Point-of-Collection Whole Blood Fixation Enables Robust Phosphoprotein Profiling by Mass Cytometry for Longitudinal Clinical Trials

Lily Shang, PhD; McKenna Coughlin; Kristina Magee, MD, PhD; Carly Lancaster, PhD; Sacha Martiny; Landon Wellendorf; Justin Jarrell, PhD; Ramji Srinivasan; Li-Chun Cheng, PhD
Teiko, Salt Lake City, UT, and San Mateo, CA, USA

Introduction

Phosphoprotein profiling by cytometry is increasingly used as a pharmacodynamic readout in clinical trials, yet sample handling remains a critical and under addressed challenge. Annual JAK inhibitor trial counts increased from 61 in 2015 to 206 in 2024. Phosphoprotein signaling states are highly labile *ex vivo*, and without immediate stabilization, phosphorylation can shift from its *in vivo* state within minutes of collection. Conventional PBMC isolation and cryopreservation compound this problem. Ficoll centrifugation introduces handling-induced phosphosignaling artifacts, and freeze-thaw stress depletes monocytes and other fragile populations. Together, these effects suppress basal signals and often make *ex vivo* stimulation necessary to detect any signal at all. These limitations are particularly acute in longitudinal clinical trials, where samples must be shipped from remote clinical sites to central labs before processing. Here we describe a workflow that integrates point-of-collection whole blood fixation using TokuKit with phospho-mass cytometry, enabling phosphoprotein profiling across peripheral blood immune cell subsets.

PBMC & Whole Blood Sample Prep and Collection

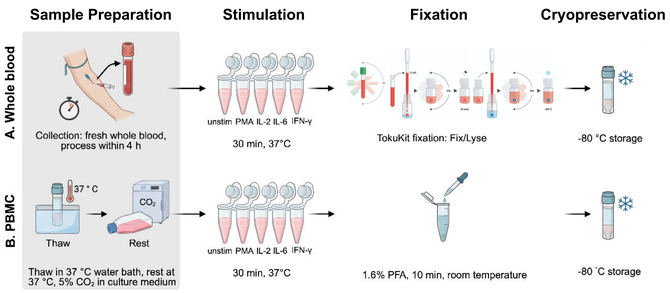


Figure 1. Sample Prep and Collection Workflows for PBMCs and Whole Blood.
Parallel processing of whole blood and PBMC. Both sample types are stimulated for 30 min at 37°C with PMA/ionomycin, IL-2, IL-6, IFN-γ, or left unstimulated. Whole blood samples are fixed in TokuKit with concurrent red blood cell lysis. PBMCs are fixed in 1.6% PFA for 10 min at room temperature. Fixed samples are cryopreserved at −80°C prior to barcoding and staining.

Sample Processing

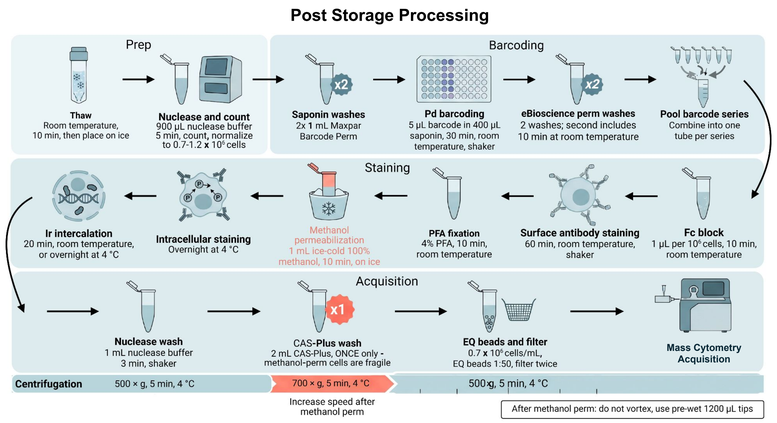


Figure 2. Post-Collection Sample Processing Workflow.
Post-storage workflow from thaw through mass cytometry acquisition, including palladium barcoding, surface and intracellular staining, methanol permeabilization, and iridium intercalation. Centrifugation speed is increased to 700 × g after methanol permeabilization because permeabilized cells are fragile. The post-permeabilization CAS-Plus wash is limited to one wash to minimize cell loss.

Gating Strategy

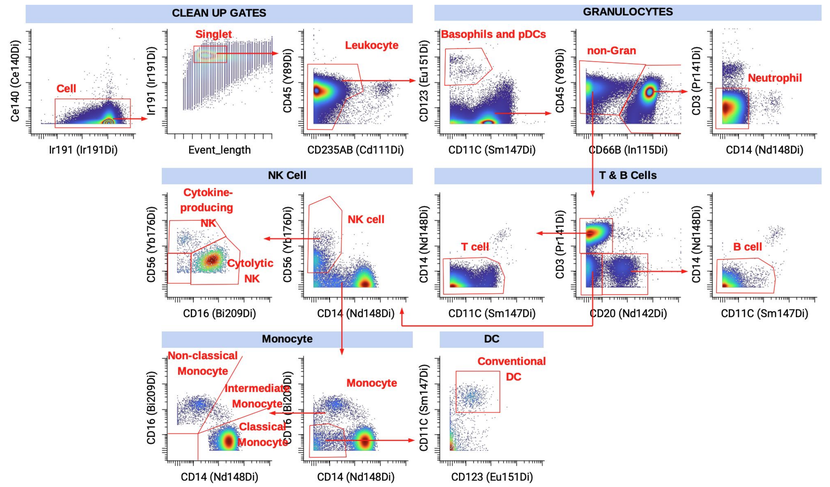


Figure 3. Gating scheme.
Representative gating strategy for major immune subsets by mass cytometry. Clean-up gates (cell, singlet, leukocyte) are followed by gating of basophils and pDCs and of neutrophils using CD123, CD11c, CD45, and CD66b, and gating of non-granulocyte leukocytes into T cells, B cells, cytokine-producing and cytolytic NK cells, conventional DCs, and classical, intermediate, and non-classical monocytes using CD3, CD20, CD56, CD11c, CD123, CD16, and CD14.

TokuKit Enables Fixation Of Phosphosignals

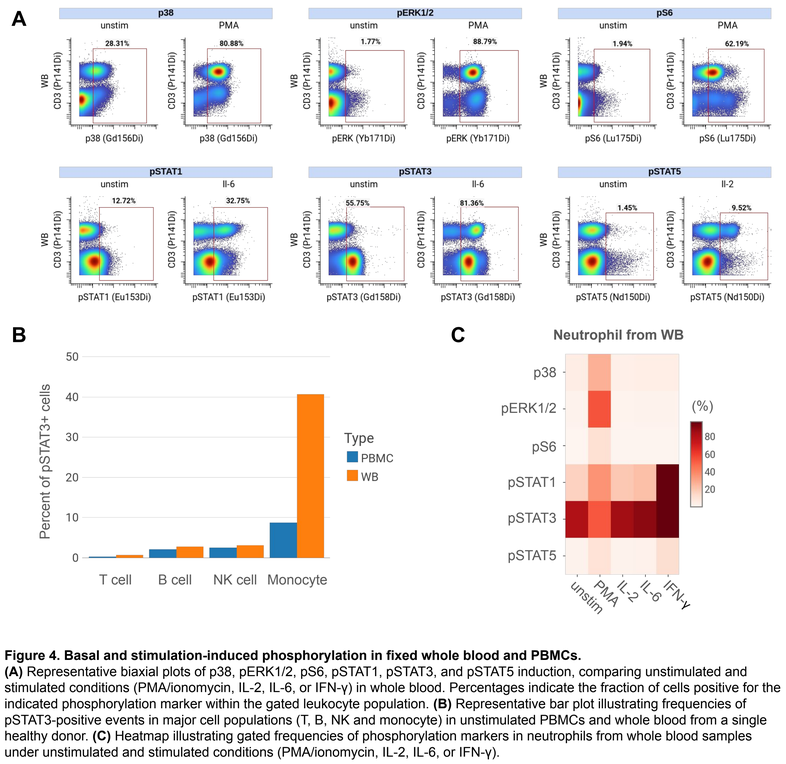
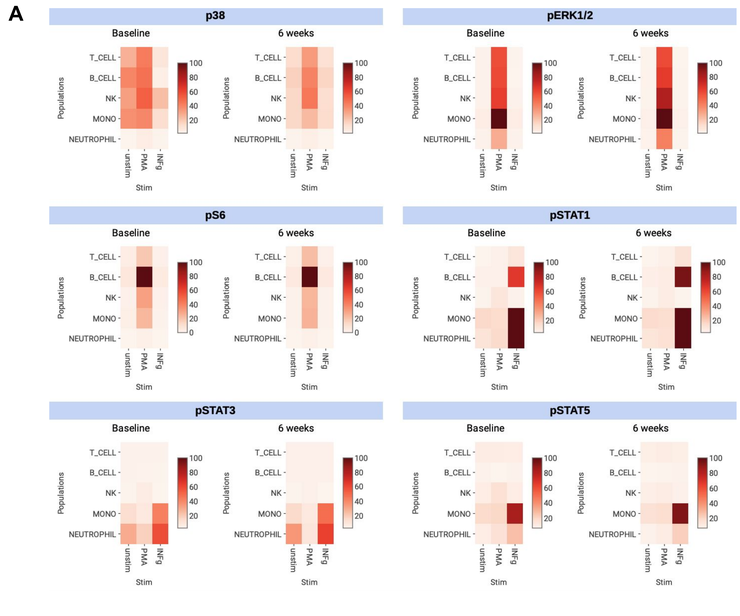


Figure 4. Basal and stimulation-induced phosphorylation in fixed whole blood and PBMCs.
(A) Representative biaxial plots of p38, pERK1/2, pS6, pSTAT1, pSTAT3, and pSTAT5 induction, comparing unstimulated and stimulated conditions (PMA/ionomycin, IL-2, IL-6, or IFN-γ) in whole blood. Percentages indicate the fraction of cells positive for the indicated phosphorylation marker within the gated leukocyte population. **(B)** Representative bar plot illustrating frequencies of pSTAT3-positive events in major cell populations (T, B, NK and monocyte) in unstimulated PBMCs and whole blood from a single healthy donor. **(C)** Heatmap illustrating gated frequencies of phosphorylation markers in neutrophils from whole blood samples under unstimulated and stimulated conditions (PMA/ionomycin, IL-2, IL-6, or IFN-γ).

Phosphosignals Are Stable Over 6 weeks



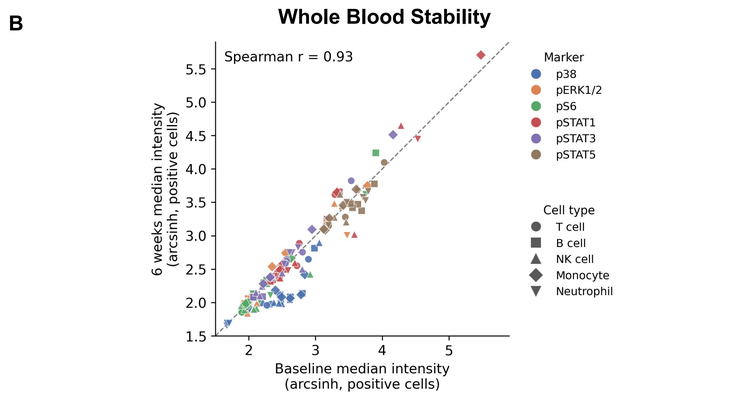


Figure 5. Storage stability of stimulation-induced signal.
(A) Heatmaps show the percentage of cells positive for p38, pERK1/2, pS6, pSTAT1, pSTAT3, and pSTAT5 across five immune populations (T cells, B cells, NK cells, monocytes, and neutrophils) and five stimulation conditions (unstimulated, PMA/ionomycin, IL-2, IL-6, and IFN-γ) in whole blood at baseline and after 6 weeks of storage. **(B)** Median signal intensity of phosphoprotein-positive cells (arcsinh-transformed, cofactor 5) at 6 weeks versus baseline for all 150 marker, population, and stimulation combinations in (A). Colors indicate markers and shapes indicate cell types. Dashed line, identity.

Conclusion

TokuKit whole blood fixation preserves basal *in vivo* phosphoprotein states across multiple signaling pathways, maintaining signal integrity for downstream profiling without the monocyte depletion and pathway perturbations associated with PBMC cryopreservation. Because fixation happens immediately at the point of collection, this approach is particularly well-suited for longitudinal clinical trials, where variable transit times and storage conditions make immediate processing impractical. These data support TokuKit-based whole blood collection as a practical platform and workflow for pharmacodynamic monitoring and immune profiling in multisite studies.

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