Eur Respir Rev 2006; 15: 101, 157–161
DOI: 10.1183/09059180.00010106
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Quantitative morphology using bronchial biopsies

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ABSTRACT: Bronchoscopy with bronchial biopsies allows sampling of airway tissue in prospective clinical studies and is increasingly applied for the study of airway inflammation and remodelling.

It is important to recognise that there are limitations inherent to the use of bronchial biopsies, including the inability to randomly sample the airway and susceptibility to the ''reference trap''. Nonetheless, certain stereological principles can be applied to the analysis of these specimens, which may improve the validity of the results obtained. These principles relate to choice of reference space, adequacy of sampling and treatment of tissue orientation in the estimation of thickness and surface area.

With attention to these principles, the present authors have developed protocols for the assessment of multiple measures of airway remodelling in bronchial biopsies, including airway epithelial mucin content, epithelial goblet cell size, reticular basement membrane thickness and smooth muscle morphological outcomes. They have also developed protocols for the enumeration of inflammatory cells important in the study of asthma and chronic obstructive pulmonary disease, including eosinophils and neutrophils.

In the present article, relevant stereological principles and the details of these specific protocols will be reviewed.

KEYWORDS: Airway remodelling, bronchial biopsy, inflammation, morphometry, stereology

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SUPPORT STATEMENT
P.G. Woodruff and A.L. Innes were supported by the National Institutes of Health, grants K23 RR17002 and HL-07185, respectively.

Bronchoscopy with bronchial biopsies allows sampling of airway tissue for research into the mechanisms of airway disease and has been applied to study airway inflammation and some aspects of airway remodelling. Sampling airway tissue by endobronchial biopsy has advantages over strategies that employ surgical and autopsy specimens. For example, endobronchial biopsies allow greater flexibility in subject selection and sampling can be repeated over time. Thus, the approach allows prospective studies, allows recruitment of subjects with specific characteristics, including those with relatively mild disease, and enables investigators to repeat the sampling procedure under specific conditions (*e.g.* before and after an intervention or therapy). However, there are inherent limitations in the study of airway morphology using bronchial biopsies that relate to the fact that the samples are small and not full thickness, that sampling is limited to the larger airways and that the biopsies are limited to the carinae. Methods for biopsy analysis have been developed to address some of these limitations but they cannot be entirely eliminated.

THE TECHNIQUE OF BRONCHIAL BIOPSY

Methods for research bronchoscopy have been reviewed in detail elsewhere [1–3] and are beyond the scope of the present article. However, certain important points merit emphasis. First, safety is paramount in research bronchoscopy and attention to adequate informed consent, adequate training of physician bronchoscopists, medication dosing (particularly lidocaine), and subject monitoring during and post-procedure is mandatory. Secondly, bronchial biopsies obtained at fibreoptic bronchoscopy sample specific levels of the airway, typically subsegmental, segmental and lobar carinae. In the present authors' laboratory, usually six to 10 bronchial biopsies are performed at second-order (lobar) through to fifth-order (subsegmental) carinae. Sampling is begun in the lower lobe basilar segments and then proceeds apically, sampling only one lung per bronchoscopy. Thus, samples are obtained from the carinae of large and small cartilaginous airways only.

INHERENT LIMITATIONS OF BRONCHIAL BIOPSY

One major limitation of bronchial biopsy is that random sampling of the airway is not possible.

European Respiratory Review
Print ISSN 0905-9180
Online ISSN 1600-0617

As a consequence, estimates made using bronchial biopsies predominantly reflect the morphology of the carinae of the larger airways. In addition, because it is not possible to sample the entire airway, the reference space representing the entire airway cannot be estimated, and the interpretation of stereological ratios in the absence of an estimate of the entire reference space is susceptible to a ''reference trap'' [4]. In other words, an important change in the volume of the reference space might go undetected in a disease state or after an intervention and this change would have important implications as to how a stereological ratio is interpreted. Using an example, the numerical density of goblet cells per volume of airway epithelium might not differ in bronchial biopsies from two groups but if the total volume of epithelium in the airway in one group has increased, then the total number of goblet cells will have increased in that group. This could have biological significance and could be missed if awareness of the increase in the reference space is lacking. As will be discussed later, there are strategies to detect common changes in reference spaces in the airway and reference spaces that may be used that are less susceptible to change. Using these approaches, the probability of falling into a reference trap in the analysis of bronchial biopsies may be decreased but this may not be eliminated entirely, due to the inherent limitations of these biopsies.

A second limitation of bronchial biopsy is that there can be significant variability in morphological characteristics between biopsies. This is in part due to biological variability (as result of different morphologies at different sites in the airway). However, there is also significant variability in the degree to which each biopsy adequately samples the epithelium (due to desquamation by the forceps) or the subepithelial compartment (variability on depth of each biopsy). This limitation is best handled by insisting that quantitative morphology be performed using multiple biopsies from any given subject. Recommendations on the number of biopsies will be provided later.

APPLICATION OF STEREOLOGICAL PRINCIPLES TO BRONCHIAL BIOPSIES

Of the stereological principles that are presented elsewhere in the present issue of *European Respiratory Review*, there are three that are particularly important in the study of bronchial biopsies: 1) attention to the reference space; 2) attention to sampling; and 3) handling of orientation-sensitive measures. The present authors have established general approaches in their laboratory that address each of these considerations.

Reference space

Since it is not possible to sample the entire lung using bronchial biopsies, the morphometric data must be normalised to a specific reference space. The choice of the reference space for measurements is particularly important. In general, a space that is invariate across groups or experimental conditions is preferred. The present authors have chosen to use three reference spaces for their morphometric measures, depending on the specific application, as follows: 1) volume of epithelium; 2) volume of submucosa; and 3) surface area of basal lamina. For example, normalising smooth muscle measures to the volume of submucosa studied has the advantage of yielding an easily understandable ''volume fraction'' of smooth muscle in the airway. However, in conditions accompanied by oedema of the submucosa, it is possible that the volume of the reference space could change, thereby influencing the measured volume fraction. The surface area of the basal lamina may provide a more invariate reference space under these conditions. In practice, both a volume-based reference space and the surface area of the basal lamina are measured to verify that inferences made using one reference space are consistent with inferences using the other reference space.

Sampling

Prior data from previous studies of reticular basement membrane thickness in asthma indicate that there is significant within-biopsy and between-biopsy variability in morphological measures made using systematically random samples of microscopic fields [5]. As the morphological characteristics between biopsies are variable for any given subject, a sampling scheme that employs multiple biopsies will be superior to the use of single biopsies, irrespective of whether stereological approaches are used. Within-biopsy variability is addressed by the use of multiple microscopic fields per biopsy. In general, the present authors have found greater variability due to sampling in the measurement of submucosal airway features (such as smooth muscle) as compared with surface features (such as goblet cells).

Orientation

The study of three-dimensional structures using two-dimensional sections poses some specific problems that are handled by techniques developed in design-based stereology. One such problem is that measurement of surface area is sensitive to the orientation of the object being measured [6–8]. A solution to this problem is to generate random orientation in the samples of interest so that isotropic uniform random (IUR) sections are produced. IUR sections allow the application of a specific estimator for surface area that will produce unbiased estimates of surface area. In order to produce IUR sections, each biopsy is embedded in paraffin in a spherical mould (an ''isector''), the sphere is rolled to randomise orientation, then all six biopsies from each subject are re-embedded into a single traditional mould (fig. 1) [9]. When this block is sectioned, all six biopsies appear in the same sections, with each biopsy in an IUR orientation (fig. 1).

PROTOCOLS FOR MEASUREMENT OF AIRWAY REMODELLING

In the present authors' laboratory, bronchoscopy has been performed with bronchial biopsy to obtain samples for the measurement of several outcomes of airway remodelling, including measurement of epithelial mucin content [10, 11], epithelial goblet cell size [10, 11], reticular basement membrane thickness [5] and smooth muscle morphological outcomes that reflect the mass of smooth muscle in the airway, the number of airway smooth muscle (ASM) cells and the mean size of ASM cells [12].

General approach to biopsy preparation

Six endobronchial biopsies are obtained from second- through to fifth-order carinae, examined under a dissecting microscope in the bronchoscopy suite for adequacy. They are then fixed

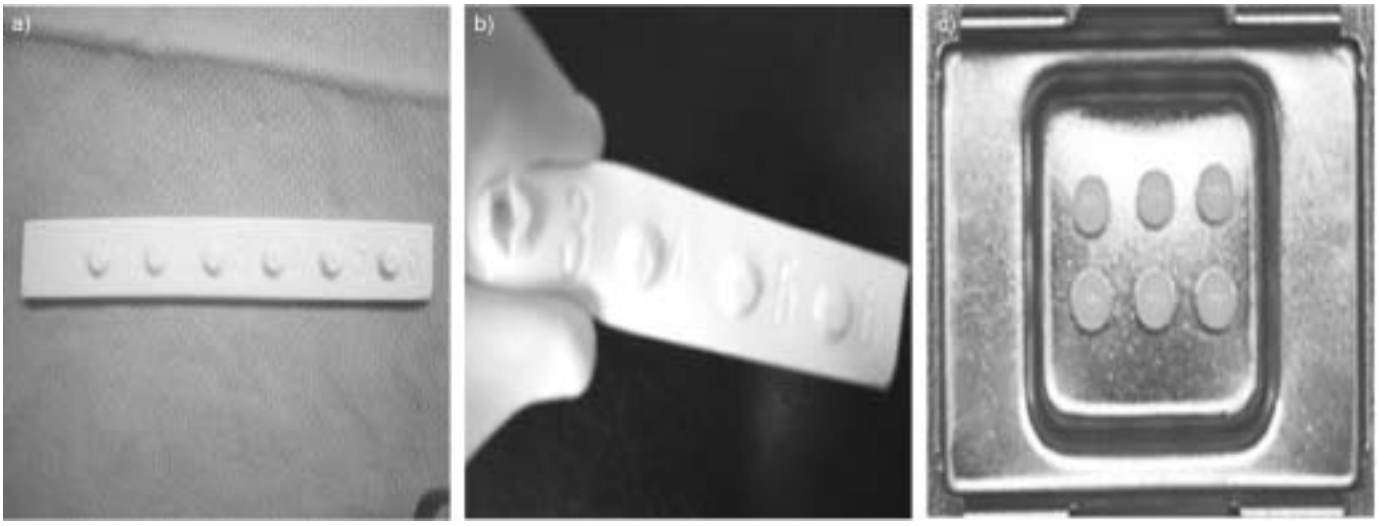

FIGURE 1. Use of the isector mould to embed bronchial biopsies in isotropic uniform random orientation. The isector is a flexible mould (a) with a 4-mm spherical cavity (b). After embedding, the spheres are rolled to randomise orientation and then each biopsy from a given subject is re-embedded together in a single rectangular mould (c).

using the following protocol. Biopsies are first fixed for 4 h in fresh 10% neutral buffered formalin at 4°C (replaced with fresh formalin after the first hour). After 4 h of fixation, the formalin is replaced with 70% alcohol and stored at 4°C until ready for paraffin processing. These biopsies can sit in 70% alcohol for several weeks. When they are ready to be processed, biopsies can be dehydrated and paraffinised *via* the following steps: 1) 70% alcohol for 15 min; 2) 80% alcohol for 15 min; 3) 95% alcohol for 15 min; 4) 95% alcohol for 15 min; 5) 100% alcohol for 15 min 3×; 6) CitriSolv Clearing agent (Fisher Scientific, Pittsburgh, PA, USA) or xylene for 15 min 3×; and 7) warm paraffin (55°C) for 1 h 2×. Each specimen is then individually embedded in paraffin in a 4-mm spherical isector mould to introduce IUR orientation. The six spherical moulds from each subject are then re-embedded in paraffin in a single conventional mould.

Epithelial mucin content

For morphometric analyses of airway epithelial mucin stores [10, 11], 3-μm thick sections are de-paraffinised and stained sequentially with alcian blue and periodic acid Schiff (AB/PAS) reagent (Sigma, St. Louis, MO, USA). To ensure adequate epithelial tissue for sampling, it is required that at least two biopsies per subject contain full-thickness, ciliated epithelium yielding ⩾10 microscopic fields. Next, the volume of AB/PAS stain, the volume of airway epithelium and the surface area of the epithelial basal lamina are determined by point- and intersection-counting using an integrated microscope (Olympus, Albertslund, Denmark), a video camera (JVC Digital Color; JVC A/S, Tatstrup, Denmark), an automated microscope stage and a computer (Dell Optiplex GS270 PC running Computer-Assisted Stereology Toolbox (CAST) Software; Olympus). A line segment grid is superimposed on systematically randomly selected microscopic fields. Points overlying positive AB/PAS stain, unstained goblet cells and other epithelial cells are counted, along with intersections of test lines with basal lamina. The measurements are recorded by blinded investigators using a 40× or 60× objective lens. The volume density of stored epithelial mucin and of specific mucin proteins is calculated by quantification of the volume of stained epithelium referenced to the following two different reference compartments: total epithelial volume and surface area of the basal lamina. The mean height of the airway epithelium can be calculated by dividing the volume of epithelium by the surface area of basal lamina for each subject.

Epithelial goblet cell size and numeric density

For quantification of goblet cell number and mean goblet cell size [10, 11], sections stained with AB/PAS are used and the ''rotator'' technique [13], using a 60× lens, is employed. The volume of individual goblet cells is estimated in systematically randomly selected microscopic fields (yielding a volume-weighted volume measurement). The total volume of epithelium and the surface area of basal lamina are measured using the point- and line intersect-counting technique. The mean number of goblet cells per volume of epithelium is then calculated from the mean goblet cell volume in each subject and the volume fraction of goblet cells per volume of epithelium for that subject. Similar calculations are made to estimate the mean number of goblet cells per surface area of basal lamina.

Reticular basement membrane thickness

For morphometric analyses of reticular basement membrane (RBM) thickness [5], 3-μm thick sections are de-paraffinised and stained with Gomori's trichome stain (Fisher Scientific). Microscopic fields are then systematically and randomly sampled within the epithelium using a 100× objective and a random start. In order to identify systematic random measurement points along the epithelial RBM, a line grid is superimposed onto each randomly selected high-power field. The RBM thickness is then measured at each intercept of the line grid with the epithelial surface of the basal lamina by measuring the length of a line extended perpendicularly from the epithelial surface across the thickness of RBM (measurement is made perpendicular to the surface of the RBM and not to the grid line). This measurement is termed the ''orthogonal intercept'' [14, 15]. The arithmetic mean thickness ($\bar{t}$) and harmonic mean thickness of the RBM can then be calculated

from the orthogonal intercept lengths for any given subject using the following formulae, which account for tangential cuts that occur when a layered structure such as the RBM is sectioned:

$$\bar{\tau} = \pi/4 \times \text{arithmetic mean of orthogonal intercepts} \quad (1)$$

$$\text{Harmonic mean layer thickness} = 8/3\pi \times \text{harmonic mean of orthogonal intercepts} \quad (2)$$

where the harmonic mean is the number of measurements/sum of the reciprocal of orthogonal intercept lengths.

$\bar{\tau}$ has the advantage that it is more easily understood by general readers. The harmonic mean thickness has the advantage that it has generated more normally distributed data in prior studies [14, 15] and may have greater physiological relevance if the diffusion of substances across a tissue layer is of interest [16].

Submucosal ASM content and numeric density

For morphometric analyses of ASM content [12], 3 μm-thick sections are de-paraffinised and immunostained for α-smooth muscle actin (Clone 1A4; NeoMarkers, Fremont, CA, USA). Serial sections are stained with Gomori's trichrome stain (Biochemical Sciences, Inc., Swedesboro, NJ, USA) to verify the adequacy of the α-smooth muscle actin immunostain and to differentiate smooth muscle from adjacent connective tissue. ASM cell number and size are measured using serial sections stained with haematoxylin and eosin. To adequately account for between-biopsy variability, a typical requirement is that each subject should have three or more biopsies. Microscopic fields are then systematically and randomly sampled within the submucosa. For measurement of volume fraction of ASM in the airway submucosa, the number of points overlying smooth muscle and other submucosal structures and the number of lines intersecting basal lamina are counted using a 20× lens. These counts allow calculation of volume fraction of smooth muscle in the submucosa and volume of smooth muscle per surface area of basal lamina. For measurement of ASM number and mean volume, paired serial sections stained with haematoxylin and eosin and the physical disector technique (described in more detail later in the present article and elsewhere [17]) using a 20× lens are employed. ASM cell nuclei are counted to enumerate ASM cells, given that these cells have only one nucleus. The volume of ASM surveyed is measured using the point-counting technique. The number of cells per volume of ASM, the mean volume of individual ASM cells and the number of cells per surface area of airway basal lamina are then calculated using these data.

PROTOCOLS FOR THE ENUMERATION OF INFLAMMATORY CELLS

Enumeration of eosinophils and neutrophils in bronchial biopsy specimens has been approached using immunohistochemical stains [10, 18]. Eosinophils are labelled with an antibody specific for eosinophil cationic protein (EG-2; Pharmacia Upjohn, Kalamazoo, MI, USA) and neutrophils are labelled with an antibody to neutrophil elastase (NP57; Dako, Carpenteria, CA, USA) using an immunohistochemistry protocol that is published elsewhere [18]. The physical disector method is used to measure the numerical density of neutrophils and eosinophils thus identified in bronchial biopsies. The rationale for use of the physical disector to estimate numeric density is described in detail elsewhere [17]. In brief, the practice of counting cell number using profiles seen in two-dimensional tissue sections is subject to bias because the cell profile number in a two-dimensional section is a function not only of the cell number but also of the cell size. Larger cells are more likely to appear in the profile and therefore be counted. The physical disector method avoids this bias by counting the cell number in a pair of serial sections a known distance apart (usually 30% of the diameter of the object of interest). Using this three-dimensional counting system, two serial sections are inspected simultaneously and a cell is counted only if it is seen in one section (the ''reference'' section) and not the next (the ''look-up'' section).

In this application of the physical disector, an integrated set of components are used, including a microscope (Olympus), a video camera (JVC Digital Color; JVC A/S), an automated microscope stage (Prior Scientific, Rockland, MA, USA), a computer (IBM 300PL; IBM Corp., Armonk, NY, USA) and CAST Grid software. Three serial sections, each 2-μm thick, are cut. The first and third sections are paired and used as the reference and look-up sections. Thus, the total thickness of the physical disector is 4 μm. The reference and look-up sections are then mounted side by side on the microscope stage. Microscopic images of the sections are examined using the 20× objective in reference and look-up sections that are aligned so they can be examined simultaneously. Using a point and line grid, points overlying epithelium and submucosa and lines intersecting basal lamina are counted simultaneously with enumeration of neutrophils and eosinophils in each field. These counts yield estimates of the number of eosinophils and neutrophils per volume of epithelium and submucosa (N_V) using the following formula.

$$N_V = 1/((a/f)\cdot h)\cdot Q/P \quad (3)$$

In this formula, a/f is the area of field, h is the distance between biopsies, Q is the sum of the cell counts, and P is the sum of points counted in the reference space. The surface area of the basal lamina per volume of epithelium or volume of submucosa are also estimated in the samples, which allows estimation of the cell number per surface area of basal lamina of the airway.

CONCLUSIONS

Quantitative morphology using bronchial biopsies poses specific challenges, some of which may be addressed using the approaches described previously, and others of which are limitations inherent to the technique. In general, the most important technical consideration is the use of an adequate number of biopsies and microscopic fields to account for between-biopsy and within-biopsy variability. This fundamental principle can also be incorporated into other approaches but its importance should not be underestimated. Other principles that are incorporated by the specific approaches presented here include attention to reference space, orientation and the application of unbiased counting rules. The intent of these approaches is to seamlessly incorporate as many as possible of the protections against bias that are afforded by design-based stereology, while maintaining efficiency in sample evaluation.

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