Published in final edited form as:

Methods Mol Biol. 2017 ; 1510: 287–293. doi:10.1007/978-1-4939-6527-4_21.

Proximity Ligation Assay to Quantify Foxp3 Acetylation in Regulatory T Cells

Jing Jiao¹, Rongxiang Han², Wayne W. Hancock², and Ulf H. Beier^{1,3}

¹Division of Nephrology, Department of Pediatrics, Children's Hospital of Philadelphia, Philadelphia, PA 19104, University of Pennsylvania, Philadelphia, PA 19104;

²Division of Transplant Immunology, Department of Pathology and Laboratory Medicine and Biesecker Center for Pediatric Liver Disease, Children's Hospital of Philadelphia, Philadelphia, PA 19104.

³Correspondence: Dr. Ulf H. Beier, 916C Abramson Research Center, The Children's Hospital of Philadelphia, 3615 Civic Center Boulevard, Philadelphia, PA 19104-4318, USA., Telephone (215) 590-2449; Fax (215) 590-7384; beieru@email.chop.edu

Summary

I.

Determining protein acetylation by immunoprecipitation and immunoblotting can be challenging, especially if the tissue of interest is low in quantity, and when good quality acetylation-site specific antibodies are not available. Proximity ligation assays allow a sensitive and quantitative method to assess Foxp3 acetylation in regulatory T cells, with as little as 1.5×10^5 cells within two days turnaround time. This method is of potential use in other similar scenarios, when post-translational modifications of a protein of interest needs to be determined with only a small amount of sample and in the absence of specific antibodies that can assess the post-translational modification in the protein of interest.

Keywords

Protein acetylation; Immunoprecipitation; Immunoblot; Proximity ligation assay

1. Introduction

In order to study post-translational modifications of proteins, either immunoblotting or a combination of immunoprecipitation and immunoblotting are considered as standard experimental procedures. These techniques are common and practical when quality antibodies are available and the protein of interest is abundant. However, in the absence of either, post-translational modifications can be much harder to assess. The discovery that inhibition of several histone/protein deacetylases (HDACs) favors the generation and suppressive function of regulatory T cells (Tregs) by increasing the abundance and acetylation of Forkhead box P3 (Foxp3) led to substantial interest in Foxp3 acetylation (1). Foxp3 is a key transcription factor of Tregs and essential to their suppressive function (2). A study by van Loosdregt et al. reported that p300 promotes, and Sirtuin-1 reduces Foxp3 acetylation, utilizing tagged Foxp3 vector transfected HEK 293 cells (3). These studies

allowed important insights into Foxp3 biology, suggesting that blocking deacetylation by Sirtuin-1 increases Foxp3 acetylation and protein level. To investigate the role of Sirtuin-1 in vivo, we had mated floxed-Sirtuin-1 mice with CD4cre and Foxp3cre mice, observed increase in Foxp3 protein expression and improved Treg function (4). However, measuring Foxp3 acetylation in Tregs isolated from Sirt1^{fl/fl}CD4^{cre} or Sirt1^{fl/fl}Foxp3^{cre} mice proved to be methodologically difficult due to the limited amount of Tregs, especially in the absence of acetylation site-specific antibodies at that time (5). This prompted a search for alternative methods to assess protein acetylation.

Proximity ligation assay is a technique developed by Landegren et al from the University of Uppsala, Sweden (6). It allows detection of trace amounts of protein *in situ* by a localized signal amplification, which triggers if two different types of secondary antibodies are close enough together (6). The reaction has been used to detect protein bound to DNA (6), protein-protein interactions (7), as well as post-translational modifications such as tyrosine phosphorylation (8). With regards to Foxp3, van Loosdregt et al used this method to document interactions of Sirtuin-1 and Foxp3 (9). We have adapted this method to determine Foxp3 acetylation in Tregs isolated from HDAC6^{-/-}, HDAC9^{-/-} and Sirt1^{fl/fl}CD4^{cre} mice (10). In our approach, we used primary antibodies of different species against acetyl-lysine residues and against Foxp3, and combined them with matching secondary antibodies (Fig. 1A). The species-specific secondary antibodies each carry a DNA stand, which if in close proximity to each other form a joint DNA segment that can be amplified using a polymerase chain reaction. The replicated DNA can be detected by fluorescence labeled complementary oligonucleotides, which will create fluorescence in the close proximity of Foxp3 and acetyl-lysine antibodies (Fig. 1A). Thus, proximity ligation assay is an alternative procedure that is feasible and sensitive to assess protein acetylation.

2. Materials

2.1 Cell Isolation, Cytospin

1. Spleens and lymph nodes from C57BL/6 and HDAC6^{-/-}, HDAC9^{-/-} and Sirt1^{fl/fl}CD4^{cre} mice.
2. Magnetic beads (catalog #130-091-04, Miltenyi Biotec) to process tissue into single-cell suspensions and to separate conventional T cells (CD4⁺CD25⁻) and Tregs (CD4⁺CD25⁺). Other modalities of purifying Tregs can be used, as well.
3. Shandon™ Double Cytoslides™ (catalog #5991054, Thermo Scientific).
4. Shandon Cytospin 3 centrifuge (Thermo Scientific). Other centrifuge and cytopsin slide products with comparable parameters maybe used, as well.
5. Phosphate buffered saline with 2% fetal bovine serum (pH 7.4).

2.2 Antibodies and Reagents

1. Antibodies against acetyl-lysine and Foxp3, were chosen to be matching mouse and rabbit based antibodies (i.e. Foxp3 mouse and acetyl-lysine rabbit). We

purchased mouse monoclonal anti-Foxp3 (catalog #14-7979-80, eBioscience) and rabbit anti-acetylated-lysine antibody (catalog #9441, Cell signaling).

2. Double-stranded DNA was visualized with Hoechst 33342 (Invitrogen). We used a Duolink® in-situ proximity ligation assay kit “Orange” (Olink Bioscience, Uppsala, Sweden), with corresponding secondary antibodies (PLUS-rabbit, MINUS-mouse), according to the manufacturer’s instructions.
3. Normal goat serum to be used as blocking solution.
4. Triton X-100 was used for permeabilization (Sigma Aldrich or other manufactures).
5. Periodate-lysine-paraformaldehyde (PLP) for fixation, which can be purchased or mixed as reported (11).

2.3 Microscope and Image Analysis Software

1. Images were obtained with an Olympus BX51 Brightfield microscope (Olympus) at 100× magnifications.
2. For image analysis and processing, we used ImageJ version 1.45s, available at the National Institutes of Health, <http://rsbweb.nih.gov/ij/> (12) as well as BlobFinder by Allalou and Wählby (Centre for Image Analysis, Uppsala, Sweden, <http://www.cb.uu.se/~amin/BlobFinder/>) (13). Both image analysis applications are in the public domain.

3. Methods

3.1 Cytospin

1. Assemble purified regulatory T cells (e.g., CD4⁺CD25⁺ using Miltenyi magnetic columns) and suspend the cells in phosphate buffered saline (PBS) with 2% fetal bovine serum at 1×10^6 cells per ml (*see* Note 1).
2. Add the cells to the cytopsin slide. For a double cytoslide, we use 150–200 µl of the cell solution per sample.
3. Centrifuge at $\sim 112.9 \text{ g} \times 5 \text{ min}$. The g force was derived from converting the centrifuge’s rounds per minute value (1,000). A similar setting on a different centrifuge is likely to produce comparable results.
4. After centrifugation, allow the cells to dry for ~ 2 hours at room temperature. Slides not to be immediately used can be wrapped in aluminum foil and stored at -80°C for later use (*see* Note 2).

3.2 Blocking and primary antibodies

1. To achieve fixation, apply periodate-lysine-paraformaldehyde (PLP) solution and incubate for 5 min at 4°C .
2. Wash the slides with PBS for five minutes at room temperature.

3. Pre-treat samples with a blocking solution composed of 10% normal goat serum and 0.2% Triton X-100 for one hour at room temperature.
4. During incubation with the blocking solution, prepare primary antibodies in 1% normal goat serum with 0.2% Triton X-100. Plan to use 80 μ l final volume per sample (*see* Note 3 & 4).
5. In addition, add Hoechst 33342 dye to the primary antibodies at a final concentration of 1 μ g/ml (*see* Note 5).
6. Tap off the pre-treatment solution. Draw a relatively tight circle around the sample using a liquid blocker pen. This aids in orientation and allows preserving antibodies and reagents.
7. After tapping off the pre-treatment solution, apply 80 μ l primary antibody solution to each sample. Now cover the samples in a dark humidity chamber, and incubate the samples overnight at 4° C (*see* Note 6).

3.3 Proximity ligation assay probes (secondary antibodies)

1. One the next day, mix the two proximity ligation assay probes at 1:5 in 1% normal goat serum with 0.2% Triton X-100, and incubate the solution for 20 min at room temperature.
2. Tap off the primary antibody solution.
3. Wash the cells with 1% normal goat serum with 0.2% Triton X-100. Washing should be performed in a staining jar, with a minimum volume of 70 ml on a shaker with gentle orbital shaking. Bring the wash buffers to room temperature before use.
4. After tapping off the 1% normal goat serum with 0.2% Triton X-100 solution, add the diluted proximity ligation assay probes, and incubate the slides in a pre-heated humidity chamber for 1 hour at 37° C.

3.4 Detection protocol

1. During incubation, mix ligation stock and purified water. Hold off on adding ligase to the mix, which is to occur just before application. However, account for the volume of ligase to be later added when making a 1:5 dilution of the ligation stock solution, by using the following ratio: 1 μ L ligase, 8 μ l 5 $\times$ ligation stock and 31 μ l high purity water per sample.
2. Tap off the proximity ligation assay probe solution from the slides.
3. Wash the slides in wash buffer A for 5 min under gentle agitation twice.
4. Add ligase to the ligation solution at above noted ratio (*see* step 3.4.1). Add the ligation-ligase solution to each sample.
5. Incubate the slides in a pre-heated humidity chamber for 30 min at 37° C.
6. Mix amplification stock and purified water. Hold off on adding polymerase until immediately before adding the amplification solution to the samples. However,

account for the volume of polymerase when making a 1:5 dilution of the amplification stock solution, by using the following ratio: 0.5 μ L polymerase, 8 μ L 5 $\times$ amplification stock and 31.5 μ L high purity water per sample.

7. Tap off the ligation-ligase solution from step 3.4.4.
8. Wash the slides in 1 $\times$ wash buffer A for 2 $\times$ 2 min under gentle agitation. Tap off all wash solution after the last washing.
9. Add polymerase to the amplification solution at above noted ratio (*see* step 3.4.6). Add the amplification-polymerase solution to each sample.
10. Incubate the slides in a pre-heated humidity chamber for 100 min at 37° C.
11. Tap off the amplification-polymerase solution from step 3.4.9.
12. Wash the slides in 1 $\times$ Wash buffer B for 10 min twice.
13. Wash the slides in 0.01 $\times$ Wash buffer B for 1 min.
14. Let the slides dry at room temperature in the dark.

3.5 Microscopy and Image processing

1. Mount slides with a cover slip using a minimal volume of Duolink in Situ Mounting Medium with DAPI, ensuring no air bubbles get caught under the cover slip. Use nail polish to seal the edges. Wait for approximately 15 min before analyzing in a fluorescence or confocal microscope (*see* Note 7).
2. Images can be processed and analyzed using Image J analyzed using BlobFinder software by Allalou and Wählby (Centre for Image Analysis, Uppsala, Sweden, <http://www.cb.uu.se/~amin/BlobFinder/>) (13), allowing quantification of cytoplasmatic and nuclear events per cell and per photograph (Fig. 1B).

4. Notes

1. Adding 2% fetal bovine serum to the phosphate buffered saline solution reduces clumping of T cells, and helps prevent the cells from stacking in clusters on the microscopy slide.
2. If the cell isolation ends late in the day, it is possible to preserve the slides after the cytopspin by allowing the slides to dry at room temperature for 1 hour, and than wrapping them in aluminum foil and a closed Ziploc bag to store them at –80° C, where they can be stored long term, even years. Prior to use, the slides should be allowed to warm to room temperature before being unwrapped from the aluminum foil.
3. The volume of the primary (and secondary) antibody solution applied to the samples depends upon the surface area of the sample. If the surface area is 2 cm², this is approximately 80 μ L. The general idea is that each sample should be fully covered by a drop, and has an equal amount and exposure to the antibody solution. To that end, prior solutions (e.g., blocking solution) should be

completely removed, however, without allowing the sample to dry, which can cause artifacts.

4. Typical dilution of primary antibodies in 1% normal goat serum with 0.2% Triton X-100 ranges from 1:100 to 1:1,000. For both the Foxp3 and acetyl-lysine antibodies indicated here, we used 1:100. This may be variable based upon the antibodies and samples used.
5. Other modalities of DNA staining can be used, as well, including 4',6-diamidino-2-phenylindole. The DNA staining can be done with either the primary or the secondary antibody staining.
6. Add wet tissue paper to the chamber to minimize evaporation of the primary antibody solution. Avoid shaking or unnecessary moving the chamber to prevent spilling of the primary antibody solution outside of the lipid markings drawn by the liquid blocker pen.
7. After the images are obtained, the slides can be stored at -20°C in the dark for several months.

Acknowledgements

This work was supported by Award Number AI095353 (to U.H.B.), AI073489, and AI095276 (to W.W.H.) from the National Institute of Allergy and Infectious Diseases. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Allergy and Infectious Diseases or the National Institutes of Health.

References

1. Tao R, de Zoeten EF, Ozkaynak E, Chen C, Wang L, Porrett PM, Li B, Turka LA, Olson EN, Greene MI, Wells AD, & Hancock WW (2007) Deacetylase inhibition promotes the generation and function of regulatory T cells. *Nature medicine* 13(11):1299–1307.
2. Feuerer M, Hill JA, Mathis D, & Benoist C (2009) Foxp3+ regulatory T cells: differentiation, specification, subphenotypes. *Nature immunology* 10(7):689–695. [PubMed: 19536194]
3. van Loosdregt J, Vercoulen Y, Guichelaar T, Gent YY, Beekman JM, van Beekum O, Brenkman AB, Hijnen DJ, Mutis T, Kalkhoven E, Prakken BJ, & Coffier PJ (2010) Regulation of Treg functionality by acetylation-mediated Foxp3 protein stabilization. *Blood* 115(5):965–974. [PubMed: 19996091]
4. Beier UH, Wang L, Bhatti TR, Liu Y, Han R, Ge G, & Hancock WW (2011) Sirtuin-1 targeting promotes Foxp3+ T-regulatory cell function and prolongs allograft survival. *Molecular and cellular biology* 31(5):1022–1029. [PubMed: 21199917]
5. Kwon HS, Lim HW, Wu J, Schnolzer M, Verdin E, & Ott M (2012) Three novel acetylation sites in the Foxp3 transcription factor regulate the suppressive activity of regulatory T cells. *J Immunol* 188(6):2712–2721. [PubMed: 22312127]
6. Fredriksson S, Gullberg M, Jarvius J, Olsson C, Pietras K, Gustafsdottir SM, Ostman A, & Landegren U (2002) Protein detection using proximity-dependent DNA ligation assays. *Nature biotechnology* 20(5):473–477.
7. Soderberg O, Gullberg M, Jarvius M, Ridderstrale K, Leuchowius KJ, Jarvius J, Wester K, Hydbring P, Bahram F, Larsson LG, & Landegren U (2006) Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nature methods* 3(12):995–1000. [PubMed: 17072308]
8. Jarvius M, Paulsson J, Weibrecht I, Leuchowius KJ, Andersson AC, Wahlby C, Gullberg M, Botling J, Sjoblom T, Markova B, Ostman A, Landegren U, & Soderberg O (2007) In situ detection of phosphorylated platelet-derived growth factor receptor beta using a generalized proximity ligation method. *Molecular & cellular proteomics : MCP* 6(9):1500–1509. [PubMed: 17565975]

9. van Loosdregt J, Brunen D, Fleskens V, Pals CE, Lam EW, & Coffey PJ (2011) Rapid temporal control of Foxp3 protein degradation by sirtuin-1. *PloS one* 6(4):e19047. [PubMed: 21533107]
10. Beier UH, Wang L, Han R, Akimova T, Liu Y, & Hancock WW (2012) Histone deacetylases 6 and 9 and sirtuin-1 control Foxp3+ regulatory T cell function through shared and isoform-specific mechanisms. *Science signaling* 5(229):ra45. [PubMed: 22715468]
11. McLean IW & Nakane PK (1974) Periodate-lysine-paraformaldehyde fixative. A new fixation for immunoelectron microscopy. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* 22(12):1077–1083. [PubMed: 4374474]
12. Schneider CA, Rasband WS, & Eliceiri KW (2012) NIH Image to ImageJ: 25 years of image analysis. *Nature methods* 9(7):671–675. [PubMed: 22930834]
13. Allalou A & Wahlby C (2009) BlobFinder, a tool for fluorescence microscopy image cytometry. *Computer methods and programs in biomedicine* 94(1):58–65. [PubMed: 18950895]

Figure 1.

Proximity ligation assay to measure Foxp3 acetylation in Tregs. (a) Experimental design: Regulatory T cells are fixated and permeabilized on a cytospin slide and incubated with primary of different species antibodies against Foxp3 and acetylated lysine. Subsequently, secondary antibodies specific to the species of the primary antibodies are applied. Each of the two different secondary antibodies have a short specific DNA strand attached. If two different secondary antibodies are in close proximity to each other, their DNA oligonucleotides undergo a reaction that forms a circular DNA oligonucleotide, which can then be amplified using a polymerase. Subsequently, the local accumulation of the DNA oligonucleotide can be detected using fluorescent probes, which will be detectable only in the presence of two opposite secondary antibodies. Therefore, fluorescence represents Foxp3 protein that is acetylated. (b) Examples of immunofluorescence images from cytospin samples of Treg demonstrating Hoechst DNA staining (left) and the DNA oligonucleotide fluorescence reaction (right, each at 100× magnification). The DNA staining is used to define nuclei, and overlaid with the proximity ligation assay images derived from from Anti-Foxp3 and Anti-Acetyl-lysine (AcK) co-localizing antibodies, and analyzed using BlobFinder software (screenshot). Overlay of DNA staining and the oligonucleotide fluorescence allows assessment of nuclear acetylated Foxp3.