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# Confocal laser scanning fluorescence microscopy of intact unfixed rat lungs

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**Abstract**

Fluorescence microscopy is a potentially powerful tool for assessing the pulmonary disposition of drugs. Difficulties maintaining lung tissue in its native hydrated state, sectioning, and other disruptions of the internal milieu create artifacts and complicate data interpretation. Confocal laser scanning fluorescence microscopy, which obviates many of the liabilities of conventional microscopy, was used to image intact, fully-hydrated, unfixed rat lung at depths up to 25 $\mu$m beneath the outer pleural surface. Rhodamine B, a relatively lipophilic fluorescent marker introduced ex vivo either intratracheally or by vascular perfusion, was used to help define endothelial and epithelial barriers to drug transport, macrophages, interstitial spaces, and Type II alveolar epithelial cells. The technique was used to characterize the disposition of the fluorescent 'model drug' 6-carboxyfluorescein at a single time point following its intratracheal instillation into anesthetized rats. Dual wavelength excitation of both rhodamine B and 6-carboxyfluorescein resulted in 'multi-colored' images showing the latter within pulmonary interstitial spaces and localized within punctate regions along alveolar epithelial surfaces. © 1998 Elsevier Science B.V. All rights reserved.

*Keywords:* 6-Carboxyfluorescein; Rhodamine B; Alveolar epithelial cells; Pulmonary disposition

## 1. Introduction

Historically, studies of the pulmonary absorption and disposition of drugs and toxicants have relied upon such quantitative techniques as kinetic and scintigraphic analyses (Enna and Shanker, 1972; Effros and Mason, 1983; Huchon et al., 1987; Newman, 1993) in addition to qualitative imaging methods such as electron and light microscopies (Schneeberger, 1978; Williams, 1984). Recent advances in light microscopy, especially the development of confocal laser scanning fluorescence microscopy (CLSFM), have dramatically improved the resolution, contrast and rejection of out-of-focus noise historically associated with

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light microscopic techniques (Rojanasakul et al., 1990; Smith et al., 1991).

While pioneers such as Weibel (Crapo et al., 1982; Weibel, 1983) have combined electron microscopy with sophisticated morphometric methods to both quantitatively and qualitatively elucidate the principal design features of the lung as they relate to airway function, many studies of the characteristics and time course of pulmonary drug disposition would benefit from an ability to observe drugs within native lung tissue in real time and space. Thus the development of a technique which provides for the identification and definition of relevant regions of interest (ROI) within native lung tissue while simultaneously revealing the location of drug relative to those regions over time, seems worthwhile.

Physically, lung tissue is relatively intractable and difficult to maintain in its normal physiological state of hydration, conformation and inflation during imaging. In addition, the close apposition of its circulatory and airway components; thin epithelial interfaces; small interstitial and intracellular spaces; and dynamic character present imposing challenges to those employing imaging techniques in the study of pulmonary absorption and distributive processes. Conventional tissue preparation for light and electron microscopies is often lengthy and may require, among other things, sectioning, fixation, dehydration and embedding. These processes often profoundly disrupt the native internal milieu of the lung. Dehydration during sample preparation for electron microscopy, for example, will obliterate pulmonary interstitial spaces as meaningful ROI for absorption and disposition studies.

The present report details a useful technique for following the disposition of intratracheally-administered fluorescent compounds in native rat lung using CLSFM. The technique permits imaging at considerable depths below the surface of intact, inflated lungs using optical, rather than physical, sectioning. It requires neither tissue fixation nor dehydration, and permits revisitation/examination of specific volume elements or areas over time. Using a lipophilic dye (rhodamine B, RB) the optical definition of important morphologic 'landmarks' within the alveolar region such as endothelial and epithelial barriers to drug transport, interstitial spaces and Type II alveolar epithelial cells was greatly improved. It is also possible to create 3-dimensional reconstructions of lung architecture from such 2-dimensional images obtained serially at various depths beneath the tissue surface (Schotton and White, 1989; Cookson et al., 1993). In addition, in order to gain a better perspective on how the technique might apply to the study of a fluoresceinated drug, the technique was used to examine the pulmonary disposition of a fluorescent 'model drug', 6-carboxyfluorescein (6-CF), following its intratracheal instillation into rats in vivo. Based on these results, it seems clear that the method should be capable of providing previously unavailable spatial and real-time kinetic information in native lung tissue to support studies on pulmonary absorption and disposition of drugs, macromolecules and toxicants in laboratory animals.

## 2. Materials and methods

### 2.1. Animals and reagents

Fischer-344 pathogen-free rats (175–200 g) were obtained from Charles River Labs, Kingston, RI. Rhodamine B (Eastman Kodak, Rochester, NY) and 6-carboxyfluorescein (> 99%, Molecular Probes, Eugene, OR) were used without further purification. Modified lactated Ringer's solution (285 mOsmol/l, pH 7.4) consisted of lactated Ringer's (Baxter, Deerfield, IL) supplemented with 5 mM $Na_2HPO_4$ and 5.37 mM NaCl.

### 2.2. Confocal microscopy

#### 2.2.1. Instrumentation

Fluorescence images of intact lungs were obtained using an inverted confocal microscope (Zeiss Axiovert, Model 410), equipped with an argon/krypton laser and a C-Apochromat 40 × / 1.2 W Korr. water immersion objective lens. Water was the preferred immersion fluid because its index of refraction approximated that of lung

tissue, thereby reducing spherical aberration and increasing resolving power. While clear images could be obtained at depths up to 25 $\mu$m, resolution was optimal 10–12 $\mu$m beneath the outer pleural surface.

*2.2.2. Lung setup*

Inflated lungs were placed heart-side down within a specially-designed chamber, consisting of a 55-mm Petri dish adapted for microscopy by replacing a rectangular portion of its bottom with a $20 \times 40$ mm ( # 1 1/2) glass coverslip. The lower portion of the right lobe was centered on the coverslip, and all residual fluids gently absorbed with a lint-free tissue to insure direct contact of the lung with the coverslip. To maintain humidity in the closed chamber, a piece of filter paper saturated with water was placed next to the lung. The chamber was mounted in an adjustable specimen holder on the confocal microscope and the water immersion objective lens focused on the edge of the lower right lobe. Contrast and brightness settings were extended to a full grayscale intensity range of 0–255 prior to imaging. Time required for preliminary focus and orienting the laser to the correct depth within the tissue was $\sim$10 min, and all images were taken within 1 h of animal death.

*2.2.3. Image acquisition*

Laser excitation wavelengths of 488 nm (emission = 515–545 nm) and 568 nm (emission > 630 nm) were used to excite 6-CF and RB, respectively. Grayscale images ($512 \times 512$ pixels) were taken at depths of 10–12 $\mu$m and stored independently for each emission wavelength. When both 6-CF and RB were present, color images were made from two grayscale images (one from each emission wavelength) taken of the identical area within the lung. These images were pseudocolored green (6-CF) and red (RB), then overlaid to form a multicolored image using Zeiss confocal software. The resultant images were further refined and formatted using Adobe Photoshop® software (Version 3.0, Adobe Systems, Mt. View, CA). Because of the wavelengths involved the nominal lower limit of resolution of the technique was $\sim$0.1 micron. Fluorescent elements of smaller dimension were, of course, discernible, but their size is indeterminate.

*2.3. Lung preparation*

Rats were anesthetized with ketamine (80 mg/kg)/xylazine (10 mg/kg) and, after remaining anesthetized for the duration of any disposition period which may have been involved, exsanguinated. The thoracic cavity was opened and heparin (1000 units as 0.1 ml of 10000 U/ml) injected into the right side of the heart. The lungs were then excised, the pulmonary artery and left atrium cannulated, and an endotracheal tube inserted into the trachea. Lungs were then inflated with air through the endotracheal tube and maintained near physiological capacity (15 cm water pressure). The pulmonary vasculature was then briefly perfused by gravity-feeding modified lactated Ringer's solution (15 cm $H_2O$ pressure) through the pulmonary arterial cannula. Perfusion was terminated when most erythrocytes had been flushed from the vasculature, resulting in almost complete blanching of the lung.

*2.4. Ex vivo administration of RB*

RB was administered either i.t. or by vascular perfusion. The latter involved slowly introducing RB (10 $\mu$g/ml in modified Ringer's solution) into the pulmonary artery until it appeared in the left atrial cannula. For i.t. administration of RB, the endotracheal tube was removed (following termination of blanching perfusion) and lungs allowed to deflate. A tracheal cannula was then inserted and its tip directed to the lower portion of the right lobe; 200 $\mu$l of RB solution (10 mg/ml) were injected i.t., followed by a 200 $\mu$l air bolus to clear the cannula of residual RB. The cannula was then replaced by the original endotracheal tube (attached to a compressed air tank) and lungs were gently re-inflated to their previous physiological volume. For both routes of administration, lungs were tied off during removal of the endotracheal tube to maintain inflation during imaging.

### 2.5. Intratracheal administration of 6-CF in vivo

Anesthetized rats were secured at a 30° angle from horizontal and the trachea exposed. A small incision was made between the 5th and 6th tracheal rings and a cannula (PE-90) inserted into the respiratory tract to a depth of 3.5 cm; 0.1 ml of 6-CF solution (4.5 $\mu$M in phosphate-buffered saline, pH 7.4) was rapidly injected into the lungs, followed by a 200 $\mu$l air bolus to clear the cannula of residual 6-CF solution. The cannula was immediately replaced by an endotracheal tube and, after 1 h, the rats were sacrificed and lungs removed.

## 3. Results

### 3.1. Enhancement of lung images with RB

Autofluorescence of lung tissue was examined following excitation at either 568 or 488 nm. Whereas very little autofluorescence was observed at 488 nm, a few isolated autofluorescent cells were evident with excitation at 568 nm. In neither case were details of the capillary endothelium and alveolar epithelium visually apparent (Fig. 1).

Two-to-three minutes after ex vivo perfusion of RB into the vasculature of the lung, many elements within the tissue became fluorescent (Fig. 2). Structures appearing to represent the alveolar capillary endothelial network were enhanced, displaying sharp fluorescent margins surrounding a non-fluorescent (dark) interior (*). Also apparent were moderately-fluorescent cells, probably tissue macrophages, displaying a homogeneous cytoplasmic fluorescence (**). The most brightly illuminated cells were cuboidal, and appeared to be located in the corners of alveoli (+) where Type II alveolar epithelial cells are found. These cells displayed a dark, non-fluorescent nucleus and intense, punctate cytoplasmic fluorescence. Alveolar septa fluoresced and were clearly distinguishable from adjacent, non-fluorescent airspaces (++). Interstitial spaces (◇), although somewhat difficult to distinguish, were discernible.

In contrast, when RB was instilled intratracheally ex vivo (Fig. 3a), capillary endothelia (*) did not appear to be as clearly defined. Cells displaying non-fluorescent dark nuclei and homogeneous, moderately fluorescent cytoplasm (probably alveolar macrophages) were clearly evident within the airspaces (**), in contrast to images obtained following vascular perfusion of RB where such cells were not evident. As with vascular perfusion of RB, cuboidal cells containing intense punctate fluorescence within their cytoplasm were again visible in the corners of the alveoli (+). Their pattern of fluorescence within the cytoplasm is consistent with what one might expect from a Type II alveolar epithelial cell containing lamellar bodies. Under high magnification (Fig. 3b) these cells appear to be associated with the alveolar epithelial surface, in contrast to adjacent, less fluorescent alveolar cells (**) which appear to be alveolar macrophages located within the airspace.

### 3.2. Intratracheal administration of 6-CF in vivo

The alveolar disposition of the fluorescent probe 6-CF, 1 h after its i.t. administration to rats, was assessed using ex vivo perfusion of the

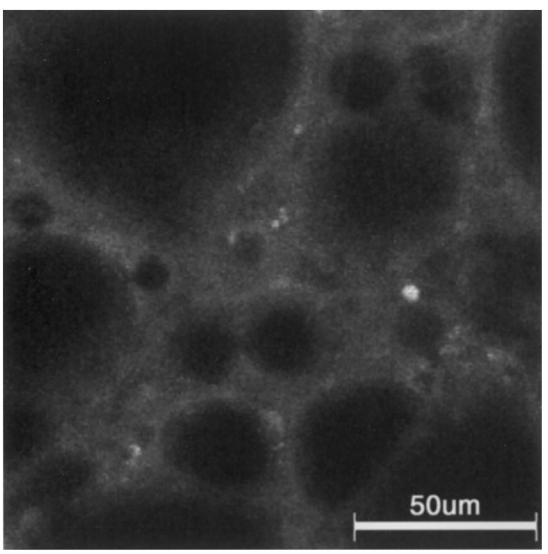



Fig. 1. Lung autofluorescence induced in untreated lung tissue by laser excitation at 568 nm at a depth of 10 $\mu$m beneath the outer pleural surface. While a few points of autofluorescence are evident, the visual field is largely non-fluorescent. Few detailed structural features are apparent.

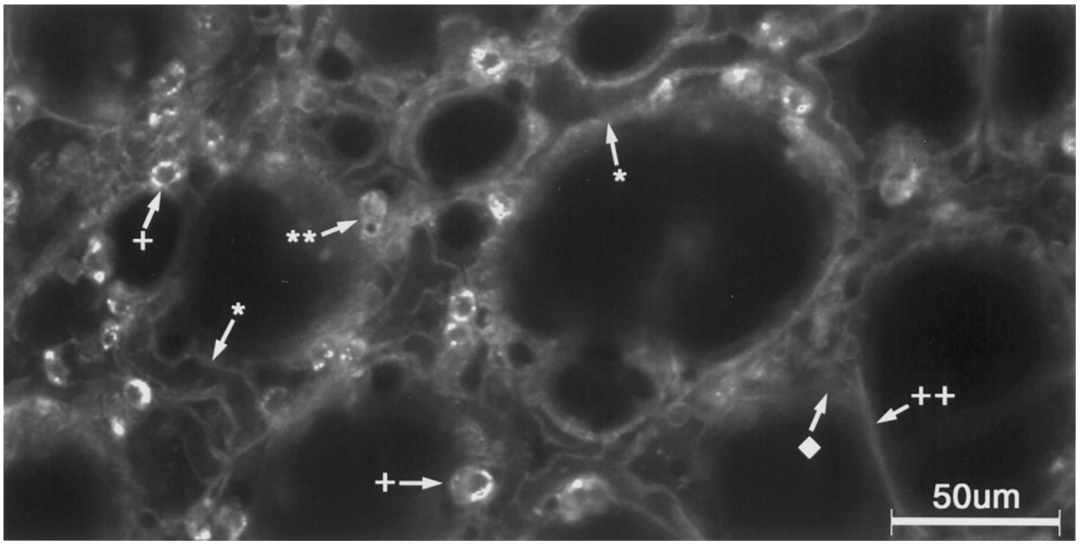


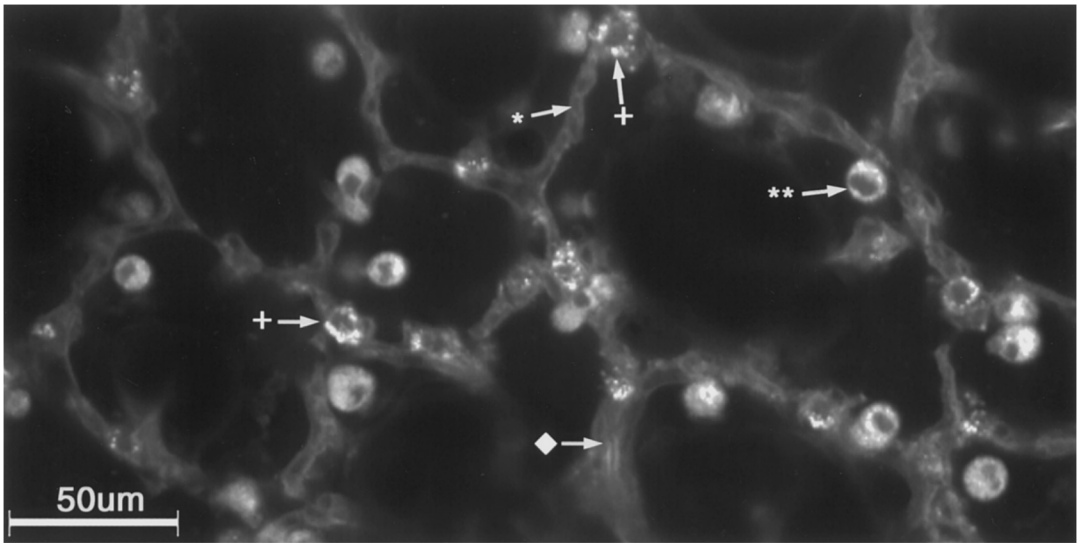

Fig. 2. CLSFM image of intact lung following ex vivo intravascular perfusion with RB (10 $\mu$g/ml). This low magnification image of the lung at a 10 $\mu$m depth shows structural features that appear to include the capillary network (*), interstitial spaces ($\diamond$), alveolar septa ( + + ) and tissue macrophages (**). Type II alveolar epithelial cells located in the corners of the airspaces are characterized by intense, punctate fluorescence within their cytoplasm ( + ).

pulmonary vasculature with RB to help define ROI and dual wavelength CLFSM to differentiate the 'model drug' from the RB. Color overlays of the 'fluorescein' (488 nm) and 'rhodamine' (568 nm) channels were used to determine the location of 6-CF within several ROI in the lung (Fig. 4). The majority of 6-CF remaining in the lung at 1 h appeared to occupy interstitial spaces at the corners of alveoli including, perhaps, trijunctional regions ($\diamond$). In contrast, cuboidal cells in the corners of alveoli ( + ), which were stained red by RB, did not appear to contain 6-CF within their cytoplasm. Intense punctate 6-CF fluorescence was also evident along selected areas of alveolar epithelial surfaces ($\square$).

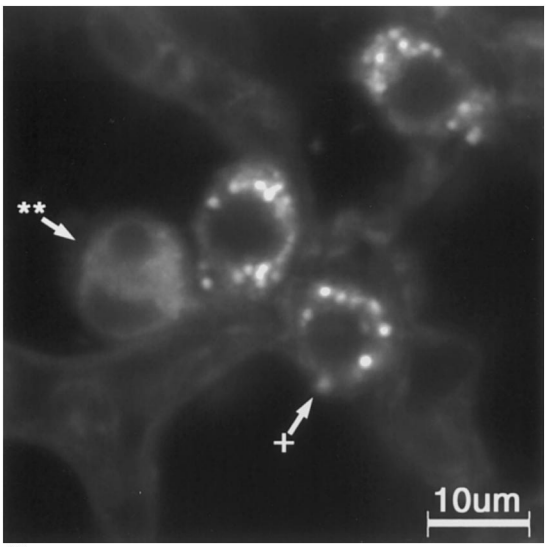
## 4. Discussion

The versatility of CLSFM has been demonstrated in several recent studies of drug transport through epithelial barriers (Rojanasakul et al., 1990; Hoogstraate et al., 1994). While images taken during the penetration of fluorescently-labeled macromolecules through corneal and buccal epithelia in vitro have provided visual information on the pathways involved in their transport across these relatively thick epithelial surfaces, no comparable data exist for the far thinner, less physically tractable alveolar epithelium. Pulmonary applications of CLSFM have included its use in characterizing the microanatomy of alveolar ducts in lung tissue (Cookson et al., 1993); determining the pulmonary deposition of aerosolized fluorescent microspheres (Pinkerton et al., 1993); examining apoprotein-A subcellular localization within isolated Type II epithelial cells (Smith et al., 1991); studying microvascular permeability in the rat lung (Pietra and Johns, 1996); mapping innervation of the bronchial tree in fetal and postnatal lung (Weichselbaum et al., 1997); and observing cigarette filter fibers in lung tissue from patients with lung cancer (Pauly et al., 1995). All except the last of these used CLSFM to examine specimens that had either been fixed, sectioned, or both, and there is virtually no information available on its use to characterize fluorescent probe disposition in fresh, intact, fully-hydrated, lung tissue.

The technique developed in the present study used CLSFM to image intact, unfixed rat lung following treatment with RB to enhance struc-




(a)




(b)

Fig. 3. (a) CLSFM image of intact lung following intratracheal instillation of RB ex vivo. (a) Low magnification image of the lung at 10 $\mu$m depth. While capillaries (*) are poorly defined, spherical cells (probably alveolar macrophages) within the airspaces display moderate, rather uniformly distributed cytoplasmic fluorescence and are very distinct (**). Interstitial spaces ($\diamond$) and Type II alveolar epithelial cells, located in the corners of airspaces and containing intense, punctate fluorescence ( + ), are also apparent. (b) Higher magnification image of cuboidal Type II alveolar epithelial cells in the corners of airspaces ( + ) more distinctly shows the punctate fluorescence within their cytoplasm as well as their association with the surface of the airspace. In contrast, the adjacent cell containing moderate uniformly distributed cytoplasmic fluorescence (**), probably an alveolar macrophage, does not appear to be attached to the surface.

tural elements within the tissue. In lieu of physical sectioning, the intact organ was optically sectioned, thereby providing images of the native alveolar environment with minimal disruption of its internal milieu.

RB is an amphoteric dye which carries no net charge at physiological pH. The molecule has a high partition coefficient (octanol/buffer = 276

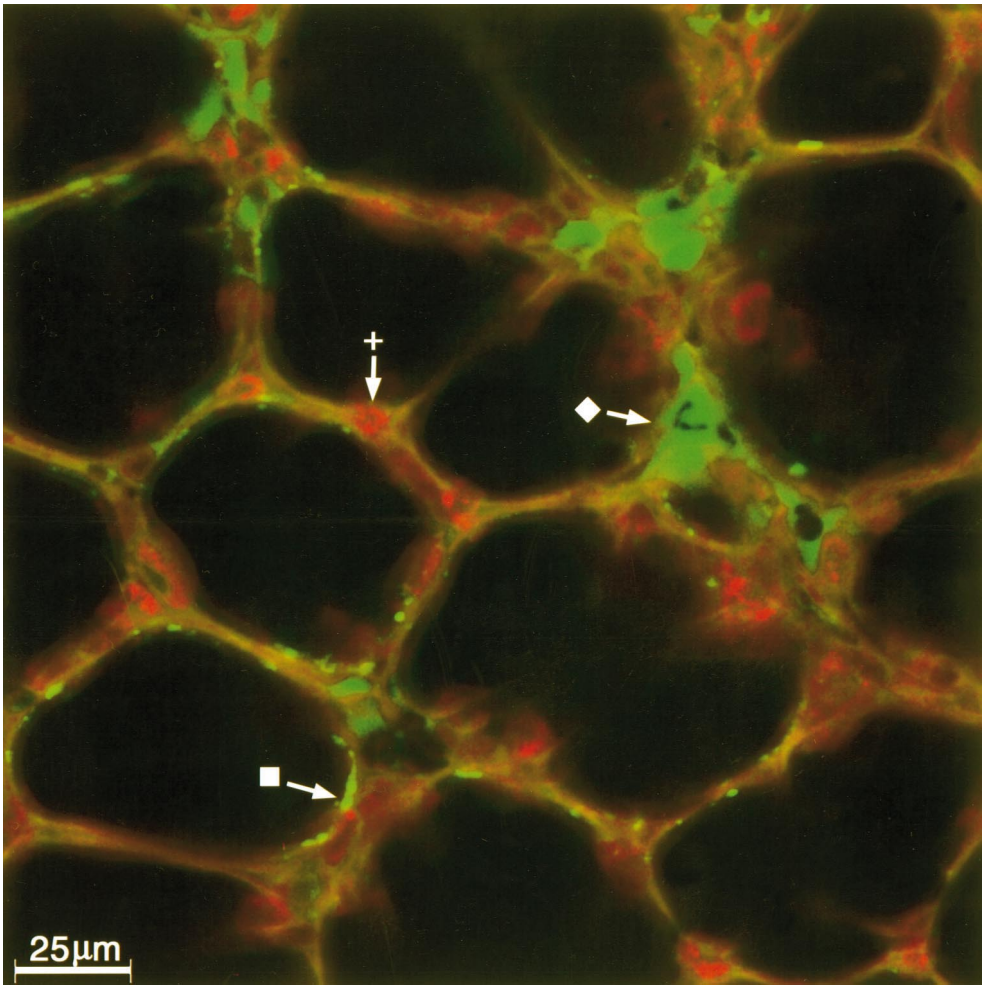



Fig. 4. Alveolar disposition of 6-CF in intact rat lung 1 h after intratracheal instillation in vivo. RB was added via intravascular perfusion ex vivo prior to imaging. This multi-colored overlay of fluorescein (488 nm excitation, green) and rhodamine (568 nm excitation, red) channels depicts the location of each fluorophore with respect to various regions of interest within the lung. Neither 6-CF nor RB appear to occupy the airspaces after 1 h. Instead the majority of the 6-CF remaining in the tissue has localized within interstitial spaces (◇). RB has intensely illuminated cuboidal Type II alveolar epithelial cells in the corners of the alveoli (+) which do not appear to contain 6-CF within their cytoplasm. Intense localized areas of 6-CF fluorescence are also evident along alveolar epithelial surfaces (□).

(Araie and Maurice, 1987) and would, therefore, be expected to readily partition into such lipophilic regions as the lamellar bodies of Type II cells and the lipid bilayers of cell membranes. It is considered to be non-toxic to cells and should not, at the concentrations used in the present study, have significantly affected the viability of the lung tissue (Guss et al., 1984).

6-CF, a mono-carboxylated fluorescein, is primarily a dianion at the pH near the alveolar surface (pH $\approx$ 6.9; Valeyre et al., 1991). It has an octanol/buffer partition coefficient of 0.0008 at pH 7.0, sufficiently low to render its partitioning into cells and transport by transcellular diffusional pathways unlikely (Grimes et al., 1982). Previous studies have demonstrated its paracellular diffusion across several epithelial surfaces including the cornea, a transepithelial pathway that

seems reasonable given the relatively small size and negative charge of the molecule (Araie and Maurice, 1987; Grimes, 1988).

When perfused into the pulmonary vasculature ex vivo, RB quickly rendered the capillary network fluorescent and appeared to be taken up by tissue macrophages. Cuboidal epithelial cells in the corners of alveoli displayed an intense pattern of punctate fluorescence within their cytoplasm which seemed to approximate in size and character the lamellar bodies that are a distinctive feature of Type II alveolar epithelial cells (Mason et al., 1982; Williams, 1987). One might expect such lamellar bodies to possess a strong affinity of RB since they contain phospholipid-containing surfactant stored in lamellar form (Ryan et al., 1975; Kalina and Socher, 1990). Thus a hydrophilic, fluorescent drug distributing into the aqueous 'hypophase' subtending the surfactant monolayer at the alveolar epithelial surface, might be taken up by Type II cells during the recycling of surfactant, thereby entering the interstices of lamellar bodies and rendering those intracellular elements fluorescent.

The ability of RB to clearly identify the location of Type II cells with alveoli may prove especially useful in studies of absorption pathways for macromolecules. Type II cell apposition to two adjoining cells at tricellular junctions is thought to be characterized by relatively large pores ( $\sim 27$ nm) through which dissolved solutes might move from airspace to interstitium (Walker et al., 1985). Thus fluorescent labeling experiments, involving simultaneous use of RB to enhance the image of Type II cells as well as various endo- and epithelial barriers along with a fluorescent drug molecule whose transport across alveolar epithelia is being investigated, may provide important information on pulmonary absorption pathways.

In contrast, when RB was intratracheally introduced into the airspaces it quickly and preferentially illuminated alveolar macrophages and alveolar epithelial surfaces. Narrow streaks of RB fluorescence observed at the periphery of alveoli following administration via this route could represent residual instillate, surfactant-associated RB, or RB involvement with the cell and basement membranes of Type I alveolar epithelial cells which are squamous and only about 0.1 $\mu$m thick (Hollinger, 1985). Since Type I cell thickness approximates the lower limit of resolution of CLSFM under the present conditions of use, the technique would not be expected to distinguish probe disposition among apical, basolateral and basement membrane elements. It should also be noted that since intratracheally-administered solutions of RB have the potential to disrupt the native air–tissue interface and its associated surfactant monolayer, it is probably preferable to administer RB via vascular perfusion for studies involving the transport of fluorescent drugs across the air–epithelial interface.

With the aid of computer-based 3-dimensional reconstruction software (e.g. VoxelView® Version 2.5, Vital Images, Fairfield, IA), vertical stacks of sequential 2-dimensional images such as those obtained in the present study may also be used to create 3-dimensional reconstructions of the interior of the lung. Images of this sort were previously attainable only by physical sectioning of the tissue. Such a procedure might prove useful in establishing more precisely the location of drugs relative to specific volume elements within their native pulmonary environment.

Despite the growing appreciation and importance of the fact that the systemic bioavailability of therapeutic peptides and proteins from the lung is remarkably high, the pathways involved in their absorption remain poorly understood. For example, it is still largely a mystery how insulin, human growth hormone and other molecules having molecular diameters in excess of 2–3 nm transit so rapidly from the airspaces into the systemic circulation (Niven et al., 1993; Patton, 1996). The technique described above may help relate structural features of the lung to possible routes of pulmonary absorption across epithelial surfaces, thereby furthering our understanding of this rather elusive process.

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