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Immunofluorescence microscopy to study endogenous TAZ in mammalian cells

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Abstract

The Transcriptional co-activator with PDZ-binding motif (TAZ), which is encoded by the *WWTR1* gene, is a key transcriptional effector of the Hippo signaling pathway. TAZ function has been implicated in a variety of developmental processes, as well as a number of diseases, most notably in driving pro-tumorigenic roles in a variety of cancers. Given that nuclear-cytoplasmic localization dynamics dictate TAZ activity, techniques for visualizing TAZ localization are critical for its study. Here we describe an immunofluorescence microscopy protocol that allows for the visualization of TAZ subcellular localization in mammalian cells, offering an approach that can aid in the analysis of TAZ regulation and function.

Keywords

TAZ; *WWTR1*; Hippo pathway; immunofluorescence microscopy

1. INTRODUCTION

The Transcriptional co-activator with PDZ-binding motif (TAZ), which is encoded by the *WWTR1* gene, has emerged as a major regulator of cell proliferation, survival, and fate in a variety of organs and tissues [1,2]. TAZ is a transcriptional regulator that functions as a key effector of Hippo pathway signaling. TAZ directs gene expression by binding to a variety of transcription factors, most notably the TEAD family (TEAD1–4) of proteins [3,4]. TAZ-regulated transcriptional programs are implicated in a variety of biological processes, which often are redundantly controlled by its paralog Yes-associated protein (YAP) [5–10]. TAZ activity is regulated in large part by its nuclear-cytoplasmic distribution, which is defined by changes in its accumulation in different cellular compartments [11].

The phosphorylation of TAZ on several conserved serine/threonine residues has been shown to contribute to the changes in TAZ nuclear-cytoplasmic distribution. For example, phosphorylation of TAZ on Serine89 in human TAZ promotes binding to 14-3-3 proteins and subsequent cytoplasmic retention [12]. Phosphorylation of this residue by the Hippo pathway kinases, LATS1 and LATS2 (LATS1/2), has emerged as a signature mode of TAZ regulation [13]. TAZ stability is also regulated by phosphorylation, contributing to the

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altered nuclear-cytoplasmic distribution of TAZ [14]. Accordingly, hypo-phosphorylated TAZ predominantly accumulates in the nucleus. TAZ nuclear-cytoplasmic distribution directs various biological events, including early cell fate determining processes [7]. Further, dysregulated nuclear TAZ activity has been implicated in a variety of human cancers [15,16]. Thus, the ability to examine TAZ localization in cells and tissues has become an important part of the study of TAZ biology.

In this chapter, we describe an immunofluorescence microscopy method for visualizing endogenous TAZ localization in mammalian cells. We demonstrate the efficacy of our method by showing the loss of immunofluorescent signal in primary mouse lung fibroblasts with *Taz* conditionally knocked out.

2. MATERIALS

2.1 Sample Preparation

1. Glass bottom microscopy slides (see Note 1)
2. Cells of interest (here we used primary mouse lung fibroblasts to validate our method, see Figure 1).
3. Phosphate Buffered Saline (PBS) (see Note 2)
4. 4% (v/v) paraformaldehyde in PBS. (see Note 3, 4)
5. Orbital shaker

2.2 Staining

1. 1% (w/v) SDS in PBS.
2. Blocking solution: 2% (w/v) Bovine Serum Albumin (BSA) in PBS.
3. Primary antibody solution: Dilute antibody (BD Biosciences mouse anti-Taz #BD560235) 1:500 in blocking solution (see Notes 4, 5)
4. PBS-T: 0.1% (v/v) Tween-20 in PBS
5. Secondary antibody solution: anti-mouse fluorescent secondary antibody (see Note 4, 6)
6. DNA stain solution: 1 µg/ml solution of DAPI (4',6-diamidino-2-phenylindole, dihydrochloride) (Thermo Fisher # 62247) in PBS, or 1 µg/ml solution of Hoechst 33342 (Thermo Fisher #H1399) in PBS.
7. Prolong Gold anti-fade reagent (Life Technologies P36930).
8. Cover slips
9. Fluorescent microscope (see Note 7)

3. METHODS

3.1 Sample Preparation

1. Grow cells to desired confluence in glass-bottom chamber slides (*see* Note 8).
2. Aspirate the growth media and add PBS to the well at room temperature (*see* Note 9).
3. Aspirate the PBS and add 4% paraformaldehyde solution to each well at room temperature. Incubate for 15 minutes at room temperature (*see* Note 10).
4. Aspirate the paraformaldehyde and wash once with PBS (*see* Note 9).
5. Add PBS to each well of the chamber slide (*see* Notes 9 & 11)

3.2 Staining

1. Aspirate the PBS from the chamber slides and add 1% SDS/PBS to each well. Incubate at 37°C for 10 minutes (*see* Note 12).
2. Aspirate the SDS/PBS buffer and add PBS to each well (*see* Note 9). Wash on a shaker at low speed for 5 minutes at room temperature.
3. Aspirate the PBS. Add blocking solution to each well and incubate on a shaker at low speed for 1 hour at room temperature (*see* Note 13).
4. Aspirate the blocking solution. Add primary antibody solution and incubate on a shaker at low speed at 4°C for 12–16 hours (*see* Note 13 & 14).
5. Aspirate the primary antibody solution. Add PBS-T to each well and wash on a shaker at low speed for 5 minutes at room temperature (*see* Note 9). Aspirate and repeat the wash for an additional 5 minutes.
6. Aspirate the PBS-T. Add secondary antibody solution to each well and incubate on a shaker at low speed for 1 hour at room temperature (*see* Notes 13 & 15).
7. Aspirate the secondary antibody solution. Add PBS-T to each well and wash on a shaker at low speed for 5 minutes at room temperature (*see* Note 9). Aspirate and repeat the wash for an additional 5 minutes.
8. Aspirate the PBS-T. Add DNA stain solution to each well and incubate on a shaker at low speed for 15 minutes at room temperature (*see* Note 16).
9. Aspirate the DNA stain solution. Add PBS to each well and wash on a shaker at low speed for 5 minutes at room temperature (*see* Note 9). Aspirate and repeat the wash for an additional 5 minutes.
10. Aspirate the PBS.
11. Mount the slides for imaging using Prolong Gold anti-fade reagent (Life Technologies P36930) (*see* Note 17).
12. Image the slides using a fluorescent microscope (*see* Notes 7, 18 & 19).

4. NOTES

1. We typically use the Thermo Scientific Nunc Lab-Tek II Chamber Slide System (Thermo Fisher 154526) or glass cover slips that fit into a 24 well culture dish (Thermo Fisher 12-545-80).
2. PBS is composed of 137 mM NaCl, 2 mM KCl, 10 mM Na₂HPO₄, Sodium Phosphate Dibasic, 1.8 mM KH₂PO₄, with the pH adjusted to 7.4 with HCl.
3. We make 4% (v/v) paraformaldehyde by diluting 5 ml 16% paraformaldehyde aqueous solution (Electron Microscopy Sciences # 15710) into 15 ml PBS.
4. The noted solutions should be made on the day that they are used and stored at 4 °C until it is time to use them.
5. Using the protocol described above, as well as variations of this protocol, we have attempted to examine the localization of TAZ using several other commercial antibodies, including: anti-WWTR1 antibody (Sigma #HPA007415), anti-TAZ antibodies (Abcam #ab84927 and #ab110239), and anti-TAZ antibody (Cell signaling #2149). The antibody we report here (BD560235) is the only antibody that we have tested that reliably shows a loss of signal in TAZ depleted or TAZ deleted cells. Some TAZ antibodies, such as the anti-WWTR1 antibody and the YAP/TAZ antibody (Cell signaling #8418), do show a loss of signal when TAZ and YAP are co-depleted or co-deleted, indicating that these antibodies also have an affinity for YAP, which may be appropriate for some experimental conditions. We therefore caution the research community in making conclusions about TAZ levels and localization without proper validation.
6. For our experiment shown in Figure 1 we made a 1:500 dilution of Invitrogen A-31571 donkey anti-mouse Alexa Fluor 647 in blocking solution.
7. For the images shown in Figure 1 we used a Zeiss AxioObserver D1 equipped with a X-Cite 120LED System.
8. For the experiment shown in Figure 1 we used 4-well chamber slides with a culture area of 1.7 cm². For larger or smaller chamber slides, adjust all volumes in this protocol accordingly.
9. For 4-well chamber slides with a culture area of 1.7 cm² we use 500 µl PBS or PBS-T per well.
10. For 4-well chamber slides with a culture area of 1.7 cm² we use 200 µl of 4% PFA solution per well.
11. Chamber slides may be stored in with PBS at 4°C for several weeks. However, if the slides are not processed right away, wrap them in Parafilm (Sigma #P7793-1) to prevent the wells from drying out.
12. We have found that using 1% SDS for cell permeabilization/antigen retrieval is an important step in our protocol. For 4-well chamber slides with a culture area of 1.7 cm² we use 200 µl 1% SDS/PBS per well.

13. For 4-well chamber slides with a culture area of 1.7 cm² we use 200 µl of blocking, primary antibody, and secondary antibody solutions per well.
14. This primary antibody incubation step may be done for 2 hours at room temperature, but we have found that reducing the time in primary antibody results in a less intense signal.
15. It is important to not incubate in secondary antibody solution for much more than 1 hour, as lengthy incubations in secondary antibody will increase the background signal. Also, we recommend covering the chamber slide with foil to protect from light during the washes and incubations for the remainder of the protocol.
16. For 4-well chamber slides with a culture area of 1.7 cm² we use 200 µl of DNA stain solution per well.
17. For the chamber slide system use the directions that accompany the slides, and for cover slips we carefully pick them up with forceps and place them on a microscopy slide (Thermo Fisher #12-550-15).
18. The highest quality images will be obtained immediately after staining. However, slides can be stored at 4°C in the dark and retain their signal for several weeks.
19. To show the efficacy of immunofluorescence staining of endogenous TAZ using the BD560235 antibody we refer the readers to our prior studies [17,18], as well as Figure 1, where we show our analysis of primary mouse lung fibroblasts isolated from *Taz-loxP/loxP* [19]; *Col1a1CreERT2* (Jax # 027751) mice. Cells isolated from these mice allow for the conditional deletion of *Taz* following treatment with 4-hydroxytamoxifen (Sigma #H7904).

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FIGURE 1. Validation of TAZ immunofluorescence staining in Taz-null mouse fibroblasts.

a. Primary mouse lung fibroblasts were isolated from *Taz-loxP/loxP*; *Col1a1-CreERT2* mice (conditional *Taz* knockout mice, Taz-null) or *Taz-loxP/loxP* mice without any Cre (wild-type). Fibroblasts were grown in the presence of 3 μ M 4-hydroxytamoxifen for 5 days to induce CreERT2-mediated knockout, then fixed and stained according to the protocol in this manuscript.

b. Intensity of nuclear Taz staining was quantified for the wild type and Taz-null fibroblasts using CellProfiler as described [20] ($\pm$ SEM, * $p < 0.01$).