Published in final edited form as:

Methods Mol Biol. 2019 ; 1922: 103–110. doi:10.1007/978-1-4939-9012-2_10.

In Vitro Analysis of Intramolecular Signaling Events in PDLSCs Using Confocal and TIRF Microscopy

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Abstract

The advances in microscopy techniques enable the detection of intracellular molecular processes to be visualized and analyzed for periodontal ligament stem cells (PDLSCs). Confocal laser scanning microscopy (CLSM) and total internal reflection fluorescence microscopy (TIRFM) are two well-studied microscopy techniques that allow an increase in the resolution and contrast of the micrographs and the capability to pinpoint events at the plasma membrane, respectively. Confocal microscopy achieves its increased resolution and contrast through a spatial pinhole that hits the plane of the image. TIRF microscopy uses the principle of incident angles and the refractive index of the substances to study the events occurring at 100–200 nm range of the cover glass with minimal background interference. Here we describe two methods for intramolecular signaling visualization upon stimulation with a ligand in normal growth conditions and mineralization-induced conditions in periodontal ligament stem cells (PDLSCs). These methods are important for visualizing the signaling events within PDLSCs at a molecular level and thereby understand the mechanisms by which these cells could be manipulated for tissue engineering and regeneration.

Keywords

Confocal microscopy; Immunocytochemistry; PDLSCs; TIRFM; Receptor; Ligand

1 Introduction

Periodontal ligament stem cells (PDLSCs) are versatile stem cells because of their capacity to differentiate into multiple lineages, such as cementoblasts, fibroblasts, and osteoblasts, making them advantageous for tissue regeneration. Periodontal disease can destroy the hard tissues of the periodontium, which can lead to bone, periodontal ligament, and eventually tooth loss in those suffering from the disease [1]. Although PDLSCs possess the ability to differentiate into the various components of the periodontium, their success in tissue regeneration has been limited due to the lack of fundamental knowledge of their molecular processes. With the use of a ligand, we can visually track the molecular processes of PDLSCs under normal growth and mineralization conditions with the sophisticated microscopy techniques of confocal laser scanning microscopy (CLSM) and total internal

reflection fluorescence microscopy (TIRFM). With these microscopy techniques, the processes can be examined on both fixed and live cells.

CLSM is a microscopy technique developed in the late 1980s to improve both the resolution and contrast of the specimens in micrographs [2]. CLSM utilizes a spatial pinhole to minimize the fluorescence emitted throughout the entire specimen in widefield view and instead focus on a single layer of the cell. Lasers penetrate the pinhole aperture to hit the cover glass at that specific focus point size of the pinhole (usually between 0.25 and 0.8 μm) [3]. This small focus point decreases the background fluorescence emission, thus producing a clear and crisp image. The advantages that CLSM provides have made it a popular microscopy technique in the life sciences field since its inception. Intracellular interactions can be visualized with CLSM by colocalization methods (*see* Fig. 1). CLSM provides clear images that can pinpoint the intracellular localization and molecular interactions within PDLSCs.

CLSM allows the user to visualize the fluorescence of one optical section of the specimen, and thus one entire focal plane is in view; however, the TIRFM technique utilizes the total internal reflection angle to visualize events occurring 100–200 nm from the cover glass [4, 5]. In TIRFM, the cells can be live or fixed to a glass bottom dish with media or 1 $\times$ PBS in the dish. A large refractive index between the two interfaces is vital to achieve a critical angle that causes a total internal reflection within the glass slide [6]. The specificity of the depth that is achieved with the TIRF angle allows events to be seen at the plasma membrane, such as receptor-ligand interactions, endocytosis, and exocytosis [4]. This sophisticated technique greatly reduces the background fluorescence, and the limited depth decreases the signal-to-noise ratio producing crisp images of the events at the plasma membrane (*see* Fig. 2). TIRFM is advantageous for studying biomineralization events such as exocytosis and endocytosis in PDLSCs because it provides evidence for the receptor-ligand interactions at the plasma membrane.

In this chapter, we present two different microscopy techniques to study ligand-receptor interactions and cellular trafficking in PDLSCs. These techniques can provide insight into the molecular processes of PDLSCs upon stimulation with a ligand or during mineralization. The methods described will provide a framework for understanding these molecular processes and thereby lead to a better understanding of how to utilize these cells in biomineralization and tissue regeneration.

2 Materials

2.1 Cell Culture

1. Periodontal ligament stem cell (PDLSC) media: α -MEM media, 500 mM glutamine, 100 $\times$ penicillin/streptomycin stock, fetal bovine serum. Add 75 mL of FBS, 5 mL of glutamine, and 5 mL of penicillin/streptomycin to 500 mL of α -MEM media. Filter media prior to use.

2. Ascorbate phosphate: Prepare 10 mg/mL of ascorbate phosphate in water to make a 100× stock. The final working concentration will be 100 µg/mL (*see* Note 1).
3. β-Glycerophosphate: Prepare 1 M of β-glycerophosphate in H₂O to make a 100× stock. The final working concentration will be 10 mM.
4. Dexamethasone: Prepare 1 µM in 1× PBS. The final working concentration will be 10 nM.
5. PDLSC mineralization media: Prepare the media as described previously. Add the ascorbate phosphate, β-glycerophosphate, and dexamethasone to their appropriate final working concentrations from the 100× stocks. Filter media.
6. PDLSC serum-free media: α-MEM media, 500 m glutamine, 100× penicillin/streptomycin. Add 5 mL of glutamine and 5 mL of penicillin/streptomycin to 500 mL of α-MEM media. Filter media prior to use.
7. 1× PBS.
8. 6-well cell culture plates.
9. 25 mm cover glass.
10. 35 mm glass bottom dish tissue culture treated.
11. Transfection reagent of choice (*see* Note 2).
12. OPTI-FREE Media.
13. 0.5% trypsin-EDTA.
14. Ligand of interest.
15. Plasmid with fluorescent marker.
16. Incubator: 37 °C and 5% CO₂.
17. Automated cell counter or hemocytometer.
18. Trypan blue.

2.2 Immunocyto-chemistry

1. 10% Formalin.
2. 0.5% Triton X-100 in 1× PBS.
3. 5% BSA in 1× PBS.
4. 1× PBS.
5. VectaShield Mounting Media for fluorescence with DAPI.

¹-L-Ascorbic acid can also be used instead of ascorbate phosphate, but it should be made fresh prior to use. Ascorbate phosphate is more stable and can be stored in aliquots at −20 °C for an extended period of time.

²For increased transfection efficiency, try using DNAfectin as the transfection reagent over Lipofectamine for stem cells, especially PDLSCs and DPSCs.

6. Glass slides.
7. Antibodies of interest.
8. Appropriate secondary antibodies.
9. Nail polish.

2.3 Processing

1. Confocal laser scanning microscope (CLSM).
2. Total internal reflection fluorescence microscopy (TIRFM).
3. 37 °C incubator adaptor for TIRF microscope.

3 Methods

3.1 Methods for CLSM

3.1.1 Cell Culture

1. Plate approximately 1.0×10^6 cells on 10 mm tissue culture plates in PDLSC media, and incubate (*see* Note 3).
2. Observe the PDLSCs under a light microscope to determine if the cells are confluent to be passaged (approximately 80–90% confluency). The PDLSCs reach this stage of confluency within 2–3 days of growth with media change every other day.
3. Prepare two 6-well plates with a cover glass in each well. Place plates under UV light for 30 min.
4. Once the cells have reached confluency, remove the PDLSC media, and add 2.5 mL of trypsin-EDTA to the plate. Incubate the cells for 4–5 min until the cells start lifting off the plate.
5. Add 7.5 mL of PDLSC media to stop the trypsin reaction, and transfer to a 15 mL conical tube.
6. Take approximately 10 μ L of the trypsin-cell media to use for cell counting (*see* Note 4).
7. Centrifuge the cells at 1000 RPM for 5 min to collect the cell pellet.
8. Remove the supernatant and resuspend the cell pellet in PDLSC media.
9. Seed 50,000 cells in each 6-well plate with 2 mL of media in each well.
10. Incubate the plates at 37 °C.

³.All PDLSC incubations are to be at 37 °C with 5% CO₂ unless otherwise stated.

⁴.Cell counting can be done with an automated cell counter. There should be a 1:1 ratio between media and trypan blue. A hemocytometer can also be used.

11. Once a plate has reached 60% confluency, remove the PDLSC media from one plate, and add 2 mL of PDLSC mineralization media to each well. Let the cells continue growing for 2 days.

3.1.2 Treatment

1. Once the cells have reached 80% confluency, remove the PDLSC media from one plate and the PDLSC mineralization media from the other, and wash with 2 mL per well of 1× PBS twice (*see* Note 5).
2. Add 2 mL of serum-free PDLSC media to each well, and incubate for 4 h (*see* Note 6).
3. Add 250 ng of the ligand to be investigated to each well at 60, 30, 15, 10, and 5 min. One well will be the control well (*see* Note 7).
4. Remove the media and wash twice with 1× PBS.
5. Fix the cells with 10% formalin for at least 2 h (*see* Note 8).

3.1.3 Immunocyto-chemistry

1. Remove the 10% formalin, and wash the cells with 1× PBS four times.
2. Permeabilize the cells with 0.5% Triton X-100 in PBS for 30 min RT.
3. Wash four times with 1× PBS.
4. Block the cells with 5% BSA for an hour at RT (*see* Note 9).
5. Wash four times with 1× PBS.
6. Make 1 mL of primary antibody in 5% BSA for each well with the proper dilution of the antibody.
7. Apply the primary antibody with the dilution recommended. Leave the primary antibody on overnight at 4 °C.
8. Wash four times with 1× PBS.
9. Make 1 mL of fluorescent secondary antibodies in 5% BSA for each well with the proper dilution of the antibody (*see* Note 10).
10. Apply the secondary antibody with the dilution recommended for 1 h at room RT.
11. Wash four times with 1× PBS.

⁵.All washing steps should be done at room temperature with light shaking for 15 min.

⁶.Prior to the treatment with the ligand, the cells should be in serum-free (no FBS) alpha-MEM period for at least 4 h. This starvation period can be extended overnight if necessary.

⁷.The amount of ligand treatment is dependent upon the ligand itself. For optimal conditions, use 250 ng as a starting point, and adjust the concentrations accordingly.

⁸.The fixation period should be at least 2 h at room temperature. The optimal results with PDLSCs have been with overnight fixation at 4 °C.

⁹.The blocking step can be anywhere between 1 and 5 h at RT or 4 °C overnight.

¹⁰.The immunocytochemistry—**step 9**—should be done in a dark room and washing steps performed in foil-covered containers due to the light-sensitive fluorescent antibodies.

12. Mount the cover glass on glass slides for imaging. Use one drop of VectaShield Mounting Media for fluorescence with DAPI prior to mounting on the coverslips.
13. Seal the glass coverslips with nail polish and allow the slides to dry. Store at -20°C until further processing.
14. Visualize and image the slides with CLSM.

3.2 Methods for TIRFM

3.2.1 Cell Culture

1. Repeat the cell culture **steps 1–8**.
2. Seed 50,000 cells in 6–35 mm dishes treated for tissue culture. Allow growth to 80% confluency (approximately 2–3 days).

3.2.2 Transfection

1. Warm the transfection reagent to room temperature prior to use (*see* Note 11).
2. Mix plasmid of interest containing GFP with the transfection reagent in approximately 50 μL of OPTI-FREE media. Let the plasmid-transfection mixture sit at RT for 20 min (*see* Notes 12 and 13).
3. Pipette the plasmid-transfection mixture into each 35 mm dish.
4. Change the PDLSC media the following morning.
5. With a fluorescent microscopy, check the transfection efficiency after 2 days (*see* Note 14).

3.2.3 Treatment for Fixed TIRF Samples

1. Remove the PDLSC media and replace with serum-free PDLSC media for at least 4 h.
2. Add 250 ng of the ligand to each well for 0, 5, 10, 15, 30, and 60 min. One well will be the control well (*see* Note 7).
3. Remove the media and wash with $1\times$ PBS twice.
4. Fix the cells with 10% formalin for at least 2 h (*see* Note 8).

3.2.4 Treatment for Live TIRF Samples

1. Remove the PDLSC media, and replace with PDLSC serum-free media for at least 4 h (*see* Note 6).
2. Place one plate into the 35 mm dish holder on the microscope in a 37°C chamber.

¹¹ Warm the transfection reagent to RT for approximately 20 min.

¹² The plasmid-transfection reagent mixture should be at RT for at least 20 min, but it can be at RT for up to 3 h.

¹³ The transfection reagent to plasmid ratio will require optimization. Our results show the most optimal transfection efficiencies have been achieved with 4 μL of 1 ng DNA plasmid with 1 μL of DNAfectin in 50 μL .

¹⁴ The most optimal transfection efficiencies were obtained after 2 days in PDLSCs.

3. Treat the plate with the ligand of interest, and capture the TIRF images at the time points of 0, 5, 10, 15, 30, and 60 min (see Note 15).

3.2.5 Processing with TIRFM for Fixed Samples

1. Wash each 35 mm dish with 1× PBS four times. Leave the remaining wash as 1× PBS prior to processing.

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¹⁵The ligand can be pipetted into the dish while it is incubating. Another strategy could be to have it injected into the dish while incubating on the microscope. This is dependent upon the microscope equipment available.

Fig. 1.

This figure represents an example of determining intracellular trafficking of vesicles in periodontal ligament stem cells with Rab7, a marker for late endosomes, and glucose-regulated protein-78 (GRP78), a receptor for dentin matrix protein-1 with confocal microscopy. Periodontal ligament stem cells were serum starved for 4 h and then treated with rDMP1 at progressing time points. Immunocytochemistry was performed with antibodies for Rab7 (TRITC) and GRP78 (FITC). The slides were mounted and processed with a Zeiss Laser Scanning Microscope 510 Meta. Within 10 min, colocalization occurs between GRP78 and Rab7, suggesting that DMP1 is transported in PDLSCs from early to late endosomes intracellularly

Fig. 2.

This is an example of TIRF images of PDLSCs. The PDLSCs were transfected with pCDH-GRP78-GFP for 2 days prior to treatment with dentin matrix protein-1 (DMP1) at various time points. The PDLSCs were fixed and washed in PBS before TIRF processing. These images represent an increase of GRP78 to the plasma membrane of PDLSCs with DMP1. These images gives insight to what happens at the membrane with DMP1 during biomineralization, and it can be used with other cell types as well