

Biopsy Techniques

Optimization for Collection and Preservation

Marina Saetta and Graziella Turato

1. Introduction

Fiberoptic bronchoscopy provides a good tool to investigate bronchial biopsies, transbronchial biopsies, and bronchoalveolar lavage (BAL) in chronic inflammatory diseases such as asthma and chronic obstructive pulmonary disease (COPD) (*1–8*). The advantage of bronchial biopsies over other sampling techniques, such as induced sputum or BAL, is that they give anatomical information on airway morphology, therefore allowing the examination of the different compartments of the bronchial wall such as epithelium, subepithelium, smooth muscle, and glands.

Bronchial biopsies can be examined with light microscopy using histochemical and immunohistochemical methods or *in situ* hybridization (ISH). Histochemical methods provide simple and inexpensive staining, which allows identification of some common cell types (e.g., eosinophils, mast cells, goblet cells) (*1*) and of some tissue components (e.g., collagen, smooth muscle) (*2*). Immunohistochemical methods are used to identify cell types and their subsets, markers of activation, cytokines, adhesion molecules, and a variety of other tissue components of interest (*3*). ISH has been used to localize messenger ribonucleic acid (mRNA) transcripts (*4*).

Light microscopic analysis can be performed either directly using an appropriate magnification (400–1000×) or by enlarging the image and transferring it to a screen or monitor and making the assessment with the aid of a computerized image system.

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Table 1
Advantages and Disadvantages of the Different Techniques
for Processing Bronchial Biopsies for Light Microscopic Analysis^a

Procedure	Advantages	Disadvantages
Snap-freezing without prior fixation	-immunoreactivity for all antibodies -ISH can be performed	-bad morphology -limited duration of immunoreactivity
Paraformaldehyde fixation and freezing	-good morphology -immunoreactivity for increasing number of antibodies -ISH can be performed	-not all antibodies are reacting
Formalin fixation and paraffin-embedding	-good morphology -immunoreactivity for increasing number of antibodies -ISH can be performed	-not all antibodies are reacting
Fixation and GMA-embedding	-good morphology -very thin sections -immunoreactivity for increasing number of antibodies	-not all antibodies are reacting -expensive instruments for cutting ultrathin sections.

^aGMA, glycol-methacrylate; ISH, *in situ* hybridization.

There are various methods of processing biopsy samples for light microscopic analysis:

1. Snap-freezing without prior fixation.
2. Paraformaldehyde fixation and freezing.
3. Formalin fixation and paraffin-embedding.
4. Fixation and glycol-methacrylate (GMA)-embedding (*see Table 1*).

Bronchial biopsies can also be examined by electron microscopy (9). Although the amount of tissue that can be examined with this technique is very small, electron microscopy allows analysis of cell ultrastructure. This is a crucial analysis, because unequivocal identification of certain cell types (e.g., myofibroblasts) and their degranulation state (e.g., eosinophils) rely on their ultrastructural characteristics.

Bronchial biopsies have also been used for study of polymerase chain reaction (PCR) (10) or for cell culture and cell cloning (11).

Although the advent of the flexible fiberoptic bronchoscope has provided a relatively safe method for sampling of the bronchial wall, bronchoscopy still remains an invasive technique. In order to maximize the information derived from each sample, it is essential to ensure that good quality biopsies are obtained. Because the method chosen for the processing of bronchial biopsies needs to be compatible with the specific objective and question of each study, careful planning and agreement of the aims and specific objectives need to be agreed on well before the study commences. The design of the study should be agreed on by all the investigators including the histopathologist, and the number of patients and biopsies should be determined in order to assess the power of the study in the detection of the differences of interest (12).

In this chapter we will describe the procedures for 1) bronchial biopsy collection and 2) bronchial biopsy preservation for light microscopic analysis. In addition, typical procedures to perform immunohistochemical analysis will be shown.

2. Materials

2.1. Snap-Freezing Without Prior Fixation

1. Thermo-flask (nitrogen-proof).
2. Optimum cutter temperature (OCT) compound: e.g., Tissue-Tek (Miles, Elkart, IN, USA).
3. Cork embedding disks.
4. Phosphate buffered saline (PBS) (Sigma P-4417): dissolve 1 PBS tablet in 200 mL distilled water to obtain 0.01 M PBS, pH 7.4 (*see Note 1*). PBS can be stored at room temperature for 2–3 d.
5. Isopentane (Aldrich).
6. Liquid nitrogen.

2.2. Paraformaldehyde Fixation and Freezing

All materials listed in **Subheading 2.1.** including PBS. In addition:

1. 2% paraformaldehyde (Sigma P-6148): heat PBS to 58–60°C on a hotplate stirrer, add preweighed paraformaldehyde powder (Sigma P-6148) to the heated PBS (2 g/100 mL) (in fumehood to avoid fumes), and stir for 60–90 min until dissolved. Cool solution and, if necessary, filter. Use the same day or store overnight at 4°C and use the day after (*see Note 2*).
2. 15% sucrose/PBS: add 15 g sucrose (Sigma S9378) and 0.01 g sodium azide (Merck 6688) 15% sucrose for every 100 mL PBS and mix; this mixture can be stored at room temperature for 2 or 3 d.

2.3. Formalin Fixation and Paraffin Embedding

1. PBS. *See Subheading 2.1.4.*
2. 10% formalin (approx 4% formaldehyde). Add 100 mL 37% formaldehyde (Sigma F-1635) to 900 mL PBS and mix. 10% formalin can be stored at 4°C.
3. 70% ethanol: add 70-mL absolute ethanol (Sigma) to 30-mL distilled water and mix. 70% ethanol can be stored at room temperature.
4. Ethanol 90%: add 90-mL absolute ethanol (Sigma) to 10-mL distilled water and mix. 90% ethanol can be stored at room temperature.
5. A safety-approved clearing medium, e.g., K Clear (Kalktek).
6. Paraffin wax (Merk).
7. Base tissue molds (Kalktek).
8. Tissue cassettes (Kalktek)

2.4. Fixation and GMA Embedding

1. Acetone (Merk).
2. Phenylmethyl sulfonyl fluoride (Sigma P-7626).
3. Iodoacetamide (Sigma I-6125).
4. Methyl benzoate (Merk 29214 4L).
5. Embedding resin: JB4 embedding kit (Park Scientific 0226), which includes GMA solution A (GMA monomer), GMA solution B, and benzoyl peroxide. GMA solution A is required more often and can be bought separately (Park Scientific 0226A).
6. Embedding capsules (TAAB C094).

3. Methods

3.1. Biopsy Collection

1. Premedicate patients with intravenous atropine (0.5–1 mg) and fentanyl (0.15–0.30 mg).
2. Spray lidocaine solution 2% onto the base of the tongue, pharynx, and nasal passages for topical anesthesia.
3. A flexible bronchoscope is introduced transorally or transnasally with the patient in a supine position and passed through the larynx.
4. Up to 10 mL of 2% lidocaine are instilled through the bronchoscope channel to provide anesthesia for the airways below the vocal cords (*see Notes 3–5*).
5. Take one to eight bronchial biopsies through the bronchoscope from the subcarina of a basal segmental bronchus of the right lower lobe (*see Notes 6–9*).

3.2. Biopsy Preservation

The biopsies obtained by bronchoscopy should be gently extracted from the forceps and immediately prepared for microscopic analysis. There are various methods of processing biopsy samples:

1. Snap-freezing without prior fixation (3).

2. Paraformaldehyde fixation and freezing (**13**).
3. Formalin fixation and paraffin-embedding (**14**).
4. Fixation and GMA-embedding (**15,16**).

Bronchial biopsies are potentially hazardous human material, and the same precautions as used in handling blood samples must apply before tissue is fixed.

3.2.1. Snap-Freezing Without Prior Fixation

1. Place biopsy in PBS.
2. Cool isopentane in a polypropylene beaker by immersing it in liquid nitrogen kept in a Dewar thermo-flask until the lower aspects and edges appear frozen and white.
3. Label the back of each cork embedding disk with information about the biopsy.
4. Put a drop of OCT compound on the cork to form a base.
5. Place the biopsy on the OCT base with a further covering drop of OCT.
6. Holding the disk with a pair of long forceps, immediately plunge the disk with the OCT-covered biopsy into the liquid nitrogen-cooled isopentane and hold it there until the tissue and medium is frozen (i.e., turns white).
7. Wrap in tin foil (with information about tissue source and so on appended) and store in a well-labeled container at -80°C until use (*see Note 10*).

3.2.2. Paraformaldehyde Fixation and Freezing

1. Place biopsy samples in freshly prepared 2% paraformaldehyde for 2 h at 4°C (in this and in the following steps use a 20-mL vial to hold the biopsies) (*see Note 11*).
2. Transfer biopsy samples to 15% sucrose/PBS for 1 h at 4°C (*see Note 12*).
3. Change into 15% sucrose/PBS overnight at 4°C .
4. Snap-freeze following the procedure described in **Subheading 3.2.1**.
5. This method can be modified for study of gene expression by ISH (*see Note 13*).

3.2.3. Formalin Fixation and Paraffin Embedding

1. Melt paraffin wax in a stove at 58°C .
2. Place biopsy samples in 10% formalin for 4 h at 4°C (in this and in the following steps use a 20-mL vial to hold the biopsies).
3. After fixation, dehydrate the biopsies by passing them through a graded series of ethanol (70%, 90%, 100%, 100%, 100%) at room temperature, 15 min each.
4. After dehydration, pass the biopsies in three changes of a safety-approved clearing medium (15 min between changes) at room temperature.
5. Place biopsies in liquid paraffin wax at 58°C for 1 h.
6. Place each biopsy in a tissue base mold containing liquid paraffin, cover with a tissue cassette, add liquid paraffin, and allow to “refresh.”
7. Remove paraffin-embedded biopsies from the tissue base mold.
8. Paraffin-embedded biopsies may be stored at room temperature for several years.
9. This method can be modified for study of gene expression by ISH (*see Note 13*).

3.2.4. Fixation and GMA Embedding

1. Place biopsies immediately into ice cold acetone containing 2 mM phenylmethyl sulfonyl fluoride (35 mg/100 mL) and 20 mM iodoacetamide (370 mg/100 mL).
2. Fix overnight at -20°C .
3. Transfer to acetone at room temperature for 15 min.
4. Immerse in methyl benzoate at room temperature for 15 min.
5. Pass in three changes of GMA monomer (GMA solution A) containing 5% methyl benzoate at 4°C , 2 h each.
6. Prepare GMA embedding resin: GMA solution A 10 mL, GMA solution B 250 mL, benzoyl peroxide 45 mg.
7. Embed biopsy in freshly prepared GMA embedding resin in a Taab flat bottomed capsule, placing biopsy in the bottom of the capsule and filling to the brim with resin and closing lid to exclude air.
8. Polymerize overnight at 4°C .
9. Blocks can be stored in airtight container at -20°C .
10. **Steps 4–7** must be carried out under a fume extraction hood.

3.3. Advantages and Disadvantages of the Different Methods to Preserve Bronchial Biopsies

Snap-freezing without prior fixation provides samples for immunohistochemical analysis of certain antigen expression, which fixation tends to mask. Moreover, sections obtained from these biopsies, after fixation in freshly prepared paraformaldehyde, can be used for subsequent studies of gene expression by ISH. A disadvantage of snap-freezing without prior fixation is that tissue structure is not well-preserved for morphometric analysis (*see Table 1*).

Immediate fixation in freshly prepared paraformaldehyde and subsequent freezing preserves good morphology and facilitates subsequent molecular analysis, but does not allow for immunohistochemical analysis of certain antigens, such as cell-surface adhesion molecules. In addition, fixation in paraformaldehyde has the advantage that these biopsies can also be used for ISH (*see Note 13 and Table 1*).

One advantage of paraffin embedding is that it provides excellent morphology and allows for a preliminary overview of the extent of mucosal inflammation using histochemical methods. A disadvantage of the technique is that it does not allow for extensive cell phenotyping by immunohistochemical means, and many of the required surface epitopes may be masked by the processing of the tissue. During fixation, formalin denatures proteins by reacting primarily with basic amino acids of the epitope to form crosslinking “methylene bridges.” Therefore, after formalin fixation, some antigens are masked and cannot be easily demonstrated. Several of these can be revealed after proteolytic digestion with trypsin (17), microwaving (18,19), or autoclaving (20) (*see Table 1*).

The advantage of GMA-embedding technique is the relative thinness of the section (1–2 μm), which gives a greater resolution or “clarity” compared with a 5- μm thick section. This is because of the thin section having fewer focal planes through which the light microscope must focus. In addition, thinness allows the possibility of more than one cut through a single cell using adjacent sections. This enables staining of two distinct epitopes on the same cell. In paraffin-embedded sections, “double labeling” techniques can be applied for the same effect, although these procedures are less easy to control (*see Table 1*).

3.4. Immunohistochemical Analysis of Bronchial Biopsies

Immunohistochemistry applied to biopsy sections is usually performed using immunoenzymatic-staining methods that give colored end-product reactions. Other methods such as fluorescence can be applied to snap frozen sections, but tissue morphology is not easily visible without phase contrast microscopy. Furthermore, fluorochromes are usually short-lived and should be captured photographically to ensure a record is kept. However, a distinct advantage of the fluorescence technique is in the form of double-labeling methods that can be applied, where red and green fluorochromes may be separately seen using selected filter blocks. By a procedure of photographic double exposures, those cells that are double-labeled appear in a resultant mixture of color (i.e., yellow) (*17*).

There are a number of immunoenzymatic staining methods that include “direct” and “indirect” immuno-methods. Direct methods are now used only rarely and have been superseded by indirect methods, which include avidin–biotin methods and soluble enzyme immune-complex methods. Both are indirect three-stage methods. In both procedures the first stage utilizes the immunochemical properties of the antibody to combine specifically with the antigen. The unconjugated primary antibody applied to sections in this stage may be polyclonal, generally raised in rabbit, or monoclonal, mainly raised in mouse. There are numerous advantages of monoclonal antibodies in immunohistochemistry over their polyclonal counterparts. These include high homogeneity, absence of nonspecific reaction, and negligible lot-to-lot variability. However, the end reaction intensity may be relatively weak compared with polyclonal antibodies. The second stage of the avidin–biotin method comprises an antibody (link antibody) labeled with the vitamin biotin that has been raised against the animal species of the primary antibody. Biotin is used as the label as it has a specific chemical affinity (not immunological) for either avidin, a glycoprotein of egg white, or streptavidin, a protein isolated from the bacterium *Streptomyces avidii*. The third stage comprises a complex of either avidin or streptavidin labeled with an enzyme-like horseradish peroxidase or alkaline

phosphatase that will bind to the biotin of the second stage. These sites are then visualized by reacting with a chromogenic substrate. In the soluble enzyme immune complex methods the general procedure differs from that for avidin–biotin methods in respect of the following:

1. An unconjugated antibody directed against immunoglobulins from the species used for the primary antibody is applied to the sections as the link antibody. The link antibody is added in excess, so one of its two binding sites (Fab sites) remains free.
2. The enzyme immune complex consists of an enzyme (peroxidase or phosphatase) and an antibody directed against the enzyme itself. The antibody of the enzyme immune complex and the primary antibody must be raised in the same species. So that, when the enzyme immune complex is added to the sections, the second free binding site of the link antibody will bind the enzyme immune complex.

For all the immunohistochemical procedures listed above, appropriate positive and negative controls must be included in each staining run. As positive controls, use can be made of tissue sections known to be positive for the antigen under study, for example, tonsil or clones producing specific cytokines or cells transfected with copy deoxyribonucleic acid (cDNA) encoding specific human cytokines. This method can also be implemented to assess the specificity of the primary antibody. To confirm the specificity, use can be made of tissue sections known to be negative for the antigen under study. Affinity absorption of the primary antibody with highly purified antigen provides the ideal negative control for differentiating specific from non-specific staining. As negative controls, use can be made of sections of the tissue under study incubated either without primary monoclonal antibody or with isotype and species-matched irrelevant primary monoclonal antibodies.

4. Notes

1. PBS may be obtained using different procedures. However, it is mandatory to use the same kind of PBS to collect all the samples of the study.
2. 2% paraformaldehyde may be frozen in 20-mL aliquots at -80°C and stored at -80°C until needed.
3. The first studies on airway inflammation in asthma used rigid bronchoscopy. Although rigid bronchoscopy has been performed under local anesthesia, it usually requires general anesthesia, and its use in research has been very limited because of considerable discomfort to the patient. In contrast, fiberoptic bronchoscopy, which can be easily performed under local anesthesia, using premedication with bronchodilators and mild sedation, has been much more acceptable in the clinical and research setting (21).
4. Premedication and sedation vary. Most investigators use premedication with bronchodilators such as salbutamol by metered dose or other inhaler, or nebu-

lizer followed by intravenous atropine, which reduce bronchial secretions and cough during the procedure. Sedation is often used during bronchoscopy in the hope that it will reduce stress level and cough and that it will induce analgesia, euphoria, and amnesia, altering the subject's perception of discomfort. The drugs used include either midazolam or diazepam intravenously (22,23).

5. Various bronchoscopes have been used, with various biopsy forceps. Some investigators used cupped forceps or alligator forceps. Clearly bigger bronchoscope channels and bigger forceps will give more tissue. Some investigators think that relatively new forceps give better biopsies. It is prudent to have several sets of forceps available at the time of bronchoscopy as this covers the possibility of malfunction (21).
6. Biopsies should be taken under direct bronchoscopic vision from segmental and subsegmental carinae. Studies that have compared different airway levels (24,25) did not find differences in immunohistochemistry, so that samples can be pooled. When taking repeated biopsies from the same subject over time, one should avoid previous biopsy sites. Video recording of the procedure may be helpful.
7. Many protocols combine bronchial biopsies with BAL, almost always with BAL performed first. This approach has the advantage of sampling large and small airways, but prior BAL may make biopsies more difficult to obtain (in the face of residual lavage fluid or local bronchospasm associated with BAL).
8. Fiberoptic bronchoscopy in association with endobronchial biopsies is usually well tolerated and does not induce significant long-term clinical sequelae. Caution has been exercised when performing bronchoscopy in asthmatic subjects because of concern about its safety in patients with hyperresponsive airways. However, several studies have reported that fiberoptic bronchoscopy can be conducted safely in asthma although caution must be exercised in those with very responsive airways because of the possibility of bronchoconstriction (21).
9. It is essential that skilled bronchoscopists carry out research bronchoscopies to expedite the procedure and, if severe patients are to be studied, this should be performed in centers with considerable experience. Resuscitation equipment (for intubation, electrocardiographic monitoring, and defibrillation) together with necessary drugs (salbutamol, adrenaline, hydrocortisone) should be available, and the subject should have an existing cannula. The procedure should be preferably in a facility dedicated to bronchoscopy (23).
10. The freezing procedure itself is a knack that once learned is not difficult. However, it must be taught and practiced in order to avoid the common (and often published) artifact of ice-crystal damage. The result of such damage is a peppering of the tissue with artifactual spaces that do not allow adequate and reliable quantification of inflammatory cells. The importance of good quality starting material and fast freezing rates cannot be overemphasized for successful results.
11. Some investigators use 4% paraformaldehyde to fix bronchial biopsies.
12. Prior soaking of the tissue in cryoprotectants such as 15% sucrose buffer can help to prevent ice-crystal formation during the freezing procedure. 0.01% sodium azide should be added to inhibit bacterial contamination and growth.

13. This method can be modified to collect bronchial biopsies for study of gene expression by ISH. Modifications are necessary to prevent RNase contamination and include:
 - i. Gloves must be worn during the handling of the reagents. Glassware used for preparation of solutions must be baked at 200°C for 2 h. Disposable weighing boats and baked lab weighing spoons must be used.
 - ii. Distilled water should be replaced with diethyl pyrocarbonate (DEPC)-treated water obtained as follows:
 - a. Add 2-mL DEPC, (Sigma D-5758) to every 2000 mL of double distilled water and let the solution stir for a minimum of 2 h in a fumehood.
 - b. Pour the solution in DEPC labeled bottles and autoclave for 25 min in order to destroy the DEPC.

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