FACS to Obtain Pure Mitotic Cell Populations

PROTOCOL FOR:

Comparative analysis of mitosis-specific antibodies for bulk purification of mitotic populations by fluorescence-activated cell sorting

Amy E. Campbell[1,2], Chris C.-S. Hsiung[1,2], and Gerd A. Blobel[1,2]
[1]Division of Hematology, The Children's Hospital of Philadelphia, Philadelphia, PA 19104; [2]Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104

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LEGEND

⇨ ***ATTENTION***

* ***HINT***

REST

REAGENTS

- **Nocodazole** (Sigma-Aldrich, St. Louis, MO, USA, M1404)
- **Dulbecco's Phosphate-Buffered Saline** (Life Technologies, Carlsbad, CA, USA, 14200)
- **37% formaldehyde** (Thermo Fisher Scientific, Waltham, MA, USA, F79)
- **Glycine** (Thermo Fisher Scientific, Waltham, MA, USA, BP3811)
- **Protease inhibitor cocktail** (Sigma-Aldrich, St. Louis, MO, USA, P8340)

ANTIBODIES

- **Anti-MPM2 antibody** (EMD Millipore, Billerica, MA, USA, 05-368)
- **Allophycocyanin-conjugated AffiniPure F(ab')$_2$ Fragment Donkey Anti-Mouse IgG (H+L)** (Jackson ImmunoResearch, West Grove, PA, USA, 715-136-150)

EQUIPMENT

- **500 mL conical tubes** (Corning, Corning, NY, USA, 431123)
- **50 mL conical tubes** (Corning, Corning, NY, USA, 352070)
- **5 mL FACS tubes with strainer cap** (VWR, Radnor, PA, USA, 352235)
- **Beckman Coulter Allegra X-15R centrifuge** (Beckman Coulter, Pasadena, CA, USA, 392932)

RECIPES

Resuspension Buffer (100 mL)

Component	Volume	Final concentration
500 mM EDTA	0.4 mL	2 mM
1X PBS	to 100 mL	

Cell Lysis Buffer (500 mL)

Component	Volume	Final concentration
1 M Tris pH 8.0	5 mL	10 mM
5 M NaCl	1 mL	10 mM
Igepal/NP-40	1 mL	0.2%
H_2O	to 500 mL	
100 mM PMSF – add fresh	1:100	1 mM
protease inhibitor cocktail – add fresh	1:500	

10X FACS Buffer (100 mL)

Component	Volume	Final concentration
FBS	20 mL	20%
500 mM EDTA	4 mL	20 mM
NaN_3	0.2 g	0.2%
1X PBS	to 100 mL	
100 mM PMSF – add fresh	1:100	1 mM
protease inhibitor cocktail – add fresh	1:500	

*To make 1X FACS Buffer, dilute 10X FACS Buffer 1:10 in PBS.

10X Slippery Buffer (100 mL)

Component	Volume	Final concentration
500 mM EDTA	0.4 mL	2 mM
Igepal/NP-40	2 mL	2%
1X PBS	to 100 mL	

PROCEDURE

⇨ This protocol is for the isolation of formaldehyde-fixed suspension cells in mitosis in sufficient quantities for a typical ChIP experiment. The protocol can be adapted for other cell types suitable for FACS.

NOCODAZOLE BLOCK

1. Grow cell cultures to ~20-25X the required amount of purified mitotic cells.
2. Set aside an asynchronous control population by transferring a desired number of cells to a new tissue culture flask. Return culture to incubator.
3. Arrest culture in mitosis by adding nocodazole at a final concentration of 200 ng/mL. Return culture to incubator for 8-12 hours.

This large amount of starting material for mitotic arrest is necessary to compensate for loss of formaldehyde-fixed cells in subsequent staining and sorting.

Optimize the duration of nocodazole treatment for each cell type used. Be sure to add nocodazole during log-phase growth.

From here on, process the mitotic arrest sample and asynchronous control sample identically, except where noted.

CROSSLINKING

4. Collect cells by centrifugation in appropriately sized conical tubes (typically 50 mL or 500 mL) at 295 g for 5 minutes at room temperature.
5. Aspirate media such that only cell pellet remains.
6. Resuspend cells in Resuspension Buffer at a density of ~1-5 million cells per mL and transfer to an appropriately sized conical or Erlenmeyer flask.
7. Add formaldehyde to a final concentration of 1% for exactly 10 minutes while mixing (either by rocking the vessel or with a stir bar) at room temperature.
8. Quench crosslinking reaction by adding glycine to a final concentration of 1 M. Mix for an additional 5 minutes at room temperature.

The high concentration of glycine (1 M) helps reduce loss of formaldehyde-fixed cells in subsequent steps.

9. Transfer cells to 50 mL conical tubes (use multiple as needed to fit entire sample volume). Pellet cells at 821 g for 20 minutes at 4 °C. Keep samples on ice or at 4 °C from here on unless otherwise noted.

From here on, minimize unnecessary contact of cells with various surfaces of vessels and serological pipettes, i.e. unnecessary pipetting and transferring between containers. For example, add buffers to sample using a serological pipette as needed, then resuspend the cell pellet using an air displacement micropipette (e.g. P1000) to minimize the surface area that comes into contact with fixed cells.

At this step, cell pellets can be stored in a small volume (e.g. 1 mL) of Resuspension Buffer (optional: supplement with protease inhibitor cocktail at 1:500 and 1 mM PMSF) overnight at 4 °C.

PERMEABILIZATION OF CELL MEMBRANES

10. Aspirate supernatant and resuspend in Cell Lysis Buffer (1 mL per 20 million cells). Incubate on ice for 10 minutes.
11. Spin at 295 g for 3 minutes at 4 °C to pellet samples.

STAINING

12. Resuspend samples in 1X FACS Buffer (1 mL per 60 million cells) containing MPM2 primary antibody (use at 1:170 dilution, or 1 µL per 10 million cells) and incubate at room temperature for 30 minutes with rotation.
13. Spin at 295 g for 5 minutes at 4 °C and aspirate supernatant.
14. Resuspend samples in 1X FACS Buffer (1 mL per 60 million cells) containing fluorescent secondary antibody (e.g. Allophycocyanin-conjugated donkey anti-mouse, used at 1:170 dilution) and incubate in the dark at room temperature for 30 minutes with rotation.
15. Spin at 295 g for 5 minutes at 4 °C and aspirate supernatant.

FACS

16. Resuspend in 1X FACS Buffer at a high cell density compatible with the specific FACS machine on which samples will be sorted (e.g. 60 million cells per mL for FACS Aria II).
17. Reduce cell clumping by passing sample 3-5 times through a 25-gauge needle using a syringe. Use the needle and syringe to transfer cells to 35 µm strainer cap FACS tube.

⇨ *Filtering the sample through the 35 µm strainer cap is necessary to avoid clogging the FACS machine.*

18. Prepare collection tubes containing 10X FACS Buffer at a volume that will be diluted to 1X by sheath fluid upon completion of sorting. This volume may depend on the specifics of the sorting procedure.
19. Use standard flow cytometry practices to gate for live single cells using FSC and SSC. Within the live single cells gate, use the appropriate fluorescence channel to gate for MPM2 high cells. Use the MPM2 high gate to sort the nocodazole-arrested sample. Use the live single cells gate to sort the asynchronous sample.
20. Optional: after sorting, add 10X Slippery Buffer at 1:10 dilution directly to collection tubes containing sorted samples before any further centrifugation or transferring of the sample to different containers.

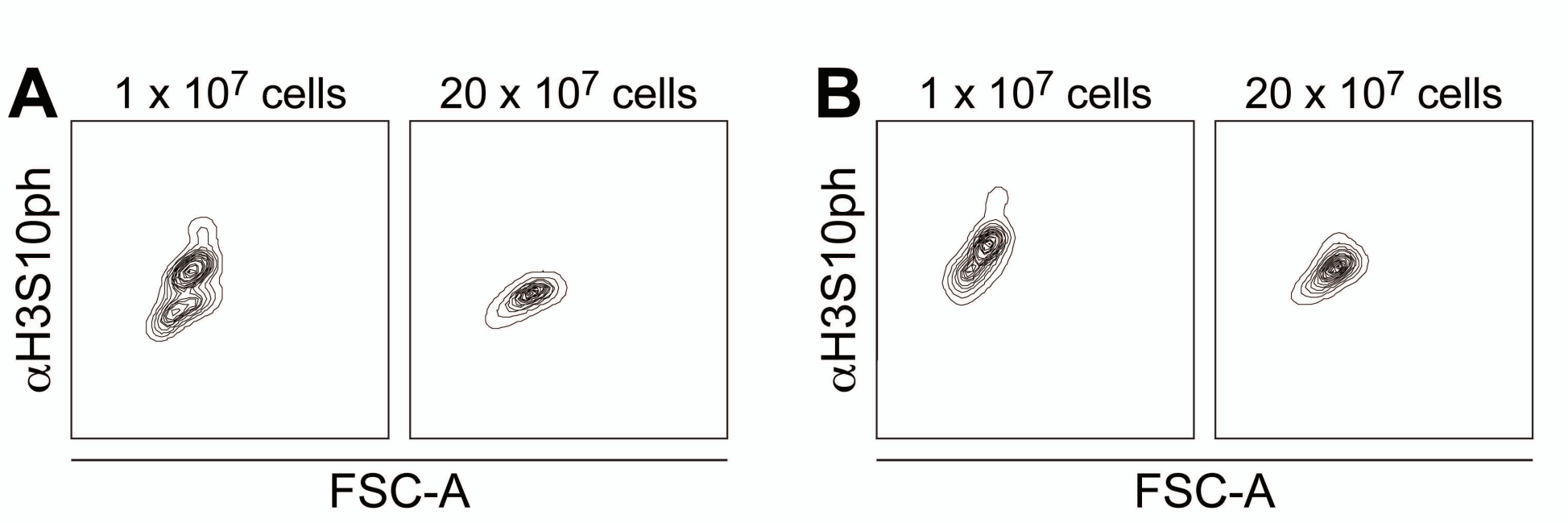
* *1X Slippery Buffer contains low concentrations of detergent that help with reducing the stickiness of fixed cells.*

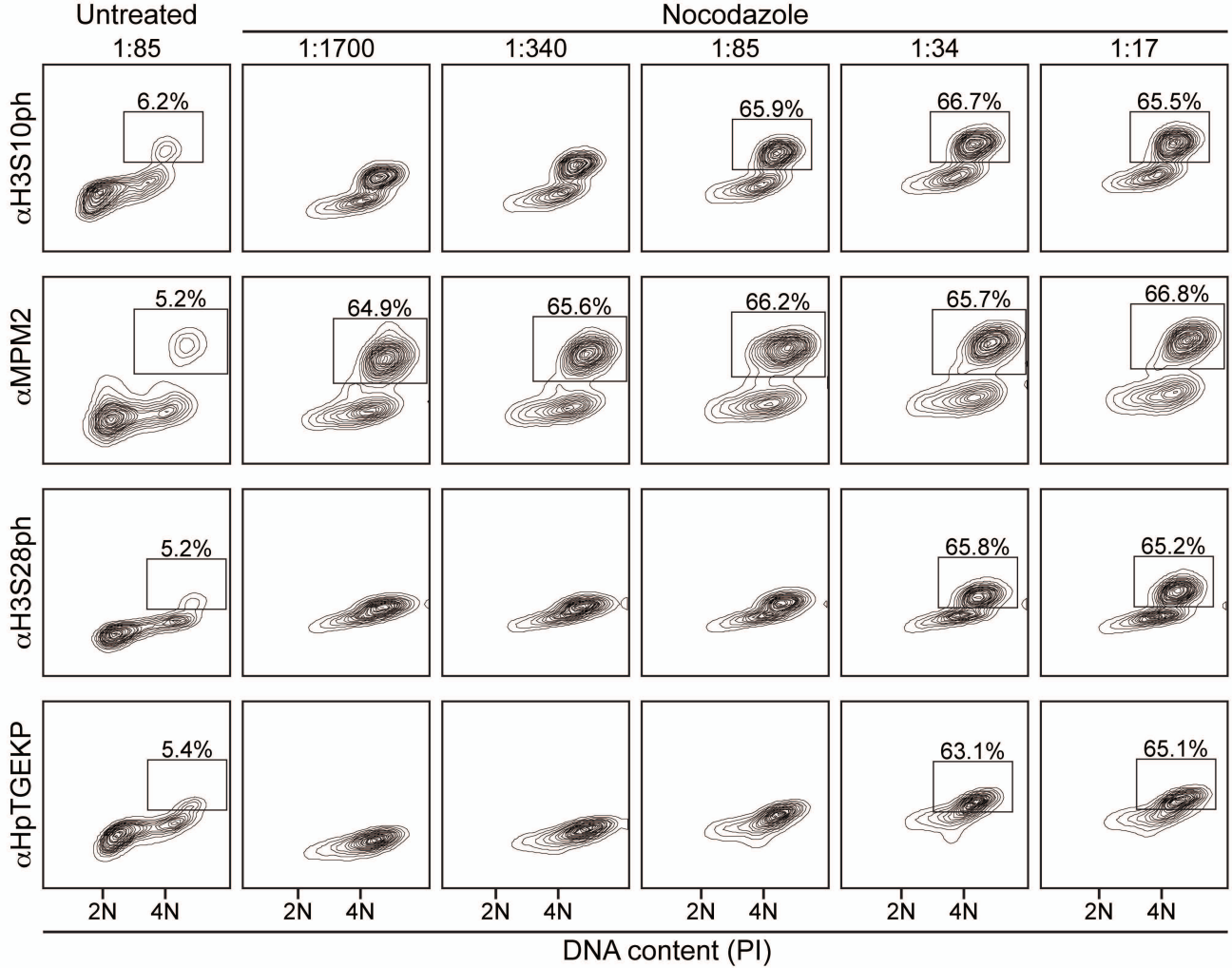
21. Proceed with typical ChIP protocol, including nuclei lysis, sonication, immunoprecipitation, and qPCR or high-throughput sequencing.

✋ *Alternatively, cell pellets may be snap-frozen with liquid nitrogen and stored at -80 °C until ready for further processing.*

* *The distribution of MPM2 epitopes on chromatin is currently unclear and may differ among cell types. Thus, MPM2 antibody staining can contribute to background fn MPM2 antibodies are captured by the pull-down method (i.e. Protein A or G beads) used in*

ChIP. We recommend adhering to standard ChIP practices of analyzing input DNA controls of MPM2-stained cells, which would produce background that can be used to calibrate signal from ChIP samples.


A
1 x 10^7 cells
20 x 10^7 cells
αH3S10ph
FSC-A
B
1 x 10^7 cells
20 x 10^7 cells
αH3S10ph
FSC-A


Untreated
Nocodazole
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1:85
1:34
1:17
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αHpTGEKP
6.2%
65.9%
66.7%
65.5%
5.2%
64.9%
65.6%
66.2%
65.7%
66.8%
5.2%
65.8%
65.2%
5.4%
63.1%
65.1%
2N
4N
DNA content (PI)