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Multifluorescence Labeling Techniques and Confocal Laser Scanning Microscopy on Lung Tissue

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Summary

Lung tissue consists of more than 40 individual cell types that might interact to produce adverse pathologies. After injury, a number of signaling proteins expressed in various epithelial and other cell types have been linked to the advent of apoptosis, compensatory proliferation, and adaptation to stress. We describe here the use of immunochemistry and multifluorescence approaches using confocal laser scanning microscopy to define the signaling pathways (protein kinases C and mitogen-activated protein kinases) activated by asbestos fibers after inhalation. Using these approaches, we are able to localize signaling events in distinct cell types of the lung and determine their status in the cell cycle (resting or nonresting). Moreover, we are able to determine whether various signaling proteins colocalize in cells and the sites affected by asbestos fibers.

Key Words: Immunofluorescence; confocal laser scanning microscopy; asbestos; extracellular signal-regulated kinases (ERKs); PKCδ; proliferation marker; Ki-67.

1. Introduction

Confocal laser scanning microscopy (CLSM) is a laser-based imaging technology widely used in pathology and cell biology research. This approach can be used to identify lesions and affected cell types. CLSM provides increased resolution over conventional wide-field microscopy and has the ability to reject out-of-focus fluorescence. It also allows a decrease in autofluorescence signal caused by collagen deposits in some tissues, such as lung, by the use of fluorescent probes that excite at higher wavelengths ***(1)***. It is a powerful technique for studying cell signaling by environmental agents in the initiation and pathology of lung disease and/or repair and adaptation. Inhaled environmental agents such as asbestos might elicit cell signaling pathways at the cell membrane. Using antibodies specific to phosphorylated (i.e., activated) signaling proteins,

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related signaling events and gene transactivation by signaling proteins can be documented in vivo. The mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinases (ERKs) as well as some isotypes of protein kinase C (i.e., PKCδ) have been linked to both cell proliferation and the development of apoptosis in response to toxic agents such as oxidants and asbestos fibers. In this chapter, we describe multifluorescence techniques to document nuclear and membrane translocation and increased activation of PKCδ and ERK 1/2 in lung tissue after inhalation of asbestos *(2,3)*. Use of a Ki-67 antibody specific for nonresting epithelial cells indicates that these protein kinases are often colocalized in proliferating cells *(4)*.

2. Materials

2.1. Processing and Sectioning

Mice are administered a lethal dose of sodium pentobarbital (Abbot Laboratories, Chicago, IL) before the chest cavity is opened, a polyurethane catheter is inserted into the trachea, and the lungs are instilled with 1X phosphate-buffered saline (PBS) at a pressure of 25 cm water. The unfixed lungs are separated by suturing, removed from the chest, snap-frozen by plunging into liquid-nitrogen-cooled isopentane, placed in OCT embedding compound (Tissue Tek, Torrance, CA), and frozen at –80°C. Sections are cut at –23°C in a cryostat using disposable knives and then retrieved onto Superfrost +/+ slides. Sections are quickly examined under the light microscope to assure that the tissue structure has not been damaged during the sectioning process. Slides are stored in a slide box at –80°C until use.

2.2. Reagents

1. 10X PBS (for 1 L): Add 2.76 g of sodium phosphate monobasic and 14.1 g of sodium phosphate dibasic (anhydrous) to 500 mL of deionized water (dH_2O). Add 90 g of sodium chloride and bring volume to 1L, followed by adjustment of the pH to 7.4. For 1X PBS, dilute 1 : 10 with distilled water (dH_2O) and adjust pH to 7.4 if necessary. Both solutions are stable at room temperature (RT).
2. PBS-PFA fixative: 3.7% paraformaldehyde (PFA), 1X PBS. PFA should be handled under a fume hood and glove protection is required. Weigh 3.7 g of PFA and add to 100 mL of 1X PBS. Heat stirring solution at 60°C until PFA dissolves. When solution becomes clear, remove flask from the heating plate, cool to RT, and filter through Whatman filter paper. This solution is made fresh each time and chilled prior to use.
3. Sodium dodecyl sulfate (SDS): 0.5% SDS, 1X PBS. Procedure should be handled with respiratory mask and gloves. Weigh 0.05 g of SDS and add to 10 mL of 1X PBS. Shake the tube until mix goes into solution. This solution is stable at RT.
4. 1% Bovine serum albumin (BSA)/PBS: 1 mg/mL BSA, 1X PBS, 0.02% sodium azide. Store solution at 4°C. This solution is stable until evidence of bacterial growth.

5. 10% Normal goat serum (from secondary antibody host) in 1X PBS. Solution is made fresh prior to each use.
6. 5% Blocking reagent from mouse-on-mouse kit (M.O.M. kit; Vector Laboratories, Burlingame, CA) in 1X PBS. Solution is made fresh prior to each use.
7. Mounting medium: AquaPolyMount (Polysciences Inc., Warrington, PA) should be stored at 4°C and warmed to RT for a few minutes before each use.

2.3. Equipment

A humid environment is necessary to prevent evaporation of reagents during prolonged incubations. Inexpensive humidity chambers can be created in the lab. For instance, a large (6-in. diameter) glass Petri dish with a moistened filter paper on the bottom can be used as a humid chamber. Because slides should not touch wet paper directly, a piece of parafilm elevated 2–4 mm above the filter paper on which place the slides can be used ***(5)***.

2.4. Controls

Staining controls omitting primary antibodies are required for each of the secondary antibodies for every staining. Negative controls using isotype control antibodies or nonimmune serum from the same species as the primary antibody are also helpful for validating the specificity of staining results.

2.5. Antibody Sources

1. PKCδ: rabbit polyclonal nPKC δ (C-20) antibody (Santa Cruz, cat. no. sc-937).
2. p-ERK: rabbit polyclonal Phospho-p44/42 MAP Kinase (Thr202/Tyr204) (Antibody, Cell Signaling Inc., cat. no. 9101).
3. p-JNK: rabbit polyclonal JNK (SAPK) [pTpY183/185] (Biosource, cat. no. 44-682).
4. Cytokeratin: pan antibody produced in mouse (Sigma, cat. no. C2562).
5. MAC3: rat anti-mouse antibody (BD Biosciences Pharmingen, cat. no. 553322).
6. proSP-C: rabbit anti-human prosurfactant protein C polyclonal antibody (Chemicon, cat. no. AB3786).
7. Ki-67: monoclonal rat anti-mouse, clone TEC3 antibody (DAKO, cat. no. M7249).

3. Methods

3.1. Single Immunofluorescence Labeling

3.1.1. MAPK and PKC

Because anti-p-ERK and anti-p-JNK antibodies used in our laboratory are all derived from rabbits, they can be detected using the same secondary antibody. Therefore, an identical procedure can be used to successfully obtain each labeling. The anti-PKCδ antibody is a mouse monoclonal; thus, it should be used in conjunction with the mouse-on-mouse kit.

3.1.1.1. p-ERK, p-JNK Antibodies, Rabbit Polyclonal

1. For fixation, slides are placed into a Coplin jar filled with fresh 3.7% PFA for 10-min at RT followed by two 5-min washes in 1X PBS (*see* **Note 1**).
2. Wash slides in 1X PBS twice for 5 min.
3. Place slides into –20°C methanol for 10 min for permeabilization. Permeabilization is advised to ensure free access of the antibody to its antigen. Storage of methanol as well as incubation of the tissue should take place at –20°C.
4. Wash slides in 1X PBS twice for 5 min.
5. Circumscribe the tissue section with a Pap-pen. Using a Q-tip, dry around the section and circumscribe the tissue section with a Pap-pen to create a hydrophobic border. This hydrophobic border allows the use of small volumes of solutions to cover the entire section without wasting unreasonably large amounts of expensive reagents.
6. Place the slides in a humid chamber.
7. For antigen retrieval, sections can be treated with 1% SDS in 1X PBS for 5 min at RT to enhance antibody staining ***(6,7)***. Apply 1% SDS for 5 min on each section.
8. Wash sections twice for 5 min in 1X PBS in a Coplin jar.
9. Block in 10% normal goat serum in 1X PBS for 1 h at RT. Serum used for the blocking step has to be from the animal species in which the secondary antibody was raised in to prevent nonspecific binding. Therefore, for primary antibodies raised in other animal species than mouse, 10% normal serum diluted in 1X PBS should be used for 1-h incubation.
10. Wash twice in 1X PBS for 5 min.
11. Apply rabbit polyclonal antibody diluted in 1% BSA/1X PBS (PKCδ, 3 µg/mL; p-ERK, 1 : 250; p-JNK, 2 µg/mL). In most instances, we recommend applying primary antibodies overnight at 4°C to enhance staining intensities while lowering nonspecific background staining.
12. Wash slides twice in 1X PBS for 5 min.
13. Cover sections with biotinylated goat anti-rabbit IgG (Vector Laboratories) for 1 h at RT (*see* **Notes 2–4**).
14. Wash slides twice in 1X PBS for 5 min.
15. Incubate sections in strepavidin Alexa 568 conjugate at 1 : 400 dilution in 1X PBS for 1 h at RT in the dark. During the incubation with fluorophore-conjugated secondary antibodies, the humid chamber should be covered with aluminum foil to avoid exposure to room light and potential fluorophore photobleaching. Secondary antibodies are chosen with the desired conjugated fluorophore and to bind to the primary antibody.
16. Rinse slides in 1X PBS for 5 min at RT.
17. Rinse twice with dH_2O for 1 min.
18. Mount section with cover slip using AquaPolyMount. Use no. 1-½ cover slips. Apply a drop of AquaPolyMount to cover the section and attach a cover slip. Using forceps, apply minimal pressure to the top of the cover slip to remove air bubbles from underneath and to create a tight seal (*see* **Notes 6,7**).
19. Store slides in a lightproof slide box at 4°C until analysis.

3.1.1.2. PKCδ Antibody, Mouse Monoclonal

1. Take slides out of –80°C and immediately place them in 4% PFA for 10 min at RT.
2. Wash slides in 1X PBS twice for 5 min.
3. Place slides into –20°C methanol for 10 min.
4. Wash slides in 1X PBS twice for 5 min.
5. Circumscribe the tissue section with a Pap-pen.
6. Place the slides in a humid chamber.
7. For antigen retrieval, apply 1% SDS for 5 min on each section.
8. Wash sections twice for 5 min in 1X PBS in a Coplin jar.
9. To minimize nonspecific antibody binding of mouse monoclonal antibodies on murine tissue, slides are incubated in 5% blocking reagent from a mouse-on-mouse kit (Vector Laboratories, Burlingame, CA) in 1X PBS for 1 h.
10. Wash twice in 1X PBS for 5 min.
11. Pre-incubate sections in 10% concentrated protein from a mouse-on-mouse kit in 1X PBS for 5 min.
12. Apply 1 : 250 dilution of PKCδ antibody in 10% concentrated protein from a mouse-on-mouse kit in 1X PBS overnight at 4°C.
13. Wash twice in 1X PBS for 5 min.
14. Apply 1 : 400 dilution of goat anti-mouse IgG conjugated to Alexa 647 in 1% BSA/1X PBS for 1 h at RT in the dark.
15. Rinse slides in 1X PBS for 5 min at RT.
16. Rinse with dH_2O for twice for 1 min.
17. Mount section with cover slip using AquaPolyMount.

3.1.2. Cell Type Markers

To establish whether the protein of interest is specific to bronchiolar and alveolar epithelial cells vs macrophages that infiltrate lung after an injury, lung sections can be colabeled with cell-type-specific markers.

3.1.2.1. Anti-Cytokeratin; Epithelial Cell Marker; Mouse Monoclonal Pan (Mixture) Antibody

1. Take slides out of –80°C and immediately place them in 4% PFA for 10 min at RT.
2. Wash slides in 1X PBS twice for 5 min.
3. Place slides into –20°C methanol for 10 min.
4. Wash slides in 1X PBS twice for 5 min.
5. Circumscribe the tissue section with a Pap-pen.
6. Place the slides in a humid chamber.
7. For antigen retrieval, apply 1% SDS for 5 min on each section.
8. Wash sections twice for 5 min in 1X PBS in a Coplin jar.
9. Block in 5% blocking reagent from a mouse-on-mouse kit in 1X PBS for 1 h at RT.
10. Wash twice in 1X PBS for 5 min.
11. Pre-incubate sections in 10% concentrated protein from a mouse-on-mouse kit in 1X PBS for 5 min.

12. Apply 1:100 dilution of anti-cytokeratin in 10% concentrated protein from a mouse-on-mouse kit in 1X PBS overnight at 4°C.
13. Wash twice in 1X PBS for 5 min.
14. Apply 1:400 dilution of goat anti-mouse IgG conjugated to Alexa 647 in 1% BSA/1X PBS for 1 h at RT in the dark.
15. Rinse slides in 1X PBS for 5 min at RT.
16. Rinse twice with dH_2O for 1 min.
17. Mount section with cover slip using AquaPolyMount.

3.1.2.2. MAC-3; Rat Anti-Mouse M3/84 Antibody That Reacts With Mac-3 Antigen Expressed on Mouse Mononuclear Phagocytes

1. Take slides out of –80°C and immediately place them in 4% PFA for 10 min at RT.
2. Wash slides in 1X PBS twice for 5 min.
3. Place slides into –20°C methanol for 10 min.
4. Wash slides in 1X PBS twice for 5 min.
5. Circumscribe the tissue section with a Pap-pen.
6. Place the slides in a humid chamber.
7. For antigen retrieval, apply 1% SDS for 5 min on each section.
8. Wash sections twice for 5 min in 1X PBS in a Coplin jar.
9. Incubate sections in 10% normal goat serum in a humid chamber on orbital shaker for 1 h at RT for blocking.
10. Wash twice in 1X PBS for 5 min.
11. Apply 20 µg/mL solution of MAC-3 antibody in 1% BSA/PBS overnight at 4°C.
12. Wash twice in 1X PBS for 5 min.
13. Apply 1:400 dilution of goat anti-rat IgG conjugated to Alexa 647 in 1% BSA/PBS for 30 min at RT in the dark.
14. Rinse slides in 1X PBS for 5 min at RT.
15. Rinse twice with dH_2O for 1 min.
16. Mount section with cover slip using AquaPolyMount.

3.1.2.3. Anti-SP-C; Rabbit Prosurfactant Protein C Polyclonal Antibody, Detects Alveolar Type II Epithelial Cells in Mouse Lung Tissue

1. Take slides out of –80°C and immediately place them in 4% PFA for 10 min at RT.
2. Wash slides in 1X PBS twice for 5 min.
3. Place slides into –20°C methanol for 10 min.
4. Wash slides in 1X PBS twice for 5 min.
5. Circumscribe the tissue section with a Pap-pen.
6. Place the slides in a humid chamber.
7. For antigen retrieval, apply 1% SDS for 5 min on each section.
8. Wash sections twice for 5 min in 1X PBS in a Coplin jar.
9. Incubate sections in 10% normal goat serum in a humid chamber on orbital shaker for 1 h at RT for blocking.
10. Wash twice in 1X PBS for 5 min.
11. Apply 3 µg/mL Solution of anti-SP-C antibody in 1% BSA/PBS overnight at 4°C.
12. Wash twice in 1X PBS for 5 min.

13. Apply 1:400 dilution of goat anti-rabbit IgG conjugated to Alexa 647 in 1% BSA/1X PBS for 30 min at RT in the dark.
14. Rinse slides in 1X PBS for 5 min at RT.
15. Rinse twice with dH_2O for 1 min.
16. Mount section with cover slip using AquaPolyMount.

3.1.3. Proliferation Marker: Ki-67; Clone TEC-3, Cell Proliferation Marker, Rat Anti-Mouse Monoclonal

1. Take slides out of –80°C and immediately place them in 4% PFA for 10 min at RT.
2. Wash slides in 1X PBS twice for 5 min.
3. Place slides into –20°C methanol for 10 min.
4. Wash slides in 1X PBS twice for 5 min.
5. Circumscribe the tissue section with a Pap-pen.
6. Place the slides in a humid chamber.
7. For antigen retrieval, apply 1% SDS for 5 min on each section.
8. Wash sections twice for 5 min in 1X PBS in a Coplin jar.
9. Incubate sections in 10% normal goat serum in a humid chamber on orbital shaker for 1 h at RT for blocking.
10. Wash twice in 1X PBS for 5 min.
11. Apply 1:25 dilution of Ki-67 antibody in 1X PBS overnight at 4°C.
12. Wash twice in 1X PBS for 5 min.
13. Apply 1:300 dilution of goat anti-rat IgG conjugated to Alexa 647 in 1% BSA/PBS for 30 min at RT in the dark.
14. Rinse slides in 1X PBS for 5 min at RT.
15. Rinse twice with dH_2O for 1 min.
16. Mount section with cover slip using AquaPolyMount.

3.2. Double and/or Triple Labeling

To simultaneously detect two or three primary antibodies raised in different host animal species (e.g., rabbit polyclonal p-ERK, mouse monoclonal PKCδ, and rat monoclonal Ki-67 (*see* **Fig. 1**; *see* Color Plate 3, following p. 274), antibodies are combined together and applied on preblocked slides (*see* **Notes 1,5,** and **8**) overnight at 4°C in a humid chamber. For detection of primary antibodies, a cocktail of two or three fluorophore-conjugated secondary antibodies can be applied to the sections or, alternatively, a sequential incubation with each of the secondary antibodies separately (*see* **Notes 3,9**). For examples of labeling techniques described above, *see* **refs.** *2* and *3*.

3.3. Fluorescent Detection of Cell Nuclei

There are a number of fluorescent dyes for cell nuclei, including SYTOX Green and propidium iodide (PI). SYTOX Green, a green nucleic acid-binding fluorescent dye, is also useful to demonstrate the general cellularity of a tissue. Diluted in 1X PBS to 5 μ*M*, it is applied directly on a tissue section for 15 min

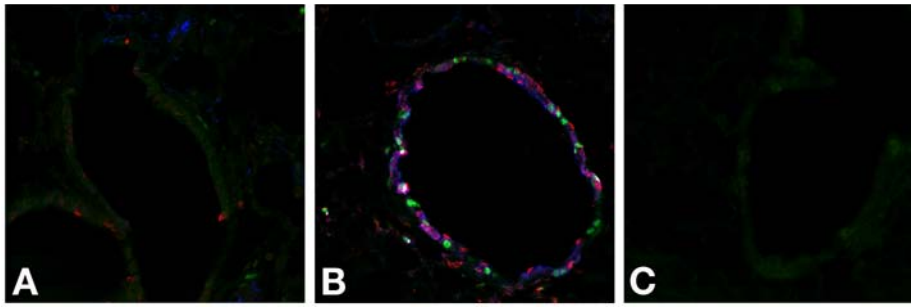

Fig. 1. Triple-labeling using mouse monoclonal PKCδ (blue), rabbit polyclonal p-ERK (red), and rat monoclonal Ki-67 (green) antibodies on sham animals **(A)** and animals exposed to crocidolite asbestos for 4 d **(B**,**C)**. White pixels appear at the areas of colocalization of all three antibodies. Colocalization of blue and red colors demonstrate colocalization of p-ERK and PKCδ. A negative control omitting primary antibody is also provided and shows no fluorescence signal **(C)**. (*See* Color Plate 3, following p. 274.)

after the excess secondary antibody has been washed off. The sections should then be washed at least two times for 10–15 min in 1X PBS. SYTOX Green is excited by 488-nm wavelength light (*see* **Note 10**), leading to a potential overlap with tissue autofluorescence. However, because the signal emission of SYTOX Green is quite intense, autofluorescence is not a major problem. In addition, this leaves two wavelengths free for staining with antibodies against proteins of interest. (*See* **Note 11.**)

Propidium iodide (PI), a nuclear stain for cells in the G1 phase of the cell cycle, can also be used as a nuclear marker of cellularity. The working solution consists of 10 µg/mL of PI and 10 µg/mL of RNase A diluted in 1X PBS. Tissue sections should be incubated for 30 min at RT in PI, followed by two 10-min washes in 1X PBS. PI is excited by yellow light, resulting in red fluorescence emission.

4. Notes

1. Depending on what staining procedures the sample is going to be used for, the tissue might receive PFA fixation prior to OCT. If this is the case, the PFA fixation step can be withdrawn from the staining procedure. No other changes are needed.
2. Always optimize antibody dilutions using recommended concentrations as a guideline. Every system is slightly different, and trial-and-error experiments might be required to optimize the antibody or a technique for your specific system. Try doing a dilution series for all primary and secondary antibodies.
3. There are a few approaches to overnight incubation with a primary antibody. Incubations can be done at 4°C in a refrigerator or in a cold room on an orbital shaker to assure even application of the antibody. If an orbital shaker is used, cover the section with a piece of parafilm to ensure even distribution and minimal

evaporation of the antibody solution. As a rule, most primary antibody incubations can also be performed for 1 h at RT on an orbital shaker. However, it is not generally recommended because of the possible loss of quality in labeling and resulting unevenness of binding.

4. The background signal can also be decreased by adding a second blocking step using the same blocking reagent after washing away the primary antibody and prior to incubation with the secondary antibody.
5. To amplify the signal, a biotin/strepavidin system can be employed. Biotin has a very specific affinity for strepavidin/avidin that allows a decrease in background fluorescence as a result of nonspecific binding of a secondary antibody. This system is especially beneficial when doing multiple labeling to match the signal intensities of different antibodies.
6. Remember to prewarm the AquaPolyMount to RT prior to use to avoid bubbles when applying to a tissue section. Other mounting media containing antifade reagents are widely available from commercial sources.
7. After the cover slip is mounted, remove excess medium around the cover slip on the slide and/or on the cover slip itself by gently blotting with a bibulous paper.
8. In the case of double or triple labeling, the possibility exists that different fixation conditions are required for preservation of the various antigens. Try to perform the entire staining procedure using a fixative that is specifically required for one of the antibodies. If a required technique is much harsher, attempt to do a sequence labeling by adding a second blocking step to decrease the fluorescent background and to even out the balance between signaling intensities.
9. When blocking sections for multilabeling using an antibody that requires specific reagents (e.g., colabeling of a rabbit polyclonal antibody with a mouse monoclonal antibody), label both probes using only reagents from a mouse-on-mouse kit needed for mouse antibody to simplify the procedure. If binding of a nonmurine-raised antibody fails, perform a sequence labeling using different blocking reagents and antibody diluents for each probe.
10. SYTOX Green can emit at the higher wavelength and be detected by the red channel. This leads to detection of nuclear labeling using a wide-band green filter. However, it does not cause any problems while imaging with the confocal microscope using the sequential scanning mode.
11. To minimize or avoid autofluorescence, use those probes that fluoresce at higher wavelengths (i.e., 568 nm and 647 nm rather than 488 nm).

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