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Technical note

Isolation of intact RNA from murine CD4$^+$ T cells after intracellular cytokine staining and fluorescence-activated cell sorting

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ABSTRACT

Intracellular cytokine staining (ICS) is a powerful method for identifying functionally distinct lymphocyte subsets, and for isolating these by fluorescence activated cell sorting (FACS). Although transcriptomic analysis of cells sorted on the basis of ICS has many potential applications, this is rarely performed because of the difficulty in isolating intact RNA from cells processed using standard fixation and permeabilization buffers for ICS. To address this issue, we compared three buffers shown previously to preserve RNA in nonhematopoietic cells subjected to intracellular staining for their effects on RNA isolated from T lymphocytes processed for ICS. Our results showed that buffers containing the recombinant ribonuclease inhibitor RNasin or high molar concentrations of salt yielded intact RNA from fixed and permeabilized T cells. As proof of principle, we successfully used the buffer containing RNasin to isolate intact RNA from CD4$^+$ T cells that were sorted by FACS on the basis of specific cytokine production, thus demonstrating the potential of this approach for coupling ICS with transcriptomic analysis.

1. Introduction

Intracellular cytokine staining (ICS) is a common method for analysis of immune cells, especially T cells. This typically requires fixation of previously stimulated cells with paraformaldehyde and membrane permeabilization using a detergent, followed by staining with appropriate anti-cytokine antibodies. Cells are then analyzed by flow cytometry to identify functional subsets. Although cells stained in this manner can be isolated by fluorescence-activated cell sorting (FACS), this is seldom done since they are no longer viable and their constituents undergo chemical modifications due to the fixation step that complicate subsequent extraction and analysis. These modifications include cross-linkage between nucleic acids and proteins and covalent modification of RNA by addition of monomethylol groups to the bases (Farragher et al., 2008). Downstream analysis of these cells including transcriptomics depends upon the development of methods that allow isolation of high quality nucleic acids from these cells. This is partly because cell surface cytokine capture assay, an alternative method that allows purification of cytokine-secreting cells, requires bifunctional antibodies and is laborious, while suffering from limited sensitivity (Kunnath-Velayudhan et al., 2017). In addition, currently available commercial cell surface cytokine capture assays are limited to a few cytokines (http://www.miltenyibiotec.com).

Research over recent decades resulted in protocols that allow successful extraction of nucleic acids from fixed cells, as shown in the context of formalin-fixed paraffin embedded (FFPE) tissue samples (Farragher et al., 2008). The most successful of these methods uses proteinase K digestion prior to acid-phenol chloroform extraction and carrier precipitation. Proteinase K readily destroys proteins despite their highly cross-linked nature. Use of proteinase K was adopted by many commercial kits that enable successful RNA isolation from FFPE samples. Transcriptomic studies performed using RNA isolated by these methods showed that while fragmentation and modifications of isolated RNA is a concern, there is high correlation of transcriptome profiles between fresh frozen and FFPE samples (Hedegaard et al., 2014). However, when one of those commercial methods was applied to perform transcriptomic studies of primary human CD4$^+$ T cells infected with HIV-1, the RNA isolated from these cells showed degradation that likely occurred during the ICS procedure (Iglesias-Ussel et al., 2013).

More recent reports have proposed modifications in the buffers used for intracellular staining in addition to the use of modified RNA isolation protocols to isolate intact RNA from fixed and permeabilized cells. One of these buffers called RNA preserving hybridization buffer, was used in fluorescent in-situ hybridization experiments of mouse induced

Abbreviations: FACS, fluorescence-activated cell sorting; ICS, intracellular cytokine staining; FFPE formalin-fixed, paraffin-embedded; RIN, RNA integrity number

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Table 1
Summary of the ICS staining buffers used and the quality of RNA obtained.

Buffer	Composition	Yield (ng)[a, b]	280/260 ratio[a]	RIN[a]
Standard permeabilization buffer	PBS containing FBS (2%), sodium azide (0.05%) saponin (0.1%) and normal rat serum (10%); pH 7.4	575.7 (477.0–615.0)	2.01 (1.81–2.13)	3.8 (2.3–5.2)
RNA preserving hybridization buffer	2× SSC containing ammonium sulfate (2.1 M), EDTA (10 mM), ultrapure BSA (1 mg/ml), formamide (25% [40% v/v]) and saponin (0.1%); pH 5.2	NP	NP	NP
High salt buffer	PBS containing sodium azide (0.05%) saponin (0.1%), purified rat IgG (0.02 mg/ml), ultrapure BSA (1 mg/ml) and sodium chloride (2.0 M); pH 7.4	585.3 (416.4–747.0)	2.03 (1.72–2.15)	9.1 (7.8–10.0)
Buffer containing RNase inhibitor	PBS containing sodium azide (0.05%), saponin (0.1%), purified rat IgG (0.02 mg/ml), ultrapure BSA (1 mg/ml) and RNase inhibitor (RNasin® Plus RNase Inhibitor, 0.5 U/ml); pH 7.4	574.9 (492.0–645.0)	1.98 (1.70–2.13)	9.0 (8.1–10.0)

NP not performed.
Results represent two independent experiments (n = 3).
[a] Average value with range in parenthesis.
[b] Total yield per million cells.

pluripotent stem cells (Klemm et al., 2014). Another buffer called high salt buffer contained 2.0 M NaCl and was used for intracellular staining for cytokeratin in a human renal cell line (Nilsson et al., 2014). A third buffer condition included a commercially available recombinant protein inhibitor to mammalian RNases (RNasin) and was used to isolate intact RNA from primary human radial glial (Thomsen et al., 2016) and pancreatic (Hrvatin et al., 2014) cells after intracellular protein staining and FACS sorting. All these studies showed that RNA isolated by these methods was suitable for downstream analysis including transcriptome profiling. In addition, a high correlation was observed between transcriptome profiles of live cells and cells which underwent fixation and permeabilization (Hrvatin et al., 2014; Klemm et al., 2014; Thomsen et al., 2016). However, these protocols were neither validated for cytokine staining nor used with hematopoietic cell types. In the current study, we assessed these buffers in the context of ICS of murine T cells and subsequent isolation of intact RNA.

2. Materials and methods

2.1. Mice

Six- to 8-wk-old female wild-type C57BL/6 mice were obtained from The Jackson Laboratory. All mice were maintained in specific pathogen-free conditions. All procedures involving the use of animals were in compliance with protocols approved by the Einstein Institutional Animal Use and Biosafety Committees.

2.2. Preparation of murine splenocytes and stimulation

Splenocyte suspensions were prepared by gently forcing spleens through a 70 μm cell strainer. RBC lysis was performed using RBC lysing buffer Hybri-Max (Sigma). The cells were washed with media [RPMI medium (Gibco) supplemented with FBS (10%; Atlanta Biologicals), penicillin-streptomycin (1%; Gibco), HEPES (1%; Gibco), beta-mercaptoethanol (0.1%; Gibco), essential amino acids (0.5%; Gibco) and non-essential amino acids (0.5%; Gibco)] and plated in 96-well round-bottom plates with 2 million splenocytes per well. For stimulation, cells were incubated in the presence of Phorbol 12-Myristate 13-Acetate (PMA,1 μg/ml, Sigma), ionomycin (1 μg/ml, Sigma), brefeldin A (5 μg/ml; Sigma) and monensin (5 μM, Sigma) for 4 h at 37 °C. In some experiments, T cells or $CD4^+$ T cells were isolated from splenocytes using Pan T Cell Isolation Kit II or $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec) respectively.

2.3. Intracellular cytokine staining and FACS analysis and sorting

After stimulation, splenocytes were washed with PBS and incubated with viability dye (LIVE/DEAD Fixable Blue (or Violet for sorting experiments) Dead Cell Stain, Molecular Probes) diluted in PBS for 30 min at 4 °C. All incubation steps were performed in tubes shielded from light. Subsequently, the cells were washed with FACS buffer (PBS containing FBS [2%; Atlanta Biologicals] and sodium azide [0.05%; Sigma]) and blocked for 30 min at 4 °C with FACS buffer containing 10% normal rat serum and 10% normal mouse serum. Antibodies specific for cell surface markers diluted in FACS buffer were added directly to this mixture and incubated for 30 min at 4 °C. The cells were then washed with PBS and fixed by incubation with paraformaldehyde (4% in PBS; Electron Microscopy Sciences) for 10 min at room temperature. The cells were washed twice with permeabilization buffer (PBS with FBS [2%], sodium azide [0.05%], and saponin [0.1%; Sigma]) and blocked for 30 min at room temperature with permeabilization buffer containing 10% normal rat serum. Antibodies diluted in permeabilization buffer were added directly to this mixture. After overnight (16 h) staining at 4 °C, cells were washed with permeabilization buffer and PBS and resuspended in FACS buffer. The cells were kept on ice until the analysis using an LSR II flow cytometer (BD Biosciences).

When alternate ICS conditions were used, the above protocol was followed until the end of the fixation step and the buffers used for subsequent steps varied. For experiments involving the RNA preserving hybridization buffer (Klemm et al., 2014), the cells were permeabilized, blocked and stained in RNA preserving hybridization buffer (Table 1). The cells were then washed with RNA preserving hybridization buffer and PBS before resuspending in FACS buffer. For experiments involving the high salt buffer (Nilsson et al., 2014), the cells were permeabilized, blocked and stained in high salt buffer (Table 1). The cells were then washed with high salt buffer and resuspended in high salt buffer lacking saponin. For experiments involving buffer containing RNasin (RNasin® Plus RNase Inhibitor, Promega) (Thomsen et al., 2016), the cells were permeabilized, blocked and stained in RNasin containing buffer (Table 1). The cells were then washed with RNasin-containing buffer and resuspended in RNasin-containing buffer lacking saponin. In all experiments, antibodies were diluted in corresponding staining buffers.

For experiments involving FACS sorting of IFNγ-producing $CD4^+$ T cells, the cells were permeabilized, blocked and stained in RNasin-containing buffer (Table 1). The cells were then washed with RNasin-containing buffer and resuspended in RNasin-containing buffer lacking saponin. Cell sorting was performed with a BD FACS Aria (BD Biosciences) and IFNγ-producing $CD4^+$ T cells were collected into a buffer which contained RNasin (0.5 U/ml), ultrapure BSA (1 mg/ml), sodium azide (0.05%), and sodium chloride (2.0 M) in PBS.

The following antibodies were used for staining: CD4-APC-Cy7 (Clone RM4–5, Tonbo Biosciences), CD8α-PE-Cy5 (Clone 53–6.7, Tonbo Biosciences), B220-PE-Cy5 (Clone RA3-6B2, BD Biosciences), MHC II-PE-Cy5 (Clone M5/114.15.2, eBioscience), IFNγ-Alexa Fluor 700 (Clone XMG1.2, BD Biosciences), TNF-Alexa Fluor 488 (Clone MP6-XT22, BD Biosciences), IL-2-PE-Cy7 (Clone JES6-5H4, eBioscience) and GM-CSF-APC (Clone MP1-22E9, BD Biosciences). For sorting