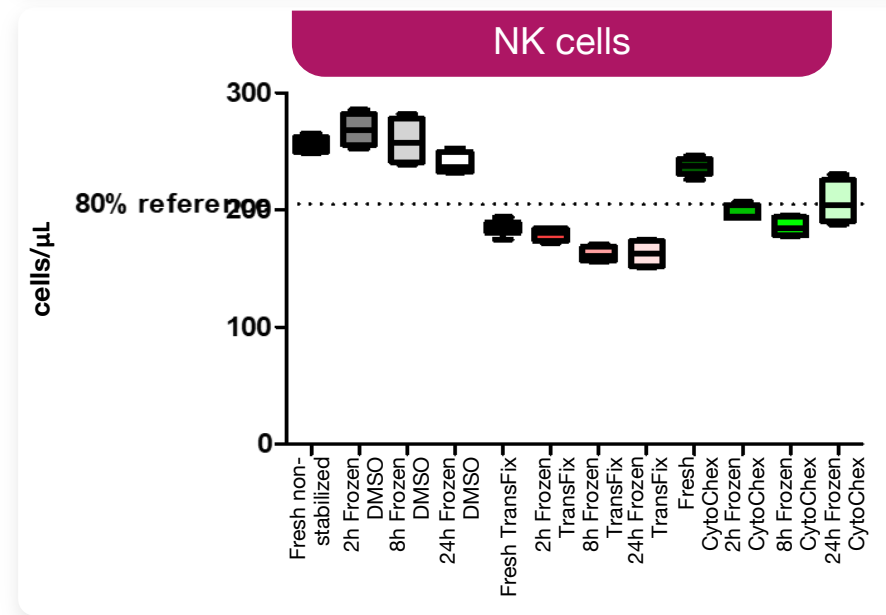
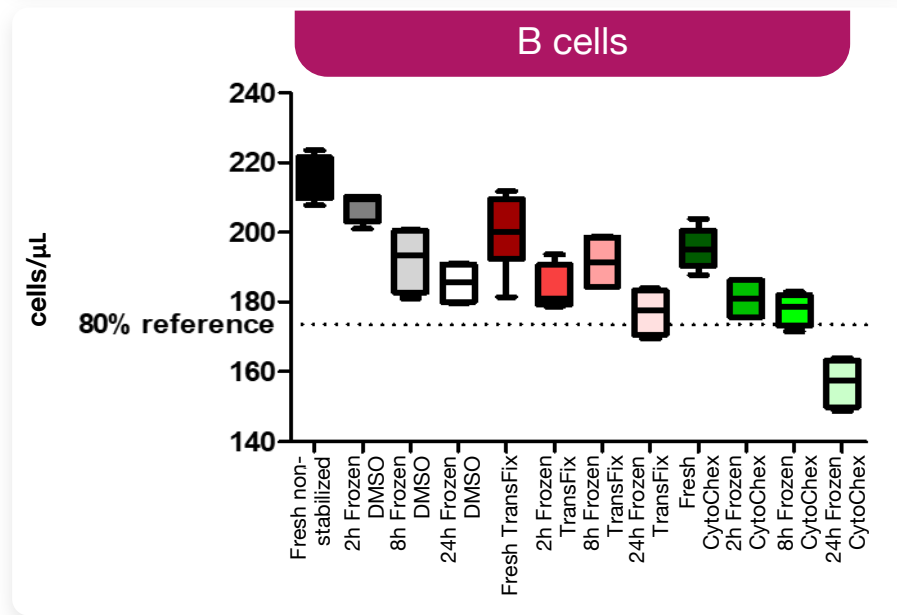
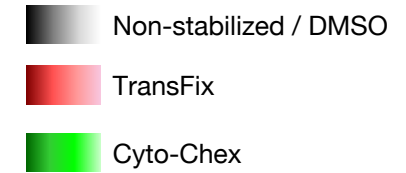
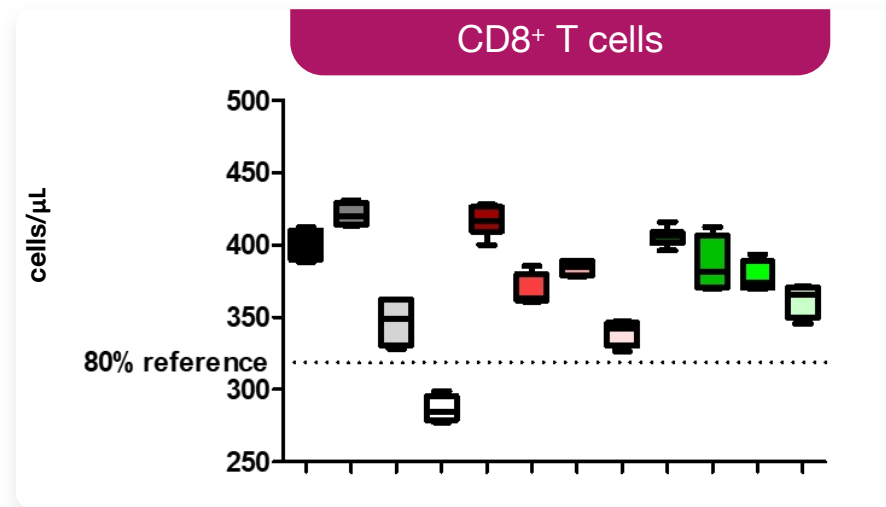
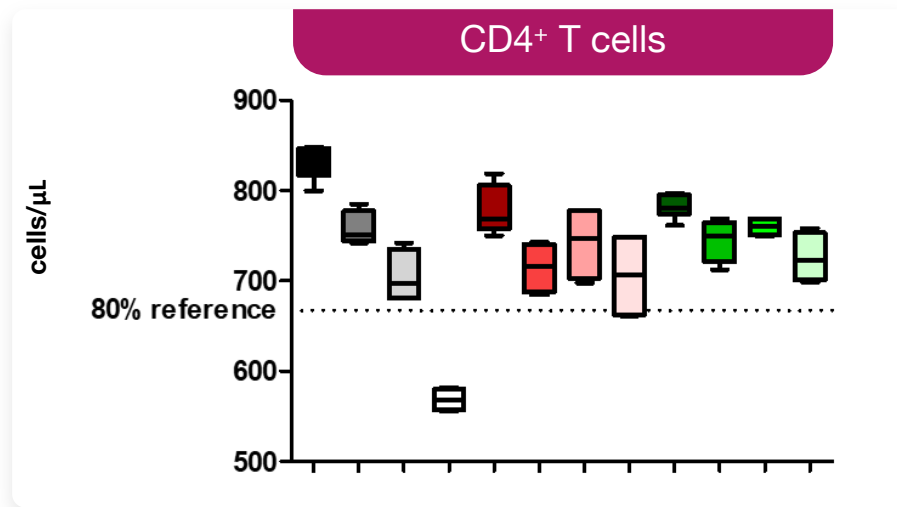
TransFix and Cyto-Chex Baseline and Stability at 5°C



Summary of TransFix and Cyto-Chex Stability at 5°C (9-12 days)

	CD4+ T cells cells/ μ L	CD4+ T cells % lymphocytes	CD8+ T cells cells/ μ L	CD8+ T cells % lymphocytes	B cells cells/ μ L	B cells % lymphocytes	NK cells cells/ μ L	NK cells % lymphocytes
Non-stabilized	4/4 donors <80% of reference	4/4 donors within 80- 120% of reference,	4/4 donors <80% of reference	3/4 donors within 80- 120% of reference	4/4 donors <80% of reference	2/4 donors <80% of reference	3/4 donors within 80- 120% of reference	4/4 donors within 80- 120% of reference
	0% pass	100% pass	0% pass	75% pass	0% pass	50% pass	75% pass	100% pass
TransFix	4/4 donors within 80- 120% of reference	4/4 donors within 80- 120% of reference	3/4 donors <80% of reference	2/4 donors <80% of reference	2/4 donors within 80- 120% . 1	4/4 donors within 80- 120% of reference	3/4 donors <80% of reference at baseline and all timepoint	1/4 donors <80% of reference at baseline and all timepoint
	TransFix does not stabilize CD8+ and NK cells at 5°C							
	100% pass	100% pass	25% pass	50% pass	75% pass	100% pass	25% pass	75% pass
Cyto-Chex	4/4 donors within 80- 120% refer	4/4 donors within 80- 120% refer	3/4 donors borderline 80% of reference	3/4 donors within 80- 120% refer	2/4 donors <80% of reference	4/4 donors within 80- 120% refer	2/4 donors <80% of reference	4/4 donors within 80- 120% refer
	Cyto-Chex does not stabilize B and NK cells absolute concentrations at 5°C							
	100% pass	100% pass	100% pass	100% pass	50% pass	100% pass	50% pass	100% pass

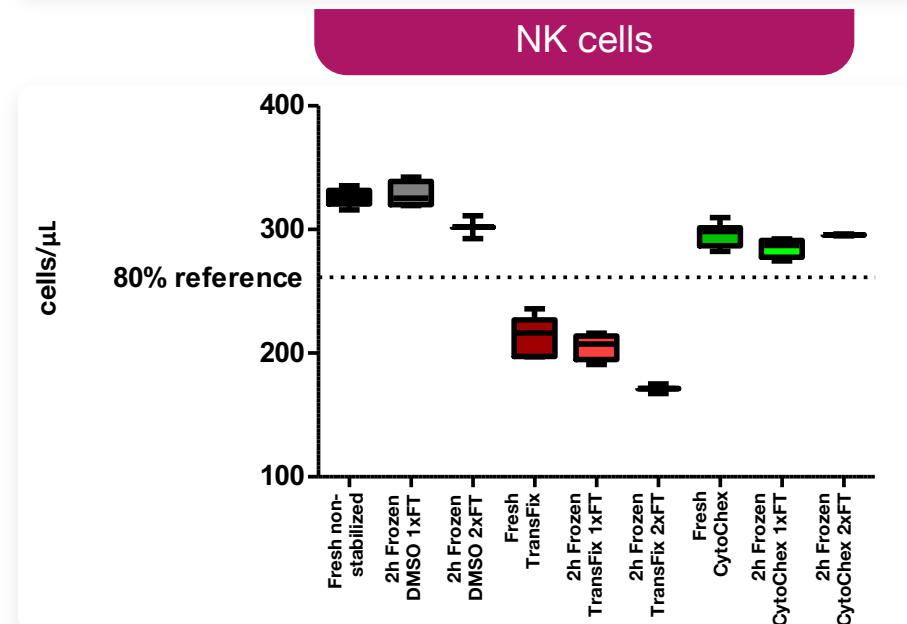
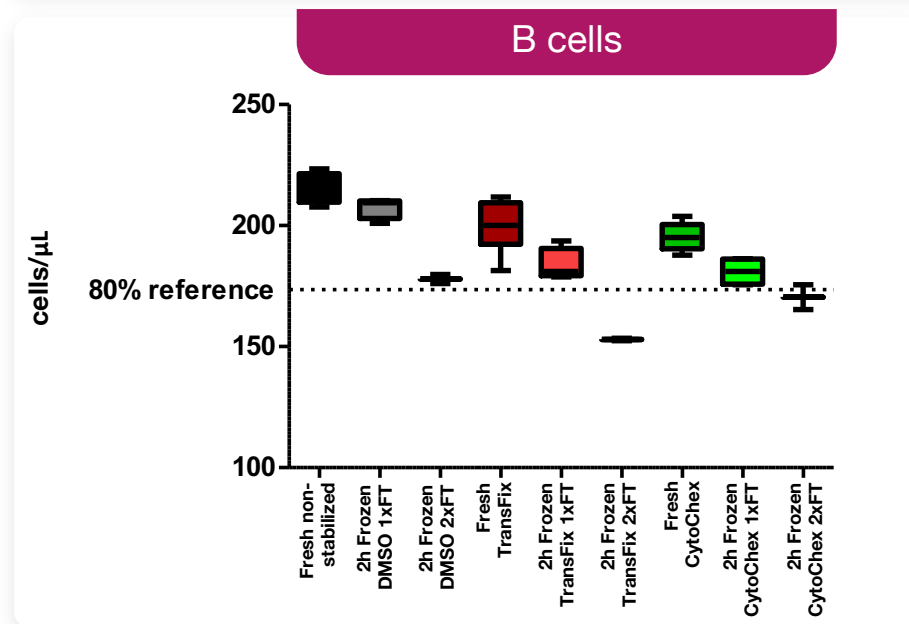
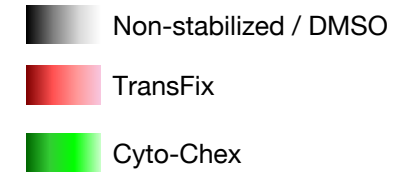
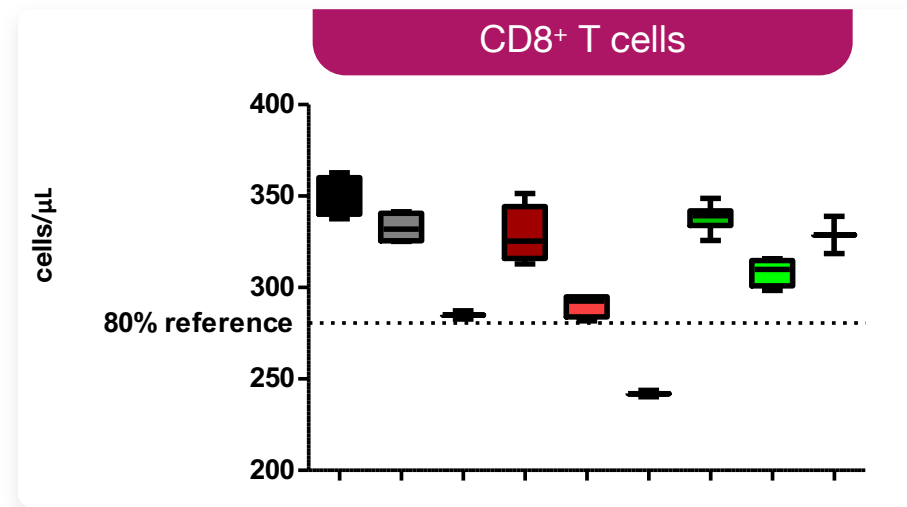
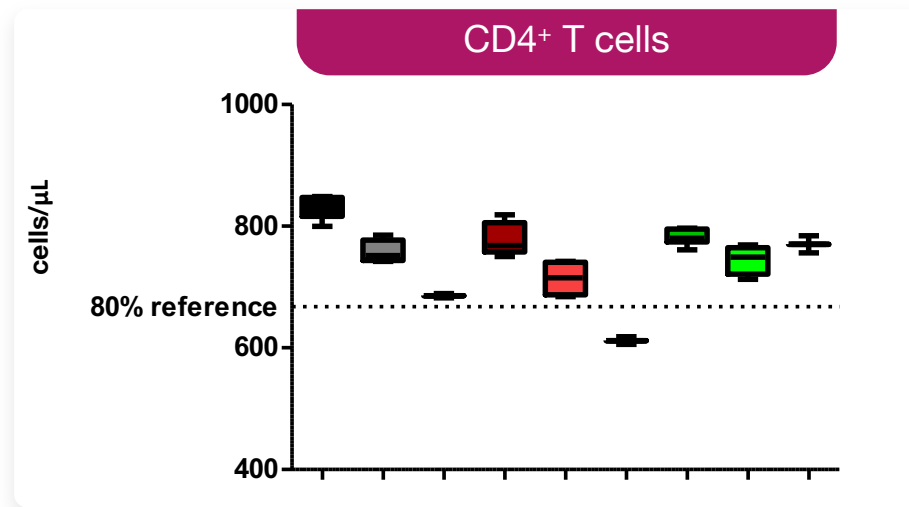
10% DMSO, TransFix and Cyto-Chex Stability at -80°C



Summary of 10% DMSO, TransFix and Cyto-Chex Stability at -80°C

	CD4 ⁺ T cells cells/μL	CD4 ⁺ T cells % lymphocytes	CD8 ⁺ T cells cells/μL	CD8 ⁺ T cells % lymphocytes	B cells cells/μL	B cells % lymphocytes	NK cells cells/μL	NK cells % lymphocytes
10% DMSO	5/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference
	10% DMSO stabilizes all cell types when stored at -80°C within 2h of collection							
	100% pass	100% pass	100% pass	100% pass	100% pass	100% pass	100% pass	100% pass
TransFix	5/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	3/5 donors <80% of reference at baseline and all timepoints	3/5 donors <80% of reference at baseline and all timepoints
	TransFix does not stabilize NK cells at baseline							
	100% pass	100% pass	100% pass	100% pass	100% pass	100% pass	40% pass	40% pass
Cyto-Chex	5/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	4/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference
	Cyto-Chex stabilizes all cell types when stored at -80°C within 2h of collection							
	100% pass	100% pass	100% pass	100% pass	100% pass	100% pass	80% pass	100% pass

10% DMSO, TransFix and Cyto-Chex 2x Freeze-Thaw Stability at -80°C



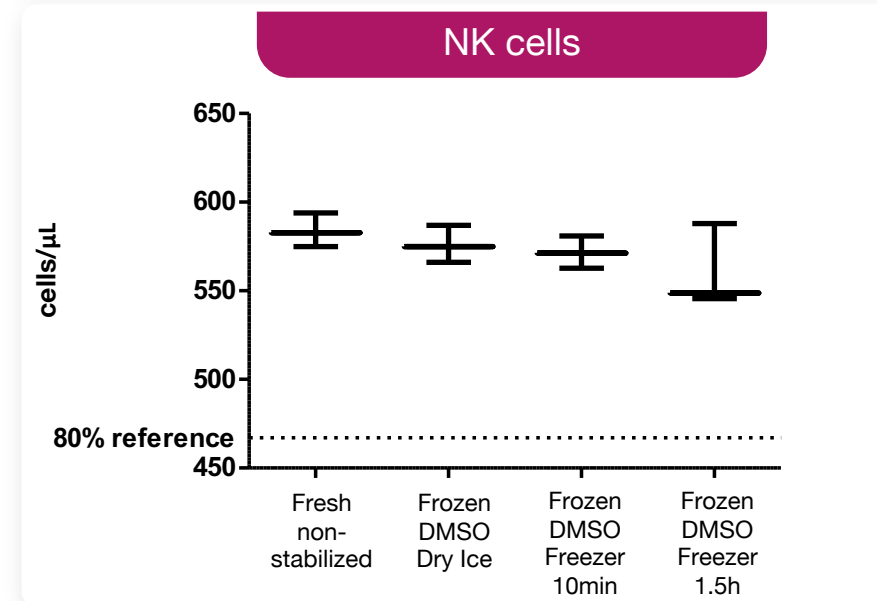
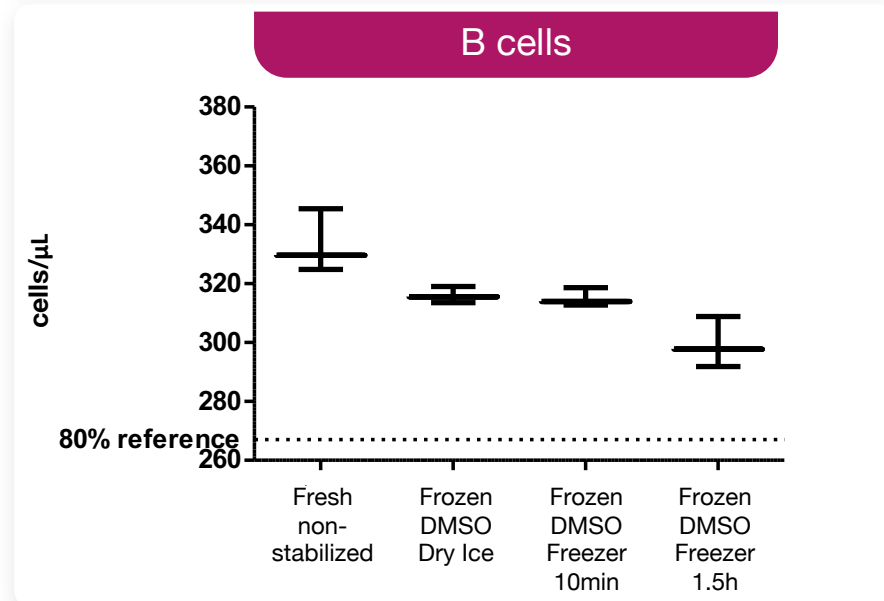
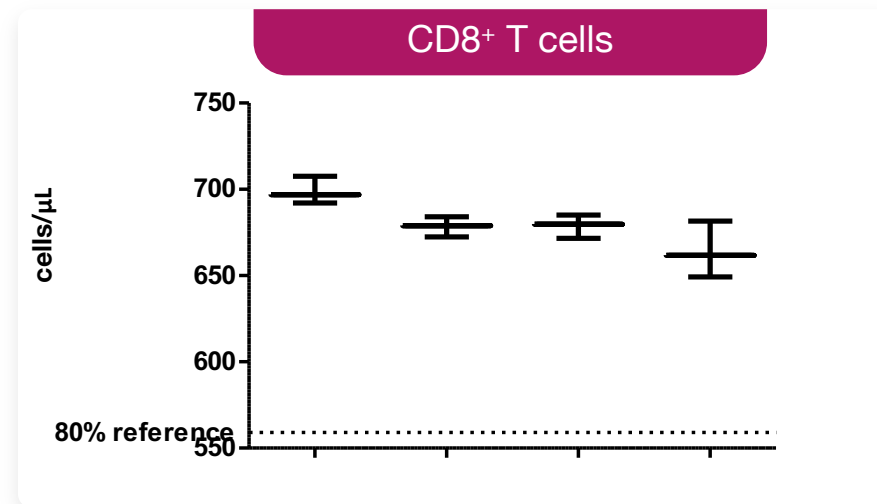
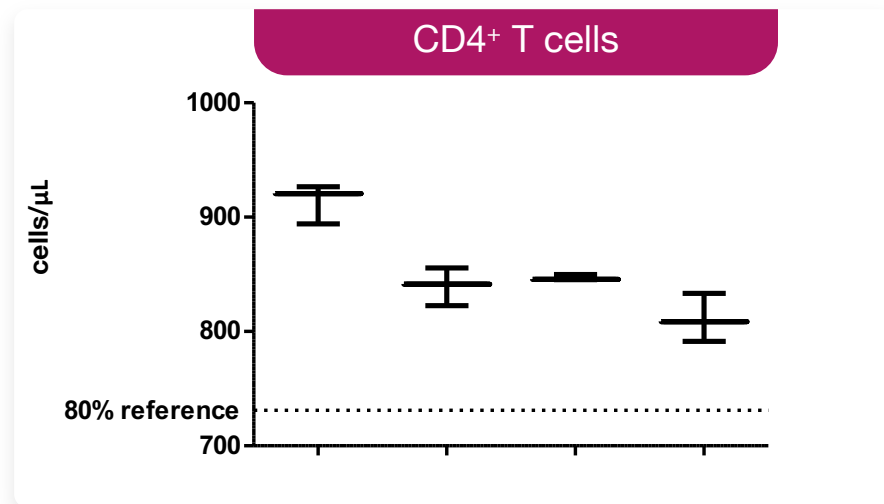
Summary of 10% DMSO, TransFix and Cyto-Chex Freeze-Thaw Stability

	CD4 ⁺ T cells cells/ μ L	CD4 ⁺ T cells % lymphocytes	CD8 ⁺ T cells cells/ μ L	CD8 ⁺ T cells % lymphocytes	B cells cells/ μ L	B cells % lymphocytes	NK cells cells/ μ L	NK cells % lymphocytes
10% DMSO	5/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference
	10% DMSO stabilizes all cell types at 2xFT							
	100% pass	100% pass	100% pass	100% pass	100% pass	100% pass	100% pass	100% pass
TransFix	4/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	4/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	4/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	3/5 donors <80% of reference at baseline and at timepoint	2/5 donors <80% of reference at baseline and at timepoint
	Transfix does not stabilize NK cells, other cell types borderline pass at 2xFT							
	80% pass	100% pass	80% pass	100% pass	80% pass	100% pass	40% pass	60% pass
Cyto-Chex	5/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	4/5 donors within 80- 120% of reference	5/5 donors within 80- 120% of reference	3/5 donors within 80- 120% of reference	3/5 donors within 80- 120% of reference
	Cytochex does not stabilize NK cells at 2xFT							
	100% pass	100% pass	100% pass	100% pass	80% pass	100% pass	60% pass	60% pass

Summary of Frozen Stabilizers Comparison

	Relative Accuracy to Fresh Non-Stabilized Samples	Freeze-Thaw Stability	Ease of Gating	Suitability for Clinical Collection
10% DMSO	<ul style="list-style-type: none"> All cell types pass when frozen within 2-8h of collection 	<ul style="list-style-type: none"> All cell types pass at 2xFT when frozen within 2-8h of collection 	<ul style="list-style-type: none"> Similar to fresh non-stabilized 	<ul style="list-style-type: none"> 10% DMSO has to be manually added to a defined volume of whole blood (or vice versa) Speed of freezing after DMSO addition critical? Cheapest option
TransFix	<ul style="list-style-type: none"> Baseline NK enumeration not accurate 	<ul style="list-style-type: none"> NK cells fail at 2xFT when frozen within 2h of collection All cell types fail at 2xFT when frozen within 8h of collection 	<ul style="list-style-type: none"> NK gating more challenging 	<ul style="list-style-type: none"> Direct collection Total volume needs to be estimated to calculate the correct dilution factor Underfilling the tube might lead to inaccurate results?
Cyto-Chex	<ul style="list-style-type: none"> All cell types pass when frozen within 2h of collection NK cells fail when frozen within 8h of collection 	<ul style="list-style-type: none"> NK cells fail at 2xFT when frozen within 2-8h of collection, other cell types borderline pass 	<ul style="list-style-type: none"> NK gating very challenging 	<ul style="list-style-type: none"> Direct collection Underfilling the tube might lead to inaccurate results?
Best Choice	10% DMSO	10% DMSO	10% DMSO	???

10% DMSO Freezing Speed Comparison



Summary of Frozen Stabilizers Comparison

	Relative Accuracy to Fresh Non-Stabilized Samples	Freeze-Thaw Stability	Ease of Gating	Suitability for Clinical Collection
10% DMSO	<ul style="list-style-type: none"> All cell types pass when frozen within 2-8h of collection 	<ul style="list-style-type: none"> All cell types pass at 2xFT when frozen within 2-8h of collection 	<ul style="list-style-type: none"> Similar to fresh non-stabilized 	<ul style="list-style-type: none"> 10% DMSO has to be manually added to a defined volume of whole blood (or vice versa) Speed of freezing not critical within ~2h of DMSO addition Cheapest option
TransFix	<ul style="list-style-type: none"> Baseline NK enumeration not accurate 	<ul style="list-style-type: none"> NK cells fail at 2xFT when frozen within 2h of collection All cell types fail at 2xFT when frozen within 8h of collection 	<ul style="list-style-type: none"> NK gating more challenging 	<ul style="list-style-type: none"> Direct collection Total volume needs to be estimated to calculate the correct dilution factor Underfilling the tube might lead to inaccurate results?
Cyto-Chex	<ul style="list-style-type: none"> All cell types pass when frozen within 2h of collection NK cells fail when frozen within 8h of collection 	<ul style="list-style-type: none"> NK cells fail at 2xFT when frozen within 2-8h of collection, other cell types borderline pass 	<ul style="list-style-type: none"> NK gating very challenging 	<ul style="list-style-type: none"> Direct collection Underfilling the tube might lead to inaccurate results?
Best Choice	10% DMSO	10% DMSO	10% DMSO	10% DMSO

Validation of a Flow Cytometric TBNK Immunophenotyping Assay in 10% DMSO-Stabilized Whole Blood

Parameter	Assessment	Acceptance criteria	Comments	Result
Overall run acceptance criteria	2 sets of 2 commercial QCs per run	% lymphocytes: 100% must be within the accepted ranges cells/ μ L: 50% per cell type and 80% overall must be within the accepted ranges	QC accepted ranges: <ul style="list-style-type: none"> Assess in 4 precision runs over 2 days If mean results outside the vendor ranges, calculate new ranges based on experimental mean + 3xSD 	All QC precision results within vendor ranges
Intra-assay and inter-assay precision	At least 5 healthy donors analysed in triplicate in 4 runs by 2 analysts over at least 2 days	$\leq 20\%$ CV for all results within and overall runs	Add-on validation of precision and stability in the respective diseased samples should be performed prior to analysing diseased samples	$<12\%$ CV pass
Instrument carryover	Sequential analysis of validation samples or QCs and «blank» buffer samples Data from the blank samples evaluated in the same gating template as the validation/QC samples.	$<20\%$ of the CD3 ⁺ CD4 ⁺ cells/ μ L of the BD Multi-Check CD4 Low Control		0.6 cells/ μ L pass
Long-term stability	At least 5 healthy donors analysed at baseline (within 2h of collection without stabilizer) and at various timepoints (1 month, 3 months, 6 months) in triplicate	80% of the results per cell type are within $\pm 20\%$ change of the baseline	Freshly collected baseline samples processed in an independent run	Assessment ongoing
Short-term & freeze-thaw stability	At least 5 healthy donors analysed at baseline (within 2h of collection without stabilizer) and at least 2x freeze-thaw cycles in triplicate	80% of the results per cell type are within $\pm 20\%$ change of the baseline	Same baseline samples as for the long-term stability	3x FT pass
Post-staining/ processed sample stability	At least 5 healthy donors and 2 QCs analyzed immediately after processing (within 1h of staining) and at various timepoints after storage at 5°C (e.g. 6h, 16h, 24h)	80% of the results per cell type are within $\pm 20\%$ change of the baseline	Precision runs used as the baseline	up to 24h pass

Conclusions & Critical Considerations



Sample stability assessment is a critical parameter in flow cytometry method development and validation



Ambient temperature, refrigerated, or frozen stability with various stabilizers must be assessed for each marker panel and Context of Use due to differential effects on cell integrity, antigen conformation and affinity, autofluorescence, and fluorochrome quenching



Stability of multiple samples should be assessed, ideally both healthy and diseased



TransFix and Cyto-Chex storage at RT/5°C approved for IVD immunophenotyping of HIV patients → different Context of Use and readout (e.g. CD4+/CD8+ ratio) compared to case study (T, B, NK cells/ μ L)



The effect of the stabilizer and storage conditions on the gating hierarchy should also be assessed and, if necessary, the gating strategy adapted



The suitability for clinical collection should also be evaluated



General stability acceptance criteria: 80% of the results *per cell type* should be within $\pm 20\%$ change of the baseline → *depends on the Context of Use*

Acknowledgements



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Andrea Maddalena, Scientist Molecular and Cell Biology



Johannes Stanta, Global Director Molecular and Cell Biology

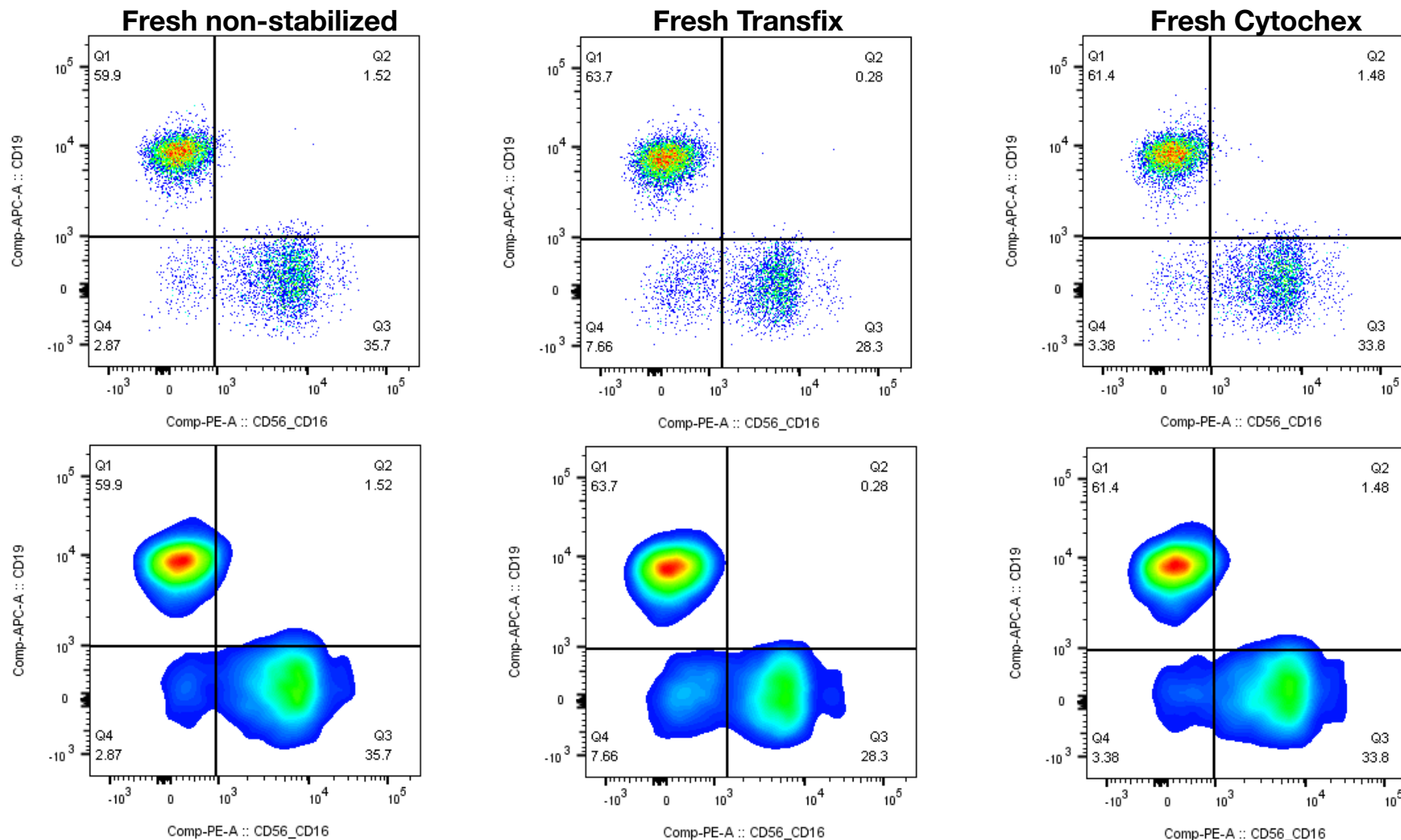


Petra Struwe, Vice President Bioanalytical Services

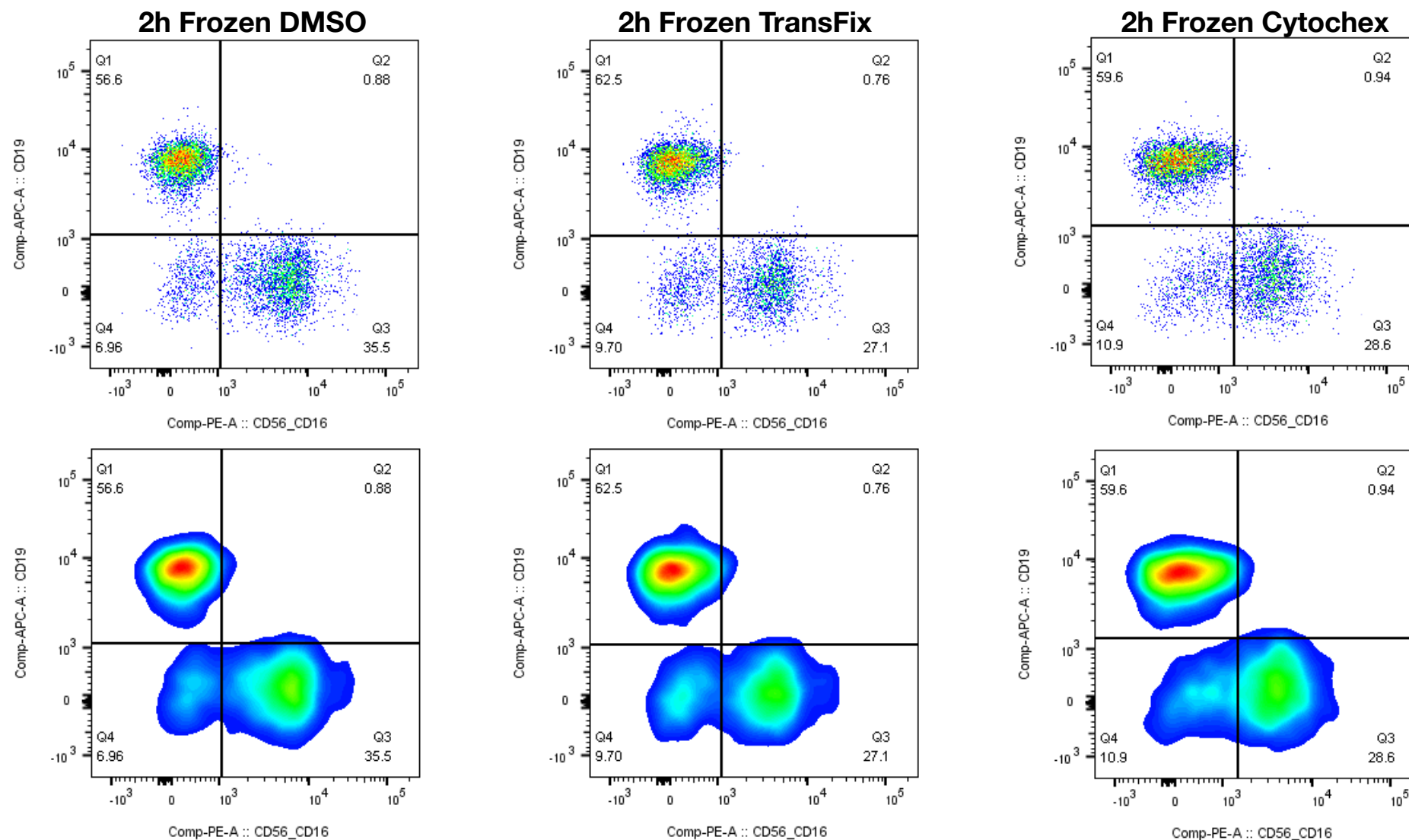
THANK YOU

Back up slides

B and NK Gating Challenges



B and NK Gating Challenges



Summary of 10% DMSO, TransFix and Cyto-Chex Stability at -80°C (8h)

parameter	CD4+ cells/ μ L	CD4+ % lymphocytes	CD8+ cells/ μ L	CD8+ % lymphocytes	B cells cells/ μ L	B cells % lymphocytes	NK cells cells/ μ L	NK cells % lymphocytes
10% DMSO	5/5 donors within 80-120% of reference 100% pass	5/5 donors within 80-120% of reference 100% pass	5/5 donors within 80-120% of reference 100% pass	5/5 donors within 80-110% of reference 100% pass	5/5 donors within 80-120% of reference 100% pass	5/5 donors within 80-120% of reference 100% pass	5/5 donors within 80-120% of reference 100% pass	5/5 donors within 80-120% of reference 100% pass
TransFix	5/5 donors within 80-120% of reference 100% pass	5/5 donors within 80-110% of reference 100% pass	5/5 donors within 80-120% of reference 100% pass	5/5 donors within 80-110% of reference 100% pass	5/5 donors within 80-120% of reference 100% pass	5/5 donors within 80-120% of reference 100% pass	3/5 donors <80% of reference at baseline and all timepoint 40% pass	3/5 donors <80% of reference at baseline and all timepoint 40% pass
CytoChex	5/5 donors within 80-120% of reference 100% pass	5/5 donors within 80-110% of reference 100% pass	5/5 donors within 80-120% of reference 100% pass	5/5 donors within 80-110% of reference 100% pass	5/5 donors within 80-120% of reference 100% pass	5/5 donors within 80-120% of reference 100% pass	3/5 donors within 80-120% of reference 60% pass	3/5 donors within 80-120% of reference 60% pass

Conclusions:

- DMSO stabilizes all cell types when stored at -80°C within 8h
- TransFix does not stabilize NK cells at baseline
- CytoChex does not stabilize NK cells when stored at -80°C within 8h

Summary of 10% DMSO, TransFix and Cyto-Chex 2x Freeze-Thaw Stability (8h)

parameter	CD4+ cells/ μ L	CD4+ % lymphocytes	CD8+ cells/ μ L	CD8+ % lymphocytes	B cells cells/ μ L	B cells % lymphocytes	NK cells cells/ μ L	NK cells % lymphocytes
10% DMSO	5/5 donors within 80-120% of reference 100% pass	5/5 donors within 80-120% of reference 100% pass	4/5 donors within 80-120% of reference 80% pass	5/5 donors within 80-120% of reference 100% pass	5/5 donors within 80-120% of reference 100% pass	5/5 donors within 80-120% of reference 100% pass	5/5 donors within 80-120% of reference 100% pass	5/5 donors within 80-120% of reference 100% pass
TransFix	3/5 donors within 80-120% of reference 60% pass	5/5 donors within 80-120% of reference 100% pass	3/5 donors within 80-120% of reference 60% pass	5/5 donors within 80-120% of reference 100% pass	3/5 donors within 80-120% of reference 60% pass	5/5 donors within 80-120% of reference 100% pass	3/5 donors <80% of reference at baseline and all timepoint 40% pass	3/5 donors <80% of reference at baseline and all timepoint 40% pass
Cyto-Chex	4/5 donors within 80-120% of reference 80% pass	5/5 donors within 80-120% of reference 100% pass	4/5 donors within 80-120% of reference 80% pass	5/5 donors within 80-120% of reference 100% pass	4/5 donors within 80-120% of reference 80% pass	5/5 donors within 80-120% of reference 100% pass	1/5 donors within 80-120% of reference 20% pass	3/5 donors within 80-120% of reference 60% pass

Conclusions:

- **DMSO stabilizes all cell types (CD8+ borderline) at 2xFT when frozen within 8h**
- **Transfix does not stabilize anything at 2xFT when frozen within 8h of blood collection**
- **Cytochex does not stabilize NK cells at 2xFT when frozen within 8h of blood collection**