Jun 30, 2025

FACS to measure epitopes in CellTrace-multiplexed samples

Nature Cell Biology

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DOI
https://dx.doi.org/10.17504/protocols.io.dm6gpdwp5gzp/v1

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Dam&ChIC

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DOI: https://dx.doi.org/10.17504/protocols.io.dm6gpdwp5gzp/v1

External link: https://doi.org/10.1038/s41556-025-01687-w

Protocol Citation: Samy Kefalopoulou, Peter Zeller 2025. FACS to measure epitopes in CellTrace-multiplexed samples . **protocols.io** https://dx.doi.org/10.17504/protocols.io.dm6gpdwp5gzp/v1

Manuscript citation:
Samy Kefalopoulou, Pim M. J. Rullens, Kim L. de Luca, Sandra S. de Vries, Tessy Korthout, Alexander van Oudenaarden, Peter Zeller, Jop Kind (2025) Retrospective and multifactorial single-cell profiling reveals sequential chromatin reorganization during X inactivation.Nature Cell Biology doi: 10.1038/s41556-025-01687-w

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Protocol status: Working
We use this protocol and it's working

Created: April 14, 2025

Last Modified: June 30, 2025

Protocol Integer ID: 126661

Keywords: FACS, Flow cytometry, CellTrace dyes, epitopes in celltrace, facs in sample, facs analysis, abundance of epitope, measurements of epitope abundance, cleaner comparisons in epitope abundance, cell types of interest, flow cytometry, celltrace, cell type, multiplexed sample, celltracer, epitope, cell sorting, epitope abundance, epitope abundance across perturbation condition, cell, facs as part, fac, using antibody, same sample preparation procedure of sortchic, multiple sample, sample preparation, antibody, expressed cell, same sample preparation procedure

Abstract

This assay enables measurements of epitope abundance via FACS in samples that have been (optionally) multiplexed with Cell-Tracer dyes. It uses the same sample preparation procedure of sortChIC and Dam&ChIC, followed by FACS-based detection of epitope(s) of interest and, if desired, single-cell sorting.

It can be used for:

(i) Flow cytometry (FACS) analyses, to evaluate epitope abundance across perturbation conditions or timecourse samples. By multiplexing with CellTracers, multiple samples/conditions can be stained and measured by FACS as part of one multiplexed "super-sample". This reduces batch effects and staining variations allowing for direct and cleaner comparisons in epitope abundance between conditions.

(ii) FACS analyses followed by single-cell or bulk sorting, to enrich for populations of interest based on abundance of epitopes. For example, cells from a given perturbation or drug treatment can be co-stained with a suitable control sample and subsequently single-cell or bulk sorted for Dam&ChIC or sortChIC. This approach can also allow to sort cell types of interest from a complex sample, using antibodies against highly expressed cell (surface) markers. For an example implementation check **Zeller et al. 2023** and the protocol described by **Gaza et al. 2024**.

Materials

Antibodies

Suitable fluorescently-conjugated primary antibodies
or
Suitable primary antibodies + fluorescently conjugates secondaries

Chemicals and Buffers

For sample preparation:

- PBS0
- Ethanol
- Tween 20 (Sigma, P9416)
- cOmplete Mini EDTA-free Protease Inhibitor Cocktail (Roche, 11836170001)
- HEPES 1M (Gibco, 15630080)
- Spermidine Solution (Sigma, S2626-1G)
- Sodium Chloride (NaCl) 5M
- EDTA 0.5M
- Hoechst 34580 (Sigma-Aldrich, 63493-5MG)

For CellTrace stainings:

- CellTrace™ CFSE Cell Proliferation kit (Invitrogen, C34570)
- CellTrace™ Far Red Cell Proliferation kit (Invitrogen, C34572)
- CellTrace™ Yellow Cell Proliferation kit (Invitrogen, C34573)
- Rat Serum (Sigma, R9759)
- DMSO

Other materials

- Polypropylene round bottom tubes 5 ml (Corning, 352002)
- Protein LoBind tubes 0.5 ml (Eppendorf, 0030108094)

FACS

- Beckman Coulter CytoFLEX
- BD LSR Fortessa

Before start

Considerations on experimental design:

- The protocol makes use of (primary or secondary) antibodies conjugated with a fluorophore.
- Stainings with CellTrace dyes can be done (optionally) to multiplex different samples/experimental conditions, as described in the Dam&ChIC protocol. Multiplexed samples with unique combinations of such dyes can be distinguished during FACS. This is useful when the goal is to directly compare epitope abundance between samples, as it allows parallel processing, and thus mitigates batch effects and staining variations.
- It is important that all fluorescent channels can be confidently measured by FACS without bleed through. This poses a limit in the amount of samples that can be multiplexed with CellTracers, and the amount of epitopes that can be stained for in the same experiment. The minimum we recommend is doing CellTrace multiplexing for samples and their respective controls, whenever this is possible with the available fluorescent channels.
- In case single-cell or bulk sorting for Dam&ChIC/sortChIC will be performed, make sure to stain with (fluorescently-conjugated) antibodies that have been tested and titrated in advance. Use a FACS sorting system instead of FACS analyzer.

Buffer preparation

1 **Wash buffer 0f (WB0f)**: the basic ChIC buffer for fixed cells

	solution	volume	concentration in ChIC buffer
	Ultra-pure water	47.5 mL	
	1M HEPES pH 7.5	1 mL	20 mM
	5M NaCl	1.5 mL	150 mM
	pure spermidine solution	3.6 uL	66.6 ug/ml
	10% Tween-20	250 uL	0.05%

Wash buffer 1f (WB1f)***: the antibody incubation buffer for fixed cells
WB0f + protease inhibitors + 4 uL/mL 0.5M EDTA
*a variation of WB1f is used specifically during Cell-Trace stainings of fixed cells, in which the spermidine is omitted

NOTES:

- Tween 10% stock solution should be put on a roller to dissolve properly and can be aliquoted and frozen at -20^{o}C.
- Buffers are preferably made fresh and used within 24 hours. Buffers made for the overnight antibody staining can be stored at 4^{o}C and used the following day.

Sample preparation for antibody staining

2 **Harvest cells and Wash**

1. Harvest cells and make a nice single-cell suspension.
2. Wash cells two-three times with room-temperature PBS0 (without Ca^{2+} and Mg^{2+})
3. Count on a cytometer.

3 **Cell fixation with Ethanol**

1. Pre-cool 100% Ethanol, by placing it at -20^{o}C a few hours beforehand.
2. Resuspend 1 million cells in 300 uL ice-cold PBS0 in a 15mL tube (ideally in a low-binding tube to prevent cell loss)
3. While vortexing, add drop-by-drop 700 uL ice-cold 100% Ethanol.
4. Fix for 1-2 hours at -20^{o}C. It is also possible to fix overnight.

5. Spin tubes at 300rcf for 4 minutes in a cooled centrifuge (4^{O}C) and remove supernatant. If none of the optional following steps are desired, proceed to *step 7*.

NOTES:

- It is important that fixation is done properly and drop-wise in order to avoid formation of clumps.
- Scale the volume accordingly if cell numbers are higher or lower. E.g. fix 0.5 million cells in 500uL total volume, 2 million cells in 2 mL total volume etc.

3.1 (optional)

Cell-Trace stainings for sample multiplexing

To enable parallel processing of multiple samples/conditions and minimize batch effects, we use a sample hashing strategy, using CellTrace Cell Proliferation kits (Invitrogen) that enable staining of fixed cells. Samples stained uniquely with CellTrace dyes can be mixed together right before antibody staining into a "super-sample". The populations of interest will be distinguished during FACS.

1. Resuspend cells in Wash Buffer 1f (WB1f), in which no spermidine is added.
2. Transfer cells in low-binding 1.5mL tubes.
3. Wash cells once more with WB1f (-sperm).
4. Stain 1 million cells in 1 mL WB1f (-sperm) with 0.25 uL Cell-Trace dye. If necessary to stain with multiple non-overlapping dyes, add 0.25 uL of each.
5. Incubate for 20-30 minutes at 4^{O}C protected from light.
6. Quench the staining with the addition of 50 uL rat serum.
7. Incubate for 10 minutes at 4^{O}C protected from light.
8. Spin down at 300rcf for 4 minutes at 4^{O}C. Remove supernatant.

NOTES:

- Two washes are required to remove as much EtOH as possible prior to stainings.
- We have successfully used Cell-tracer dyes CFSE (C34570, Invitrogen), Yellow (C34573, Invitrogen) and Far-Red (C34572, Invitrogen), and combinations of them, to multiplex up to 8 different samples together.
- The use of other colors is possible but they should not overlap with Hoechst 34580, which will be used to measure DNA content during FACS.
- Cells stained with Cell-Trace dyes can be cryopreserved (*step 3.2*), or immediately used for antibody staining (*step 4*).

3.2 (optional)

Cryopreservation of EtOH-fixed samples

1. Resuspend cells in WB1f.
2. Spin down at 300rcf for 4 minutes at 4^{o}C. Remove supernatant.
3. Repeat with a 2nd wash in WB1f.
4. If desired, make aliquots of cells in 0.5mL low-binding tubes.
5. Add the same volume of WB1f containing 20% DMSO (final 10% DMSO per sample)
6. Freeze at -80^{o}C. Cryopreservation is long-term and samples can remain in good quality for several years.

NOTE:

- If Cell-Trace stainings were done prior to cryopreservation, it is handy to make aliquots of 100-200K cells per staining. Plan this accordingly to how many samples will be mixed and stained together for FACS.

Antibody stainings

4 **Primary antibody staining**

For cryopreserved EtOH-fixed cells:

1. Thaw cells from -80^{o}C
2. Spin down at 300rcf for 4 minutes at 4^{o}C
3. Wash cells twice with WB1f to remove DMSO.
4. Resuspend in 400 uL of WB1f containing the primary antibody.
5. Incubate overnight at 4^{o}C on a roller.

For multiplexing of cryopreserved EtOH-fixed Cell-Tracer stained cells:

1. Thaw cells from -80^{o}C
2. Mix together the different populations in equal cell numbers in a 0.5mL or 1mL low-binding tube to make a "super-sample". E.g. 200K cells stained with CT CFSE + 200K cells stained with CT Far-Red + 200K cells stained with CT Yellow
3. Spin down at 300rcf for 4 minutes at 4^{o}C
4. Wash once with WB1f to remove DMSO.
5. Transfer to a 0.5mL low-binding tube (optional) and repeat wash with WB1f.
6. Resuspend in 400 uL of WB1f containing the primary antibody.
7. Incubate overnight at 4^{o}C on a roller.

For cells freshly-fixed with EtOH:
At this point EtOH-fixed cells are in a 15ml tube, spun down once and supernatant is removed.

1. Resuspend cells in WB1f.
2. Transfer 0.5 million cells to a 0.5mL low-binding tube
3. Wash cells twice with WB1f to remove all EtOH.
4. Resuspend in 400 uL of WB1f containing the primary antibody.
5. Incubate overnight at 4^{o}C on a roller.

For cells freshly-fixed with EtOH and stained with Cell Tracers:

1. Resuspend cells in WB1f.
2. Mix together the different populations in equal cell numbers in a 0.5mL or 1mL low-binding tube to make a "super-sample". E.g. 200K cells stained with CT CFSE + 200K cells stained with CT Far-Red + 200K cells stained with CT Yellow
3. Spin down at 300rcf for 4 minutes at 4^{o}C
4. Remove supernatant.
5. Transfer to a 0.5mL low-binding tube (optional) and repeat wash with WB1f.
6. Resuspend in 400 uL of WB1f containing the primary antibody.
7. Incubate overnight at 4^{o}C on a roller.

NOTES:

- The primary staining can be done with antibodies directly conjugated with a fluorophore or non-conjugated ones, in which case a secondary antibody staining is necessary.
- The staining with fluorescently-labelled primaries should be protected from light.
- We have done co-staining for multiple epitopes together overnight. Alternatively, it's possible to do the stainings sequentially (e.g. reduced to 2-3h each), with washes in between.
- The amount of antibody to be used requires optimization. We recommend preliminary testing of antibody concentrations around the recommended by the manufacturer, e.g. a series of 1:200, 1:400, 1:1000, 1:2000, together with negative (unstained) controls to define the background.
- Use of appropriate controls is critical for FACS-based quantifications; in all experiments take along a negative control (unstained sample) and, if possible, biological negative and positive controls.

5 (optional)

Secondary antibody staining

In case the primary antibody is not fluorescently-labelled, a secondary fluorescently-labelled antibody is used instead.

1. Spin the nuclei at 300 rcf for 4 minutes at 4°C.
2. Resuspend the pellet with Wash Buffer 1f to wash.
3. Spin again at 300 rcf for 4 minutes at 4°C.
4. Resuspend nuclei in 500 uL WB1f containing the secondary antibody.
5. Incubate for 1 hour at 4°C on roller, protected from light.

6 **DNA content staining with Hoechst 34580**

1. Spin the nuclei at 300 rcf for 4 minutes at 4°C.
2. Resuspend the pellet with Wash Buffer 1f to wash.
3. Spin again at 300 rcf for 4 minutes at 4°C.
4. Resuspend nuclei in 500 uL WB1f containing Hoechst 34580 at a final concentration of 2.5 ug/mL.
5. Incubate for 1 hour at 4°C on roller

7 **Washes and transfer to FACS tubes**

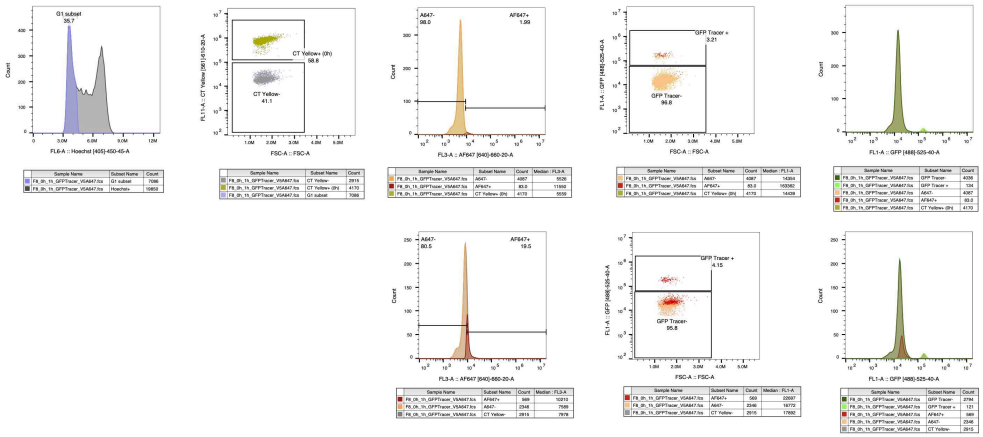
1. Wash the nuclei twice with WB1f, like above.
2. After the final wash, resuspend in 600 uL WB1f
3. Pipette the suspension in a FACS tube through a filter cap to get rid of clumps.

Flow cytometry (FACS)

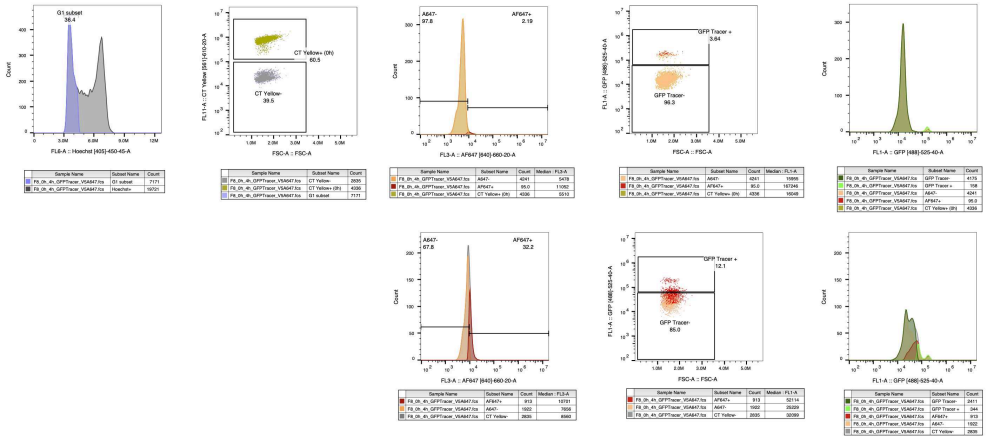
8 **Measure samples by FACS**

See below an example experiment, in which two samples (timepoint 0 and timepoint X) were multiplexed with CellTracer staining (Yellow pos/Yellow neg) and each "super-sample" was stained with Hoechst 34580 and two different antibodies (488 and 647). When mixing and measuring the samples together, we can be more confident that the shift in abundance for the different epitopes of interest (measured with 488 and 647) reflects biology and is likely not a technical artifact from staining variations.

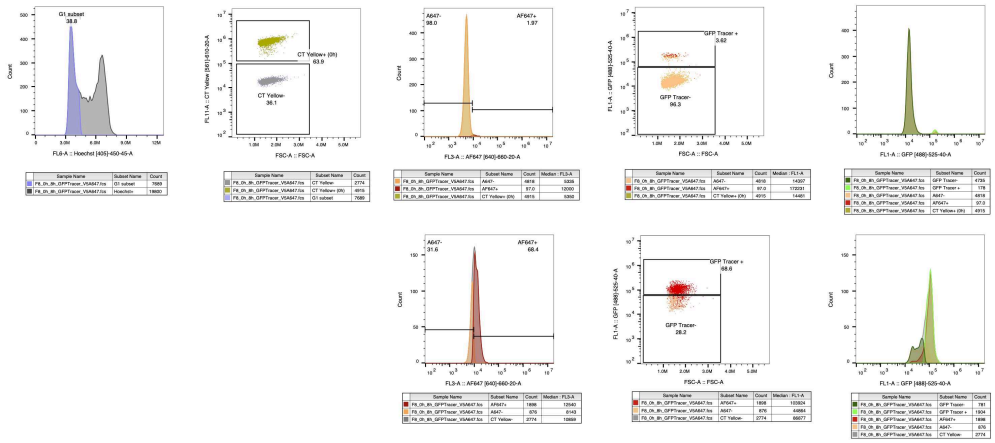
Note: this strategy still requires the use of unstained negative controls, which is essential in every FACS assay.



Example multiplexed supersample 0-1h



Example multiplexed supersample 0-4h



Example multiplexed supersample 0-8h

Gated FCS files can be extracted for the desired multiplexed populations, and further analyzed using custom scripts in R/Python

9 (optional)
Single-cell or bulk sorting
Based on your FACS setup, sort single cells or bulk populations and process samples according to the desired method. This approach is compatible with both Dam&ChIC and sortChIC, and potentially other protocols following a similar sample preparation procedure.

Protocol references

Zeller, P. *et al.* Single-cell sortChIC identifies hierarchical chromatin dynamics during hematopoiesis. *Nat Genet* **55**, 333–345 (2023).

Gaza, H. V., Bhardwaj, V. & Zeller, P. Single-Cell Histone Modification Profiling with Cell Enrichment Using sortChIC. in *Chromatin Immunoprecipitation* (ed. Greulich, F.) vol. 2846 215–241 (Springer US, New York, NY, 2024).