Fixed cell immunofluorescence for quantification of hypoxia-induced changes in histone methylation

Dilem Shakir, Michael Batie and Sonia Rocha*

Department of Molecular Physiology and Cell Signalling, Institute of Systems, Molecular and Integrative Biology, University of Liverpool, Liverpool L69 7ZB, UK

* Author to whom correspondence should be addressed: Sonia.Rocha@liverpool.ac.uk

Keywords: Hypoxia, histone methylation, immunofluorescence, immunostaining.

Running Head: IF for histone methylation measurements in hypoxia

Abstract

Hypoxia and its signalling pathway play a key role in human physiology and a variety of diseases. Alterations in histone methylation coordinate transcriptional responses to hypoxia. Here we detail a fixed cell immunofluorescence method for quantifying hypoxia-induced changes in histone methylation, exemplified by the measurement of H3K27me3.

1. Introduction

Eukaryotes possess oxygen-sensing enzymes, which can detect and transduce responses to fluctuations in oxygen availability [1]. Hypoxia (reduced oxygen availability) responses are essential in human development, normal function, and disease [2]. At the cellular level, changes in chromatin, the transcriptome, the epi-transcriptome, the proteome, and the metabolome, coordinate functions to adapt to decreased oxygen availability [3]. Among these changes are alterations in histone methylation [4]. Techniques used to study total levels of specific histone methylation in response to hypoxia in cell culture models include immunoblotting, fixed cell immunofluorescence (IF), and proteomics [5]. Each method comes with advantages and disadvantages. Immunoblotting is relatively cheap and very accessible, however, multiplexing different histone methylation modifications in the same experiment are limited, and results may be influenced by the histone solubilisation method. Proteomics is highly sensitive, is not hindered by antibody quality, and enables detection of many histone methylation modifications in tandem but requires histone proteomics expertise and access to a mass spectrometry instrument. IF allows for detection of histone methylation modifications at the single cell level and multiplexing of 2-3 histone methylation modifications, however, this technique is more time-consuming than immunoblotting, and microscope access may be a limitation.

Fluorescent microscopy allows visualisation of fluorescently labelled cell/tissue components, including proteins of interest. Fluorescent labelling is typically achieved using fluorophore-conjugated antibodies (IF), expressing the protein of interest with a fluorophore tag such as GFP, or using a specific fluorescent dye that binds to the protein of interest. Fixed cell IF uses fixation, which preserves cellular morphology and antigenicity of the targeted cellular component. As the optimal fixative can vary for different antigens, fixation optimisation may be required when using new and/or unvalidated antibodies. In our protocol, we describe methanol and paraformaldehyde (PFA) fixation approaches, which are the two most commonly used fixatives for IF experiments. Cell membrane permeabilization is required for

immunostaining. Additionally, a blocking step prior to primary antibody incubation is critical to prevent non-specific binding. Following primary antibody incubations and washing, fluorophore-conjugated secondary antibodies are used for fluorescence detection under a fluorescence microscope. In addition, staining of specific cellular structures or regions can be achieved with specific fluorescent dyes. Here we describe a fixed cell IF protocol for measuring histone methylation changes in response to hypoxia in mammalian adherent cell lines (*see* **Fig 1**).

2. Materials

2.1 Cell culture and treatments

1. Cell line (*see* **Note 1**)
2. Cell culture incubator set to 37°C, 5% CO_2, and ambient O_2 (21%)
3. 150 mm cell culture plates
4. 35 mm cell culture plates
5. Cell culture medium: Dulbecco's modified eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 10% foetal bovine serum (FBS), 50 U/mL penicillin, 50 μg/mL streptomycin
6. Dulbecco's phosphate buffered saline (DPBS)
7. 0.25% Trypsin
8. 35 mm petri dishes or 6-well plates
9. 100% ethanol
10. 19mm glass coverslips
11. Hypoxia workstation set to 1% O_2, 5% CO_2 and 37°C (*see* **Note 2**)

2.2 Immunofluorescence

1. Humidified chamber (*see* **Note 3**)
2. Tweezers
3. Benchtop rocker

4. Parafilm
5. Tissue paper
6. Aluminium foil
7. Double distilled water
8. 500 mL beaker
9. 1x Phosphate Buffered Saline (PBS)
10. PBST wash buffer: 0.05% Tween 20 in PBS
11. Blocking buffer: 1% Donkey serum in PBST wash buffer
12. 100% Methanol
13. 10 M NaOH
14. Concentrated HCl
15. 0.5% Triton X-100 in PBS
16. Primary histone methylation antibody (*see* **Note 4**)
17. Fluorophore conjugated secondary antibody (*see* **Note 5**)
18. Hoechst 33342 nuclear stain
19. Mounting medium
20. Glass microscope slides
21. Fast drying, colourless nail varnish
22. Fluorescent microscope (*see* **Note 6**)
23. 3.7% Paraformaldehyde (PFA): put 1.85 g PFA into a 50 mL falcon tube and add 3.5 mL water and 10 µL 10 M NaOH. Prepare a water bath by adding 500 mL of boiling hot water in a beaker. Gently shake the PFA containing tube in the water bath for 8 minutes. Keep releasing the lid of the tube every couple of mins to release pressure. Filter-sterilise the solution using a 0.2 µm syringe filter into a new falcon tube and dilute 10-fold with PBS and pH to 6.8 by adding 12 µL concentrated HCl.

3. Methods

3.1 Cell culture preparation and treatment

1. Cells are maintained in 150 mm cell culture plates with cell culture medium inside a cell culture incubator set to 37 °C, 5% CO_2, and ambient O_2 (21%).
2. When cells reach the desired density (e.g. 90% confluency), remove culture medium, wash cells with 5 mL DPBS and detach the cells using 5 mL pre-warmed trypsin.
3. Inactivate trypsin by adding 5 mL of culture medium. Transfer the 10 mL cell solution to a 15 mL canonical tube.
4. Sterilise 19 mm glass coverslips by dipping them into 100% ethanol using a tweezer. Place a coverslip into each 35 mm culture plate and allow the ethanol to dry for 5 minutes.
5. Seed cells at a density of $1.5x10^5$ cells (*see* **Note 7**) onto the coverslips in culture plates and make up to a total volume of 2 mL with culture medium.
6. Hypoxia treatment: put the cells into a hypoxia workstation set to 1% O_2, 5% CO_2 and 37°C for the desired incubation time (*see* **Note 2**).

3.2 Fixation and permeabilization

Discard the cell culture medium and wash cells with PBS (*see* **Note 8**). Proceed to appropriate fixation and permeabilization method (*see* **Note 9**).

3.2.1 Methanol fixation and permeabilization

1. Add 1 mL 100% ice cold methanol to coverslips (*see* **Note 8**).
2. Incubate at -20°C for 7 minutes.
3. Discard methanol and wash coverslips with 2 mL PBS on a benchtop rocker for 5 minutes.
4. Discard PBS, wash coverslips with 2 mL PBS and proceed to the blocking section (*see* **Note 10**).

3.2.2 PFA fixation and Triton X-100 permeabilization

1. Add 1 mL 3.7% PFA to coverslips (*see* **Note 8** and **Note 11**).
2. Incubate for 15 minutes at room temperature.
3. Remove PFA and wash coverslips with 2 mL PBS for 5 minutes on a benchtop rocker.

4. Discard PBS and add 1 mL 0.5% Triton X-100 in PBS to coverslips and incubate for 10 minutes on a benchtop rocker.
5. Discard 0.5% Triton X-100 in PBS and wash coverslips with 2 mL PBS for 5 minutes on a benchtop rocker.
6. Discard PBS, wash coverslips with 2 mL PBS and proceed to the blocking section (*see* **Note 10**).

3.3 Blocking

1. Prepare a fresh humidified chamber (*see* **Note 3**).
2. Add 120 µL drops (1 per coverslip) of blocking buffer onto the parafilm placed inside the humidified chamber.
3. Dry coverslips (*see* **Note 12**), then place the coverslips with the cells facing downwards onto the blocking buffer inside the humidified chamber and incubate for 30 minutes (*see* **Note 13**).
4. Put the coverslips with cells face up back into 35 mm cell culture plates containing 2 mL PBS, wash for 5 minutes on a benchtop rocker then proceed to the immunostaining section.

3.4 Immunostaining (*see* Note 14)

1. Prepare a fresh humidified chamber (*see* **Note 3**).
2. Dilute histone methylation primary antibody in blocking buffer (*see* **Note 15**).
3. Add 120 µL drops (1 per coverslip) of diluted antibody onto the parafilm placed inside the humidified chamber.
4. Dry coverslips (*see* **Note 12**), then place the coverslips with the cells facing downwards onto the diluted antibody inside the humidified chamber and incubate overnight at 4°C (*see* **Note 13**).

5. Put the coverslips with cells face up back into 35 mm cell culture plates containing 2 mL PBST and wash for 5 minutes on a benchtop rocker followed by 3 washes with 2 mL PBS for 5 minutes on a benchtop rocker.
6. Repeat step 1.
7. Dilute fluorophore conjugated secondary antibody according to manufacturer's instructions in blocking buffer (*see* **Note 5**).
8. Repeat step 3.
9. Dry coverslips (*see* **Note 12**), then place the coverslips with the cells facing downwards onto the diluted antibody inside the humidified chamber and incubate for 2 hours (*see* **Note 13** and **Note 16**).
10. Put the coverslips with cells faced up back into 35 mm cell culture plates containing 2 mL PBST and covered with aluminium foil and wash for 5 minutes on a benchtop rocker, followed by 3 washes with 2 mL PBS for 5 minutes on a benchtop rocker.
11. Repeat step 1.
12. Dilute Hoechst 33342 nuclear stain 1:15000 in water.
13. Add 120 µL drops (1 per coverslip) of diluted Hoechst onto the parafilm placed inside the humidified chamber.
14. Dry coverslips (*see* **Note 12**), then place the coverslips with the cells facing downwards onto the diluted Hoechst inside the humidified chamber and incubate for 2 minutes (*see* **Note 13**).
15. Put a drop of mounting medium onto glass microscope slides.
16. Wash coverslips by dipping them into a 500 mL beaker filled with water.
17. Dry coverslips (*see* **Note 12**), then gently place the coverslips with cells facing downwards onto the mounting medium on the microscope slides. Carefully remove excess mounting medium from sides using tissue paper, while avoiding moving the coverslips.
18. Seal the coverslip mounted on microscope slide using nail varnish.
19. Incubate microscope slides for 30 minutes in the dark to allow the nail varnish to dry.

20. Store microscope slides at 4°C for up to 4 weeks or -20°C for longer term preservation.

3.5 Image acquisition and analysis

1. Acquire images on a confocal or widefield fluorescence microscope with appropriate objectives and fluorescent channel filters (*see* **Note 6** and **Note 17**).
2. Calculate histone methylation intensity for individual cells and normalise to control condition (21% oxygen) within each biological replicate (*see* **Note 18**).
3. Plot data from step 1 as a beeswarm plot on top of a box plot.
4. Display representative images for histone methylation and nuclear staining.

4. Notes

1. Any mammalian adherent cell line is suitable, this protocol is written for HeLa cells, other cell lines may require different cell culture medium, cell detachment methods, cell culture incubator settings and cell seeding densities.
2. Other oxygen percentages can be used to induce hypoxia and should be carefully chosen along with hypoxia treatment incubation times.
3. Humidified chamber is prepared by placing a layer of water-soaked tissue paper, followed by a layer of parafilm, flat onto a sealed, lightproof box.
4. Ideally use an IF validated primary antibody. Tri-Methyl-Histone H3 (Lys27) (clone C36B11) Rabbit mAb is used in **Fig 1.**
5. Ensure that the fluorophore matches a fluorescent channel filter on the microscope. Anti-rabbit IgG (H+L), F(ab')2 Fragment (Alexa Fluor 488 Conjugate) is used in **Fig 1.**
6. A confocal or widefield fluorescence microscope with appropriate objectives and fluorescent channel filters is required. We recommend using an objective lens with a magnification of at least 40x. A Zeiss AxioObserver Z1 Microscope with x63 objective, and DAPI and FITC fluorescent channel filters is used in **Fig 1.**
7. Cells should be seeded at a density such that they are 90% confluency at the time of fixation. The cell number given here is for seeding HeLa cells 72 hours prior to fixation.

8. Perform this step inside the hypoxia chamber for hypoxia treated samples to avoid reoxygenation.
9. The Two fixation and permeabilization methods are described in this protocol, methanol fixation and permeabilization, and PFA fixation and Triton X-100 permeabilization. Which method is best is primary antibody dependent; it is recommended that both methods be tested during antibody validation/optimisation.
10. At this point coverslips can be stored in PBS at 4°C for up to 3 months.
11. PFA should be prepared fresh and used immediately or stored at 4°C for up to 24 hours prior to use. All PFA preparation steps should be carried out in a fume hood, and all PFA waste should be discarded in an appropriate PFA waste container.
12. Dry coverslips by gently dabbing on tissue paper and avoiding the cell facing side from touching the paper.
13. It is important not to move the humidified chamber during incubation periods.
14. The Multiplexing staining of 2 or 3 different histone methylation modifications on the same coverslip can be achieved using different primary antibodies raised in different species that do not cross react, and corresponding secondary antibodies with different fluorophore conjugates. Secondary antibody fluorophores should be chosen carefully as to avoid fluorescent signal crossover (spectral bleed-through) with each other and any cell compartment stain should as Hoechst. Also check that the fluorophores match the fluorescent channel filters available on the microscope.
15. For optimisation of primary antibody concentrations, it a recommended to test a range of dilutions, e.g. 1:100, 1:500 and 1:1000. The antibody supplier may also provide a recommended dilution. Using the lowest validated concentration will decrease unspecific epitope binding.
16. From this point onwards, coverslips should be protected from light where possible.
17. We recommend acquiring a minimum of 3 images with at least 30 cells imaged per sample.

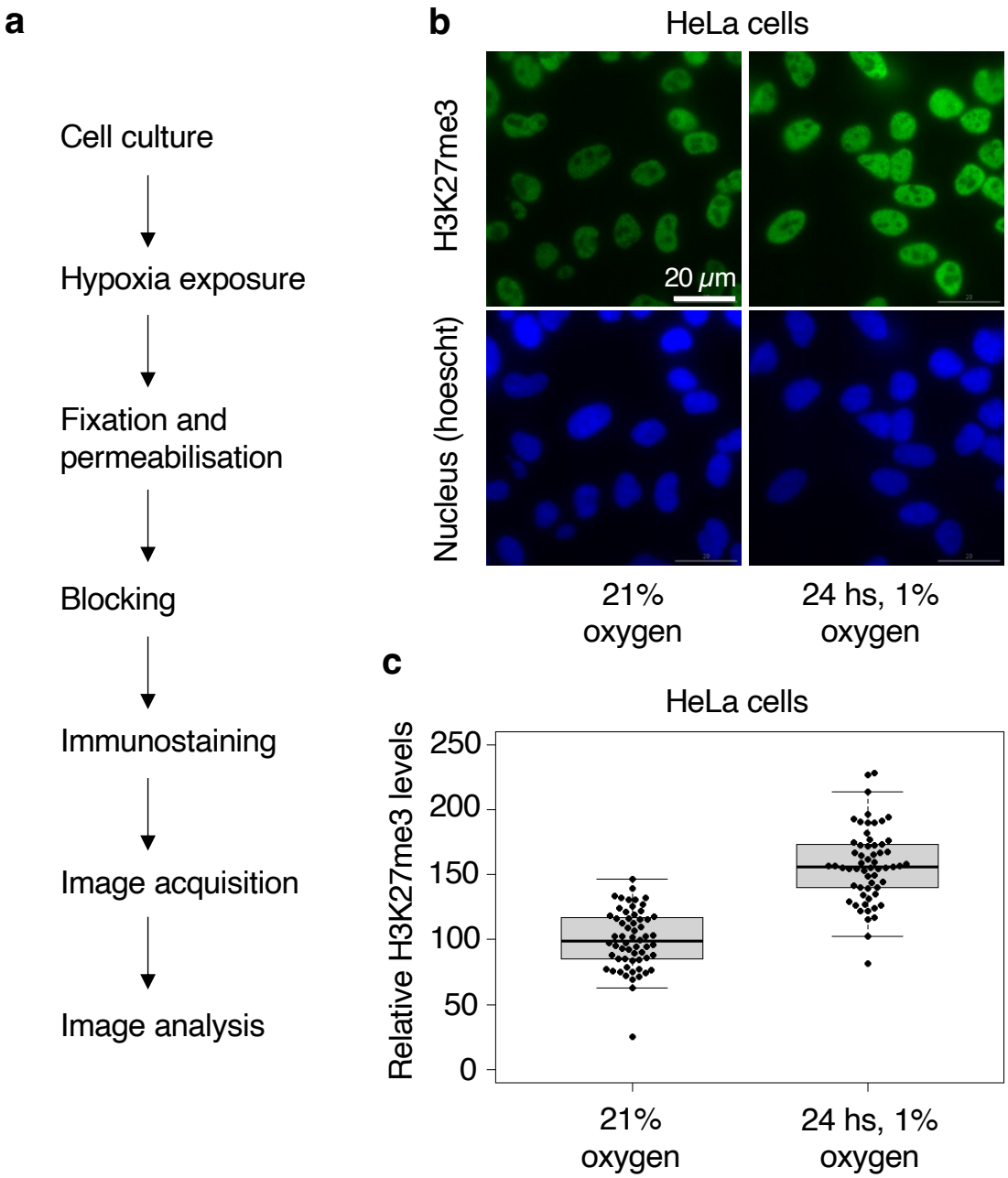
18. Image analysis software tools should be used to calculate intensities from IF images. OMERO [6] is used in **Fig 1.** Popular alternatives include IMARIS and ImageJ.

5. References

1. Hammarlund, E. U., Flashman, E., Mohlin, S. and Licausi, F. (2020) Oxygen-sensing mechanisms across eukaryotic kingdoms and their roles in complex multicellularity. Science. **370**:eaba3512.
2. Giaccia, A. J., Simon, M. C. and Johnson, R. (2004) The biology of hypoxia: the role of oxygen sensing in development, normal function, and disease. Genes Dev. **18**:2183-2194.
3. Batie, M., Kenneth, N. S. and Rocha, S. (2022) Systems approaches to understand oxygen sensing: how multi-omics has driven advances in understanding oxygen-based signalling. Biochem J. **479**:245-257.
4. Gallipoli, P. and Huntly, B. J. P. (2019) Histone modifiers are oxygen sensors. Science. **363**:1148-1149.
5. Frost, J., Frost, M., Batie, M., Jiang, H. and Rocha, S. (2021) Roles of HIF and 2-Oxoglutarate-Dependent Dioxygenases in Controlling Gene Expression in Hypoxia. Cancers (Basel). **13**:350.
6. Allan, C., Burel, J. M., Moore, J., Blackburn, C., Linkert, M., Loynton, S., Macdonald, D., Moore, W. J., Neves, C., Patterson, A., Porter, M., Tarkowska, A., Loranger, B., Avondo, J., Lagerstedt, I., Lianas, L., Leo, S., Hands, K., Hay, R. T., Patwardhan, A., Best, C., Kleywegt, G. J., Zanetti, G. and Swedlow, J. R. (2012) OMERO: flexible, model-driven data management for experimental biology. Nat Methods. **9**:245-253.

Figure legend

Fig 1. Immunofluorescence (IF) protocol for measuring histone methylation changes in response to hypoxia. **a)** Protocol flow chart**. b, c)** IF with H3K27me3 immunostaining and nuclear staining in HeLa cells cultured at 21% oxygen and exposed or not to 1% oxygen for 24 hours. **b)** Representative images. **c)** H3K27me3 levels from 60 cells per condition across two biological replicates displayed as a beeswarm plot on top of a box plot.


a
Cell culture
Hypoxia exposure
Fixation and permeabilisation
Blocking
Immunostaining
Image acquisition
Image analysis
b
HeLa cells
H3K27me3
Nucleus (hoescht)
20 μm
21% oxygen
24 hs, 1% oxygen
c
HeLa cells
Relative H3K27me3 levels
250
200
150
100
50
0
21% oxygen
24 hs, 1% oxygen