

Video Article

The Use of Flow Cytometry to Assess the State of Chromatin in T Cells

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Abstract

During a proper immune response, quiescent T cells become activated upon antigen presentation to their antigen-specific T cell receptor. This leads to clonal proliferation of only those T cells that bear a receptor that recognizes the antigen. Chromatin decondensation is a hallmark of T cell activation and is required for T cells to acquire the ability to proliferate after antigen engagement. This change in chromatin condensation can be detected using antibodies raised against histone proteins. These antibodies cannot bind to their epitopes in naïve T cells as well as they can in activated T cells. We describe how to simultaneously stain T cell-specific surface markers, track viability with a fixable dead cell stain, and measure chromatin status via intracellular staining of Histone H3 proteins. Stained cells are analyzed by flow cytometry and chromatin condensation status is measured as the mean fluorescence intensity (MFI) of the Histone H3 stain. Chromatin decondensation during T cell activation is demonstrated as an increase in the MFI.

Video Link

The video component of this article can be found at <http://www.jove.com/video/53533/>

Introduction

Flow cytometry is a laser-based technology developed for the analysis of multiple physical and fluorescent parameters in cell populations. This technology works as suspended cells in a stream of fluid encounter a laser, exciting fluorescent markers on or within the cells. These markers then emit light that is detected and quantified by photomultiplier tubes. While flow cytometry has traditionally been used to identify populations of cells, it has proven to be a useful technology when studying an array of cell properties including cell membrane integrity, protein-protein interactions and protein trafficking¹⁻³. We have developed a protocol that allows this technology to be used to detect the state of chromatin in T cells as they are activated *in vitro*⁴. We have also used this protocol to investigate the mechanism of activation-induced chromatin decondensation of T cells⁵.

T cell activation and proliferation are critical for a proper immune response. T cells, a specific subset of lymphocytes within the immune system, are required for proper immune responses and the development of immunological memory. Activation is initiated when an antigen is presented in the context of the major compatibility complex to the T cell receptor (TCR) located on the extracellular surface of quiescent T cells (reviewed in⁶). This triggers a series of dynamic and highly ordered molecular events within T cells that culminate in an increase in intracellular Ca²⁺ concentration⁷ and the nuclear translocation of transcription factors required for activation (reviewed in⁸⁻¹⁰). Once activated, T cells gain the ability to respond to Interleukin-2 (IL-2), a potent growth factor that utilizes the JAK (Janus Kinase)/STAT (Signal Transducer and Activator of Transcription) pathway to drive clonal proliferation of activated T cells¹¹. Briefly, IL-2 stimulation results in the phosphorylation of STAT proteins, a family of latent cytosolic transcription factors. Once phosphorylated, STAT proteins dimerize, translocate to the nucleus and drive expression of genes including those involved in cell cycle progression. In T cells, IL-2 signals via STAT5, which is required for T cell proliferation^{12,13}.

In order to achieve clonal expansion of activated T cells, those cells that have not experienced antigen-TCR engagement (naïve T cells), must have a mechanism to ignore the potent effects of IL-2. This is achieved via the regulation of chromatin status. Naïve T cells possess a condensed chromatin that prohibits STAT5-DNA engagement in response to IL-2 stimulation. Upon activation, chromatin decondenses and STAT5 can access the promoters of target genes, permitting clonal proliferation⁴. Interestingly, this change in chromatin status is not dependent on epigenetic modification of histone proteins (for review, see¹⁴) as we observed no global change in histone modification during T cell activation⁴.

While performing these studies, we discovered that antibodies raised against histone proteins also had difficulty accessing their epitopes in naïve cells, but that upon activation could more readily bind their epitopes⁴. Thus, antibody binding to histones serves as a readout for chromatin condensation status. Here we present the method to use flow cytometry to detect fluorescently conjugated Histone H3 antibodies in order to assess chromatin status in T cells. Chromatin condensation in a population of cells is measured as the mean fluorescence intensity (MFI) of Histone H3 staining. In the context of T cell activation, the MFI of Histone H3 staining increases, signifying the decondensation of chromatin. In

In addition to measuring chromatin status via intracellular Histone H3 staining, this protocol also incorporates surface staining and a fixable stain for live cells, permitting analysis of subpopulations of cells.

Protocol

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal protocol was approved by the Furman University Institutional Animal Care and Use Committee (Permit number: A3242-01). All materials and equipment used in this protocol can be found in the Table of Materials and Equipment, while buffers used can be found in the Table of Buffers and Solutions.

1. Single Cell Suspension of Lymphocytes from Mouse Spleens

1. Sacrifice mice via CO₂ asphyxiation. Confirm euthanasia by cervical dislocation.
Note: Use spleens from two 6-8 week old isogenic mice (e.g., C57B/6) of the same gender. Typically, two spleens are processed.
2. Spray the animals profusely with 70% ethanol solution and orient such that the head is facing left.
3. Using forceps, lift the skin of the animal upwards and away from the body. Using scissors, cut a small notch through the skin near the abdomen of the animal. Using fingers, pull each side of the notch to expose the peritoneum from the neck to the beginning of the hind legs. The spleen should be visible directly underneath the peritoneum.
4. Using forceps, lift the peritoneum and make a small incision to expose the spleen. Remove the spleen with forceps and use scissors to tease away any adipose and connective tissue.
5. Place the spleen into a 50 ml conical tube containing 10 ml PBS supplemented with 2% Fetal Bovine Serum (hereafter referred to as PBS +2%). Keep the tube on ice until ready to begin the single cell suspension.
Note: Typical recovery is between 60-90 million cells per spleen, and typically, 2 million cells are needed per sample. Perform all of the following steps in a tissue culture hood.
6. Decant the spleens into a sterile 100 mm tissue culture dish.
7. Obtain two frosted microscope slides and hold the slides so that the two rough frosted surfaces face inwards towards one another. Wet the slides in the PBS + 2% solution poured into the petri dish.
8. Hold the spleen between the frosted surfaces of the slides, with the edges still submerged, and grind the spleen gently by moving the slides back and forth against one another (**Figure 1A**). The cells will fall into the Petri dish. Continue grinding until all cells have been released and the remains of the spleen appear white (**Figure 1B**).
9. Draw up the cell suspension in a pipet and filter it slowly through a 70 µm filter into a new sterile 50 ml conical tube. Note that small pieces of red pulp will be present on the filter (**Figure 2A**).
 1. If the filter rises up, gently lift it up slightly to allow the fluid to drain through and place the filter back into the 50 ml conical tube and continue, repeating this process as necessary. If processing more than 5 spleens, it may be necessary to divide into two batches and use a new filter for each.
10. Using the stopper portion of a 3 ml syringe gently press the red pulp containing the remaining cells through the strainer (**Figure 2B**). Make sure to press both the filter bottom and sides to ensure that all red pulp has passed through the filter (**Figure 2C**).
11. Add 10 ml of PBS + 2% to the tissue culture dish and use this to wash the slides, syringe stopper, and dish to recover any remaining cells. Pass this through the same 70 µm cell strainer into the 50 mL conical tube. Repeat this step as necessary.
12. Centrifuge the filtered cell suspension at 300 x g for 5 min at 4 °C.
13. Gently decant and discard the supernatant, careful not to disturb the pellet. A trace amount of PBS + 2% will be left in the tube. Cap the tube and flick the pellet until the pellet is completely resuspended.
14. Lyse red blood cells by adding 2 mL of ACK Lysis Buffer (0.15 M NH₄Cl, 1 mM KHCO₃, 0.1 mM EDTA, pH to 7.2, store at 4 °C) per spleen to the conical tube and rotate and invert the tube to ensure that all cells come into contact with the ACK buffer. Gently swirl the tube for 1 min at RT.
15. Bring the volume of the cell suspension to 50 ml by adding PBS + 2% to the tube to neutralize the ACK buffer. Invert the tube 10 times and centrifuge for 5 min at 300 x g at 4 °C. After the centrifugation is complete, the pellet should be white (**Figure 3**).
16. Gently decant and discard the supernatant, careful not to disturb the pellet. Leave a trace amount of PBS + 2% in the tube, cap the tube, and flick the pellet to resuspend it.
17. Add 10 ml of T cell media [10% FBS, 10 mM HEPES (pH 7.0), 2 mM GlutaMAX, 1 mM sodium pyruvate, 1X non-essential amino acids, 1x penicillin/streptomycin and 50 mM β-mercaptoethanol], and further resuspend the pellet by gently pipetting up and down. Then filter the suspension through a new 70 µm filter into a new 50 ml conical tube.
18. Use another 5-10 ml of T cell media to rinse the original tube and pass this through the 70 µm filter into the tube containing the rest of the filtered cells. If processing more than two spleens, the volume of T cell media can be increased to maximize recovery.
19. Keep cells on ice until ready to be stained. Cells should be counted and viability assessed.
 1. To count cells, combine 3 ml of cells with 24 ml of PBS + 2% and 3 ml of 0.4% trypan blue. Load 10 ml of this into each chamber of an improved Neubauer hemocytometer. Viability, as assessed by trypan blue exclusion, is typically between 85-95%.

2. Viability and Surface Staining

1. Pellet cells by centrifuging for 10 min at 300 x g at 4 °C. Resuspend cells in T cell media to a concentration of 1 x 10⁷ cells/ml. Transfer 2 million cells (100 µl) into a well of a U-bottomed 96-well tissue culture plate.
2. Centrifuge the 96-well plate for 10 min at 300 x g at 4 °C.
3. Remove the supernatant from each well by flicking liquid within the well plate into the sink (the cell pellet will remain in the well) and dabbing the plate on a clean paper towel. Wash the cells by resuspending them in 200 µl PBS followed by centrifugation at 300 x g for 10 min at 4 °C. Perform all washes this way unless otherwise noted.

Note: Do not use PBS + 2% as it will interfere with the fixable PI stain.

4. Remove the supernatant by flicking the plate and add 100 ml of freshly prepared commercial stain (e.g., fixable red dead cell stain) to each well.
 1. Make the stain by diluting the reactive dye stock solution 1:10 in DMSO followed by a 1:10 dilution in PBS. Incubate the plate at 4 °C for 30 min protected from light.
5. Centrifuge the plate for 10 min at 300 x g at 4 °C. At this point forward, perform all work with the plate with the tissue culture hood light off.
6. Remove the supernatant by flicking the plate and then add 100 ml of Fc block solution. Make Fc block solution by diluting Fc block stock solution 1:100 in PBS.
7. Dilute antibodies to be used for surface staining (e.g., anti-CD4 or anti-CD8) in PBS and add 100 ml to each well. Incubate the 96-well plate for 30 min at 4 °C protected from light.

Note: Surface stain antibodies should be titrated for optimal performance. Typically use a 1:200 or 1:400 dilution, per the manufacturer's protocol.

3. Intracellular Staining for Histone H3

1. Following incubation of the surface stain, wash the cells twice in PBS.
2. After the last wash, add 100 µl of 4% paraformaldehyde to each well. Incubate the 96-well plate for 5 min at RT. Make 4% paraformaldehyde by diluting 16% paraformaldehyde in PBS. Make this fresh.
3. Wash the cells twice in PBS.
4. Make Perm/Block solution (stock Perm solution is PBS + 2% + 0.02% Triton X-100, store at 4 °C) by combining 2 ml of normal rabbit serum per 100 ml stock Perm solution. Make enough for 60 ml/sample.
5. Add 40 ml Perm/Block to each sample well and mix well by gently pipetting up and down, taking care not to make bubbles. Incubate the plate at RT for 45 min in the dark. Save remaining Perm/Block for step 3.6.
6. Dilute fluorescently conjugated Histone H3K4me1 antibody in Perm/Block solution reserved in step 3.5 and add 10 µl of this dilution to each well. Mix by gently pipetting up and down. Incubate the 96-well plate in the dark for 1 hr at 4 °C.

Note: Use an H3K4me1 antibody conjugated using the R-Phycoerythrin conjugation kit following the manufacturer's instructions.
7. Wash the cells twice in PBS + 2%.
8. Resuspend the cells in 200 µl PBS+2% and transfer the samples to FACS tubes for flow cytometry analysis. The samples can be stored at 4 °C in the dark. For optimal results analyze the samples within two days. Singly stained samples can be used as flow cytometry compensation controls.

Representative Results

Lymphocytes from a C57B/6 mouse were processed into a single cell suspension according to the protocol and counted using a standard hemocytometer. Cells were seeded in triplicate at 2×10^6 /ml in T-cell media in 15 ml conical tubes and left untreated, or stimulated with 1 mg/mL soluble anti-CD3 antibodies (clone 4C11) for 3 hr at 37 °C in a standard tissue culture incubator. Dead cells were stained and then cells were then surface stained using FITC-CD8 and APC-CD4 antibodies according to the protocol. Histone accessibility was analyzed via flow cytometry (**Figure 4**). In both naïve CD4⁺ and CD8⁺ T cells, the mean fluorescent intensity (MFI) is low, signifying a condensed chromatin state. As cells are activated with anti-CD3 antibodies the MFI increases significantly ($p < 0.001$, Student t-test) indicating that chromatin has decondensed.

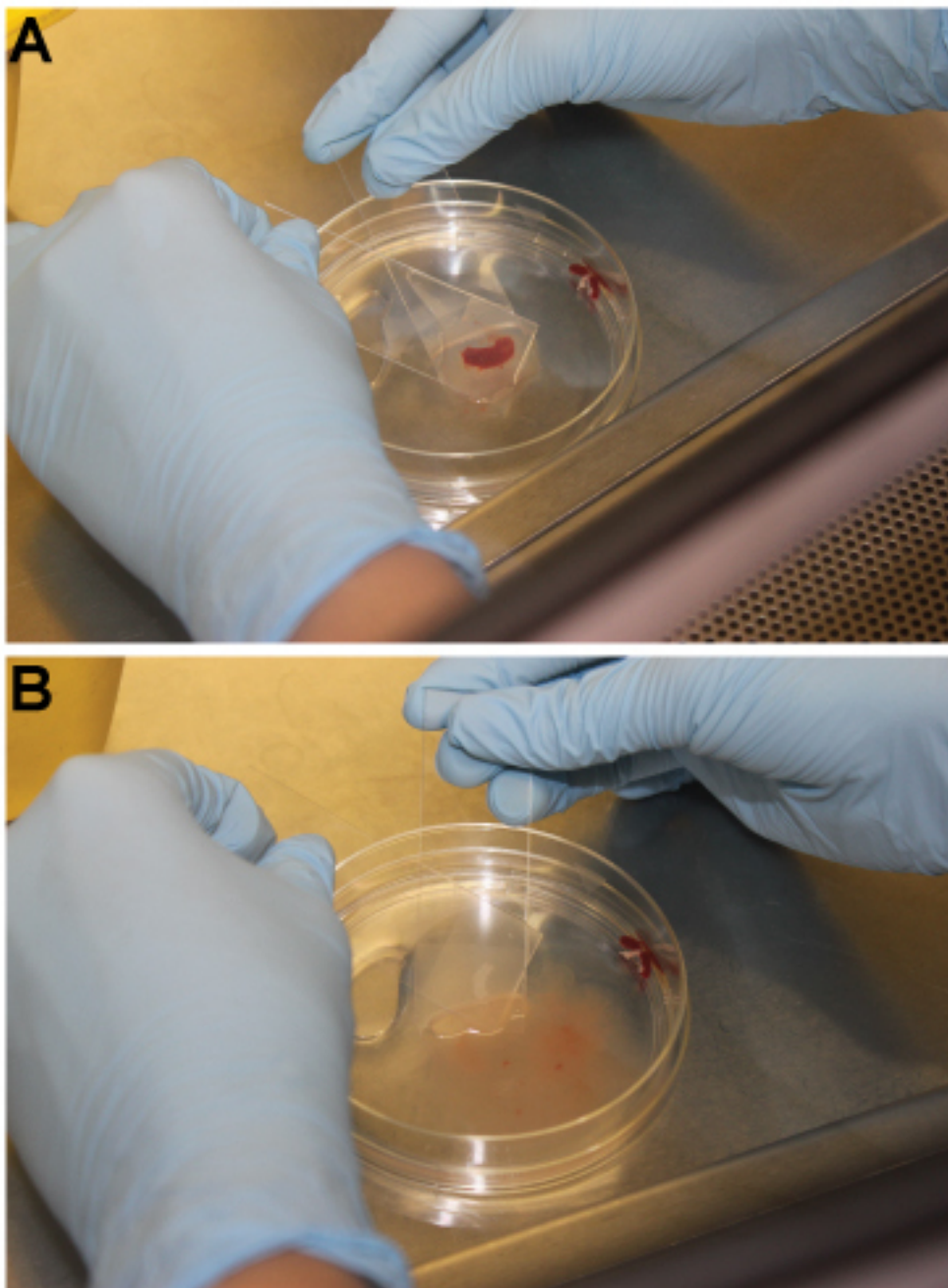


Figure 1. Technique for processing spleens into a single cell suspension using frosted microscope slides. (A) The spleen is pressed against the frosted surfaces of two microscope slides. (B) The slides are moved back and forth against each other releasing lymphocytes into a 100 mm Petri dish until the remains of the spleen are white. [Please click here to view a larger version of this figure.](#)

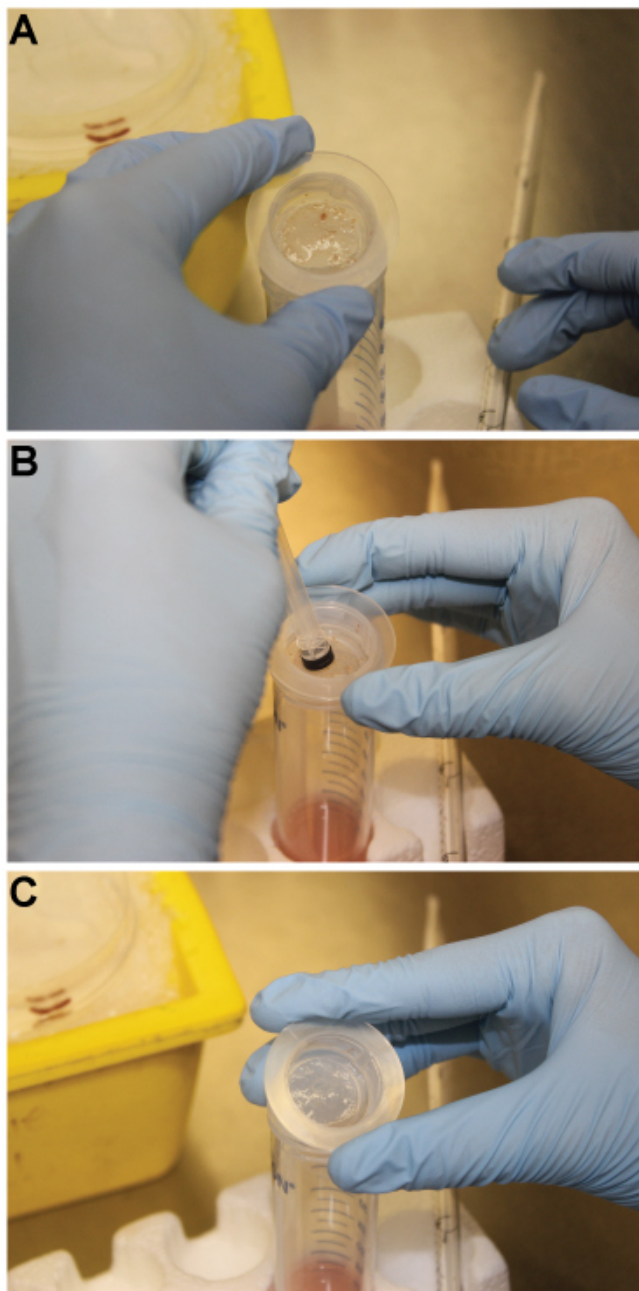


Figure 2. Using a syringe stopper to press remaining red pulp through a 70 mm cell strainer. (A) Red pulp remaining after a spleen is processed into a single cell suspension and passed through the 70 mm cell strainer. **(B)** The stopper portion of a 3 ml syringe is used to gently press remaining red pulp through the cell strainer. **(C)** After using the syringe, there should be virtually no red pulp left in the cell strainer. [Please click here to view a larger version of this figure.](#)

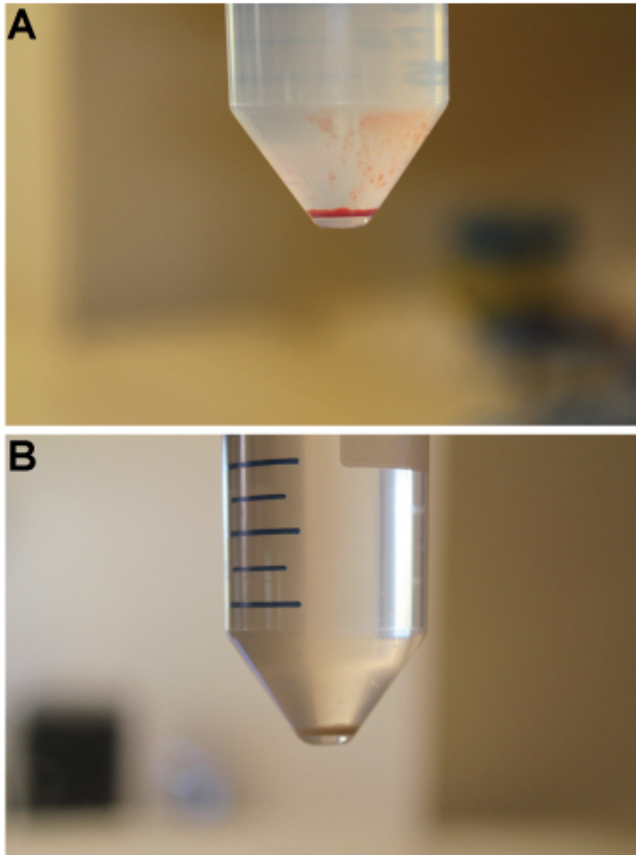


Figure 3. ACK lysis of red blood cells. (A) Centrifugation of a single cell suspension generated from a single mouse spleen prior to ACK lysis. (B) After ACK lysis of red blood cells, the cell pellet should be white. [Please click here to view a larger version of this figure.](#)

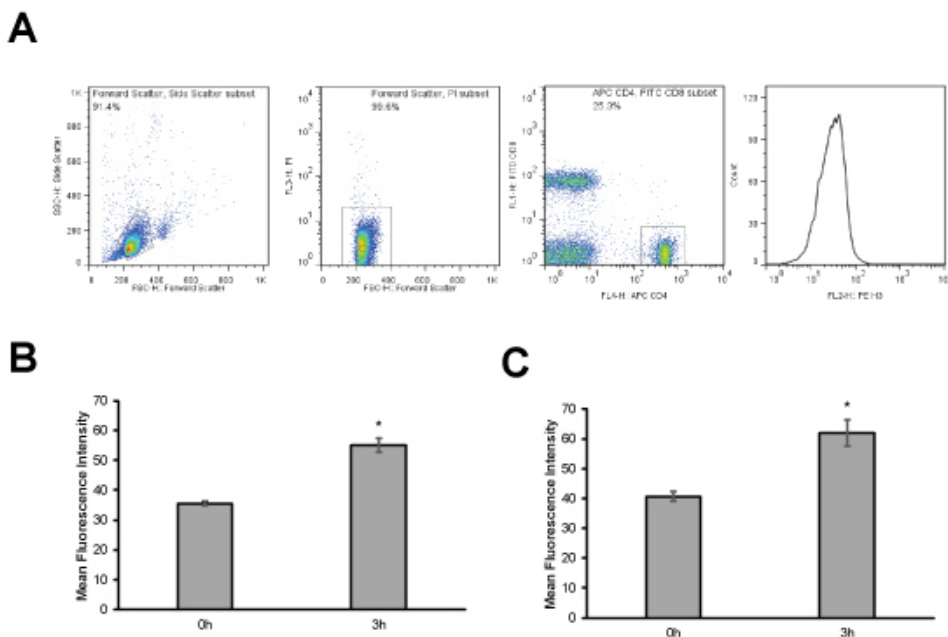


Figure 4. Representative results of the protocol. (A) Gating scheme used to analyze chromatin status in CD4⁺ T lymphocytes. (B and C) T cells were left untreated or activated with 1 mg/ml soluble anti-CD3 antibodies for 3 hr (in triplicate). Cells were then analyzed by flow cytometry

to determine chromatin condensation in CD4⁺ cells (B) and CD8⁺ cells (C). Data are the means ± Standard Deviation of the mean fluorescence intensity of H3K4me1 staining. *p <0.001 (Student's *t*-test) [Please click here to view a larger version of this figure.](#)

Output	Possible causes
Poor yield of cells following single cell suspension	•Spleen not ground sufficiently
	•Remaining cells not pressed through strainer with plunger
	•Microscope slides, plunger, and petri dish not washed well enough
Cell yield greater than 90 million cells per spleen following single cell suspension	•Incomplete ACK lysis of red blood cells
Events pressed against the axis on the H3 histogram	•Incomplete permeabilization
No change in MFI between naïve and stimulated cells	•Stimulation insufficient to induce decondensation
	•Histone H3 antibody not titered correctly
	•Perm/Block conditions not optimized

Table 1: Troubleshooting Guide. A quick reference to common issues and possible solutions.

Discussion

We developed a protocol that allows for the assessment of chromatin condensation in T cells. It relies on the simple observation that Histone H3 antibodies cannot access their epitopes readily in naïve cells, but upon T cell activation, these same antibodies are able to bind to their epitopes. By comparing the MFI of Histone H3 staining between treatment groups, the relative degree of condensation or decondensation can be determined. We have used this protocol to determine relative condensation status during thymocyte development and during T cell activation⁴. We have also used this protocol to investigate the mechanisms that control the decondensation process⁵.

This protocol relies on the use of a Histone H3 antibody to detect chromatin condensation status. Since there is no global change in histone modification during T cell activation⁴, we are able to use an H3K4me1 antibody to assess chromatin status in this protocol. We have used antibodies raised against unmodified Histone H3; however, the signal produced was much weaker overall. In our experience, antibodies raised against modified Histone H3 work better in immunofluorescence and flow cytometry assays, while antibodies against unmodified Histone H3 work better in Western blot. It must be noted that it is also possible to use antibodies raised against other histone proteins, although we have not attempted it.

The Perm/Block and Histone H3 antibody steps are the most critical steps in the protocol. The amount of Perm/Block solution used needs to be optimized each time the stock Perm solution is made. This can be done by comparing the performance of different dilutions of stock solution. A typical experiment involves comparing chromatin status in naïve T cells to those activated for 3 hr (**Figure 4**). One should choose the dilution that produces the greatest change in MFI over the time period analyzed. The stock solution can be diluted in PBS + 2% to decrease Perm/Block strength by first resuspending the cells in as much as 100 ml PBS + 2% after step 3.4 and then adding the Perm/Block in step 3.5. A similar pilot experiment should be used to test different dilutions of the fluorescently conjugated Histone H3 antibody each time a new batch of antibody is labelled. These steps are especially critical to successful detection of condensation differences early in T cell activation (e.g., within 3 hr of activation). Occasionally, there are cells that do not get permeabilized and thus will not stain with the Histone H3 antibody. This may happen if the cells are not resuspended well when adding the Perm/Block or if the Perm/Block is not strong enough. These cells will appear as events pressed against the axis when visualizing a histogram of Histone H3 staining. Since these events will skew the overall MFI, these events can be omitted from analysis by gating the normal distribution of Histone H3 positive cells. Additional assistance can be found in the Troubleshooting Guide (**Table 1**).

The inclusion of a fixable dead cell stain allows for the assessment of cell viability in the assay. This is absolutely critical when manipulating T cell activation because certain stimuli can induce cell death. In such a case, the Histone H3 antibody can bind histones in dead cells differently than live cells, leading to misinterpretation of the results.

This protocol is designed for use in 96-well plate format, permitting a high throughput analysis of chromatin status. A spleen from a 6-8 week old female mouse will typically yield between 60-90 million cells using the protocol. Since the staining protocol requires 2 million cells per sample, one can easily assay multiple treatment groups and time points in triplicate with a single spleen on a single 96-well plate. It is possible to perform

the protocol with less cells per sample; however, due to the number of centrifugation steps and the inherent loss of cells at each of these steps, it is not advisable to lower the number of cells by much. We have successfully completed the protocol with 1 million cells per sample.

We used this protocol to examine chromatin status in CD4⁺ T helper cells and CD8⁺ cytotoxic T cells. This is made possible because the protocol includes standard surface staining. The protocol could easily be adapted for the examination of chromatin in other lymphocyte subpopulations by using antibodies against population-specific surface markers. This protocol could also easily be adapted to other cell types so long as antibodies recognizing relevant surface markers are available and proper fixation/permeabilization conditions are known.

Disclosures

The authors declare that they have no competing financial interests.

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