Antibody-Driven Proximity Labeling in Fixed Tissues

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Abstract

Protein function often depends on assemblies and interactions. These show complex spatial and temporal organization within the cell. Analysis of protein function can be greatly assisted by grouping proteins with their neighbors. Rather than relying on affinity, proximity labeling targets proteins proximal to the target of interest. We describe a protocol for antibody-guided deposition of tags in fixed and permeabilized cell lines and primary human tissue samples.

Key words Proximity labeling, Antibody, Biotin, HRP, Mass-spectrometry, Protein-protein interactions

1 Introduction

Protein complexes provide much of the functionality needed for life. The kinetics of their interactions span multiple orders of magnitude in half-life and protein-protein affinity. The composition of these complexes can change with cell cycle, developmental state, and cell type. Some proteins and complexes are restricted to specific organelles or subcellular localizations, while others are more ubiquitous. Characterizing the subcellular environments where proteins of interest reside can inform us about specific functions and potential protein interactors.

1.1 Proximity Labeling

Proximity labeling relies on the deposition of a tag, typically biotin, on molecules adjacent to the target of interest [1–4] (Fig. 1). Both the labeling target and the proximal targets are usually proteins. However, proximity labeling can be used for multiple other biomolecules. Recent studies have targeted DNA either as the labeling or as the proximal target [5, 6]. Additionally, RNA was isolated following cross-linking to proximal proteins [7]. Similar approaches may be used in the future to study other biomolecules, like lipids, with subcellular resolution.

Fig. 1 Outline of the antibody-driven proximity labeling method

Proximity labeling can be used to map complexes and the immediate environment of proteins of interest [8, 9]. It may also be used to map the composition of organelles and subcellular compartments [3, 10, 11].

1.2 Limitations

Proximity labeling limitations more specific to this method include sensitivity to fixation artifacts and need for a specific antibody. More general limitations also apply. Some proteins will be harder to identify by the mass spectrometer. These include proteins that had no accessible sites for tag deposition, and proteins, typically small ones, that are harder to detect due to the biochemical

properties of their peptides. Finally, tag deposition may result in masking of some peptides from the mass spectrometer.

Not surprisingly, we found the method to work best on proteins with a clear and limited subcellular localization. By contrast, diffused cytoplasmic proteins resulted in widespread labeling.

1.3 Differential Proteomics

Many cellular proteins may have a defined function in a specific location, but are not restricted to just that compartment. The identification of a protein in an organelle is thus not always informative, as it merely implies that protein is not actively or passively excluded from it. For example, multiple proteins in the nucleoplasm are labeled by reactions targeting lamins, without having any known functional role at the nuclear envelope. It is thus often more useful to contrast two compartments or conditions. By contrasting a signal originating at the nuclear envelope with a signal originating at active chromatin marks, predominantly excluded from the nuclear envelope, an enrichment ratio can be calculated. Proteins that are identified but depleted at the nuclear envelope, compared to active chromatin, will then receive a lower priority score as nuclear envelope proteins.

Differential proteomics can also be used when comparing two conditions, for example a mutation versus wild-type tissue or cell, or the composition of a protein complex in treated versus untreated samples. However, it is still useful to identify the protein environment of the target of interest, to distinguish global and local changes between the two conditions.

1.4 Relative Quantification

Differential proteomics typically relies on relative quantification of two or more samples. Multiple methods are now capable of comparing the relative abundance of thousands of proteins from mass spectrometry samples. These include labeled and label-free methods. We use stable isotope labeling with amino acids in cell culture (SILAC [12]) and tandem mass tagging (TMT [13]), as they are easy to adapt and make the analysis straightforward. These methods can multiplex multiple samples on a single mass spectrometer run, saving machine time while eliminating between-run variability.

SILAC enables samples to be mixed earlier in the experiment, reducing experimental variability. We use SILAC for cell culture of two samples; however multiplexing more samples, typically less than four, is also possible. Alternatively, a spike-in reference, also known as Super-SILAC [14, 15], can be used for multiple cell culture samples and controls. By contrast, TMT is applied at the peptide level, and can thus be used on any mass-spec-ready sample. TMT enables to multiplex more samples (10+) on a single run. We use TMT for primary tissues.

1.5 Controls

Common sources of false positives include off-target and nonspecific antibody binding, label leakage, and nonspecific binding to the

purification beads. While the protocol is designed to minimize this background, it does not remove it completely. Quantifying proteins in samples versus controls is used to calculate enrichment over background. Good controls include proteins excluded from the target of interest, whole-tissue extracts, and unbound fraction from protein isolation. A sample without a primary antibody can also serve as a control. Controls should not be limited to a single control per sample. For example, when targeting the nuclear envelope, appropriate controls may include an inner-nuclear protein, the unbound fraction, an ER protein, and a no-antibody control.

Some controls may produce significantly more protein than the target of interest. All relative quantification methods have a limited range where comparison is accurate. It is thus important to scale the controls to roughly match the total protein content of the sample.

2 Materials

2.1 Reagents for Proximity Labeling and Validation

1. Phosphate-buffered saline (PBS).
2. PBST: PBS containing 0.1% Tween-20.
3. 4% Formaldehyde (FA): Prepare in PBST and aliquot in single-use tubes or make fresh.
4. 3% H_2O_2 : Bottles can be used for about a month from opening.
5. Blocking buffer: 1% Bovine serum albumin (BSA) in PBST.
6. Primary and appropriate secondary HRP-conjugated antibody.
7. Biotin-XX tyramide (for example Tyramide Signal Amplification kit by Thermo Fisher).
8. 500 mM of sodium ascorbate.
9. 10% (w/v) Sodium dodecyl sulfate (SDS).
10. 10% (w/v) Sodium deoxycholate.
11. 1 M NaCl in PBST.
12. Streptavidin magnetic beads.
13. Acetone containing 10 mM of NaCl.
14. Appropriate secondary antibody conjugated to a red dye.
15. DAPI.
16. FITC-streptavidin.
17. Fluorescence microscope.
18. Western blot reagents.

2.2 Mass Spectrometry

1. Sequencing-grade trypsin.
2. 500 and 100 mM of DTT in water.

3. 550 mM Iodoacetamide in water.
4. 50 mM Ammonium bicarbonate (ABC).
5. 50 mM triethylammonium bicarbonate (TEAB).
6. Trifluoroacetic acid.
7. C18 resin (Zip Tip).

3 Methods

3.1 Preparation of Cells and the Proximity Labeling

3.1.1 Preparation of Cells

For SILAC experiments, culture cells for at least five passages in SILAC medium [12, 16, 17], detaching cells from surface with an enzyme-free dissociation buffer. The number of cells needed depends on the target abundance and antibody quality. Two million cells is a good starting point, but this can be scaled down significantly for some targets.

Perfuse mice with formaldehyde [18], harvested relevant organs, wash and kept frozen until ready for use.

3.1.2 Proximity Labeling

1. On day 1, fix the samples. Fixative needs to be compatible with immunofluorescence and reversible. We use 4% FA in PBST for 10 min at room temperature (RT) for cell culture and flash-frozen samples, or formaldehyde perfusion in mouse samples. (1) For cell culture, this can be done on cells dissociated with an enzyme-free cell dissociation buffer (recommended for SILAC), washed twice with PBS. Alternatively, cells can remain adherent to the flask and scraped off later. If fixing adherent cells, wash twice with PBS before fixing. (2) For frozen unfixed primary tissues: While kept frozen, slice thin sections of the tissue block either with a cryostat or with a razor. Move the sample immediately to the fixative. Slicing thickness needs to allow for effective antibody penetration. Weigh the fixative tube before and after adding the tissue. 50 mg of tissue is a good starting point. (3) For fixed (perfused) animal tissue: Slice frozen and move to PBST.
2. Wash twice with PBST. Washes are done by spinning down the sample (2,000 rpm/370 × g for 3 min). Adipose samples may float. Make sure not to remove cells or tissue.
3. For SILAC: If using the same antibody for both samples (for example wild-type vs. mutant): calculate cell number or protein concentration and mix equal amounts into a single tube.
4. Permeabilize samples by incubating with 0.5% Triton X-100 in PBS for 7 min.
5. Wash samples with PBST.
6. Add H₂O₂ to 0.5% for 10 min, and wash twice with PBST. This will kill the endogenous peroxidases (*see Note 1*).

7. Block samples by incubating with 1% BSA in PBST for 2 h, shaking.
8. Stain with primary antibody (for example MAB3211 (1:500)) overnight at 4 °C. Antibody must be in a blocking buffer. Incubation time may be adjusted.
9. On day 2, wash samples for 20 min in PBST, and repeat three times (1 h total).
10. Add secondary HRP antibody at 1:1000 in 1% BSA for 1 h. Antibody must be in a blocking buffer.
11. Wash samples for 30 min in PBST, and repeat four times (2 h total).
12. For SILAC: If using different antibodies: calculate cell number or protein concentration and mix equal amounts into a single tube.
13. Reduce sample volume to ~50 μ L. Incubate with 3 μ L of tyramide signal amplification (TSA) reagent for 5 min (can be incubated for 30 min for hard-to-penetrated tissue samples). Add 150 μ L reaction buffer and incubate for 1–7 min (*see Note 2*). Longer incubation will result in stronger signal, but also more nonspecific proteins. Stop reaction with 1 mL of 500 mM sodium ascorbate.
14. Wash three times with PBST. If using adherent cells, scrape cells off. Optional stopping point—store at -80°C .
15. Validate labeling by immunofluorescence of subsamples. Add a red secondary fluorescent antibody, DAPI, and FITC-streptavidin and incubate for 30 min at RT. Wash three times with PBST, 20 min each. Mount on slides and image. Label worked if strong green (biotin) signal is seen co-localizing with the red signal from the secondary fluorescent antibody (*see Notes 3 and 4*). This is the most informative control. Tag staining should be strong and specific (*see Note 5*).
16. Adjust volume to 0.1 mL PBST, and add 30 μ L of 10% SDS and 20 μ L of sodium deoxycholate 10% (optional), heated for 99°C for 1 h with mild shaking. If sample does not dissolve completely (mainly hard tissues), sonicate and add 30 min of heating.
17. Centrifuge at max speed for 5 min. Optional stopping point—store at -80°C .
18. Move supernatant to a new tube and adjust volume to 1 mL with PBST. Add 250 μ L of pre-washed streptavidin beads. Incubate for 2 h at RT or overnight at 4°C . Keep unbound.
19. For beads: After incubation, wash once with PBST, twice with PBST with 1 M NaCl, and twice more with PBS (not PBST).

20. Move beads to a new tube and adjust volume to 100 μL . Subsamples ($\sim 10\%$) can be used for Western blot validations. It is better to target known interactors, and not the primary protein directly. Tag depositions can sometimes mask the epitope, particularly on the targeted protein. Antibodies from the labeling process can still be found in the mix. For blots with streptavidin-HRP, a very strong signal, either of multiple bands or of a smear, is expected.
21. For the unbound/input control: Precipitate unbound proteins by adding 6 volumes of cold acetone with 10 mM NaCl and placing in -20°C for 1 h. Centrifuge at $18,400 \times g$ for 15 min, and keep pellet. Optional stopping point—store at -80°C .

3.2 Mass Spectrometry

For TMT, Use TEAB Instead of ABC Throughout This Protocol

1. Resuspend beads/pellet in 90 μL 50 mM ABC (or TEAB for TMT) and add 2 μL of 500 mM DTT. Incubate beads for 30 min at 37°C with mixing. Incubate the unbound sample for 30 min at 56°C .
2. Spike samples with 10 μL of 550 mM iodoacetamide. Incubate for 20 min at RT, with mixing and in the dark.
3. Pellet beads and wash twice with 200 μL of 50 mM ABC (or TEAB for TMT). Spike the unbound sample with 20 μL of 100 mM DTT.
4. Resuspend beads in 200 μL of 50 mM ABC (or TEAB for TMT). Add 2 μg of trypsin and incubate overnight at 37°C with mixing. Add 2 μg of trypsin to unbound sample.
5. On day 3, add 1 μg of trypsin to each sample. Incubate for 2 h at 37°C .
6. Pellet the beads at low speed and transfer the supernatant to a fresh tube.
7. Rinse the beads twice with 150 μL of HPLC-grade H_2O , adding water to the supernatant tube. Discard the beads. We no longer recommend washing with acid (1% trifluoroacetic acid in H_2O), as it causes an unidentified polymer to leak off the beads and interfere with the mass spectrometer.
8. Add 5 μL 100% trifluoroacetic acid to the unbound sample.
9. Spin the pooled eluant at $18,400 \times g$ for 2 min and transfer supernatant to a fresh tube. This removes any leftover debris.
10. Reduce sample volume by using a heated vacuum centrifuge. Reduce samples to $\sim 30 \mu\text{L}$. Moderate heating can be used; do not overdry. Optional stopping point—store at -80°C .

11. For TMT: Label the peptides according to the kit manufacturer's instructions (for example TMT10plex™ by Thermo Fisher). Mix the labeled peptides (*see* **Note 6**).
12. Desalt and concentrate the samples using C18 resin (Zip Tip).
13. Analyze by mass spectrometry or keep at -80°C .

4 Notes

1. Make sure that H_2O_2 is potent. Otherwise, strong nonspecific staining on the tag (biotin) channel would be observed due to endogenous peroxidase activity.
2. Too short of a labeling time will result in decreased signal. Too long will result in labeling of distant proteins, and potentially generate more background that drowns the detection of less abundant proximal proteins. Both conditions would lead to known interactors not detected by mass spectrometry or ratios do not show enrichment for known interactors. Adjust labeling time to get a strong, specific, and highly localized signal when using immunofluorescence. Also make sure that fresh BSA is used for blocking with both primary and secondary antibodies.
3. No staining on any channel can be caused by primary antibody failure. Make sure that the primary antibody still works well and sample is permeabilized.
4. No staining on the tag channel only can be caused by H_2O_2 decomposition or dead enzyme in the secondary antibody. Add fresh H_2O_2 to reaction buffer and use a new secondary antibody.
5. Strong natural biotinylation may mask the signal. This can be validated by using FITC-streptavidin without the reaction. Consider using a different tag, like Azido-Cy5 tyramide (AAT Bioquest), if that is the case.
6. TMT labeling occurs after the peptides are retrieved, so no special preparation is required. However, as it relies on NHS chemistry, buffers containing primary amines must be excluded from the last processing steps.

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