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Applications of Microscopy to Genetic Therapy of Cystic Fibrosis and Other Human Diseases

Thomas O. Moninger, Randy A. Nessler, and Kenneth C. Moore

Summary

Gene therapy has become an extremely important and active field of biomedical research. Microscopy is an integral component of this effort. This chapter presents an overview of imaging techniques used in our facility in support of cystic fibrosis gene therapy research. Instrumentation used in these studies includes light and confocal microscopy, transmission electron microscopy, and scanning electron microscopy. Techniques outlined include negative staining, cryo-electron microscopy, three-dimentional reconstruction, enzyme cytochemistry, immunocytochemistry, and fluorescence imaging.

Key Words: Gene therapy; gene transfer vectors; light microscopy; confocal microscopy; electron microscopy; cystic fibrosis.

1. Introduction

Over the past 15 yr researchers at the University of Iowa have been working to identify the specific gene and cell structure that is compromised in cystic fibrosis (CF) patients. Significant effort has been expended to identify effective viral and other vectors for transfer of the normal gene construct. Light and electron microscopy are essential tools for developing strategies for genetic therapy in the treatment of disease. Negative staining and cryo-electron microscopy are critical in understanding the structure of native and hybrid vectors. Enzyme cytochemistry is used for the detection of markers that aid in determining the efficiency of gene transfer to cells. Immunocytochemistry and fluorescent proteins are employed to identify gene products, locate them within tissues and cells, and track transfected cells over time. Light, confocal, and electron microscopy are used to evaluate pathogenesis, inflammation, and cellular responses to gene transfer. This chapter will highlight some of these techniques as they have been applied in our facility to gene therapy research.

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2. Materials

The following is a partial list of microscopy-related vendors:

2.1. Transmission Electron Microscope Vendors

1. FEI Inc., Hillsboro, OR 97124, USA; (503) 640-7500.
2. Hitachi High Technologies America, San Jose, CA 95134, USA; (800) 548-9001, (408)432-0520.
3. JEOL USA Inc., Peabody, MA 01960, USA; (508) 535-5900.
4. LEO Electron Microscopy Inc., Thornwood, NY 10594, USA; (800) 356-1090.

2.2. Scanning Electron Microscope Vendors

1. FEI Inc., Hillsboro, OR 97124, USA; (503) 640-7500.
2. Hitachi High Technologies America, San Jose, CA 95134, USA; (800) 548-9001, (408)432-0520.
3. JEOL USA Inc., Peabody, MA 01960 USA; (508) 535-5900.
4. LEO Electron Microscopy Inc., Thornwood, NY 10594, USA; (800) 356-1090.
5. RJ Lee Instruments Inc., Trafford PA 15085, USA; (724) 744-0100.

2.3. Research-Grade Light Microscope Vendors

1. Leica Microsystems Inc., Bannockburn IL 60015, USA; (847) 405-0123.
2. Nikon Inc., Melville, NY, USA; (516) 547-8500.
3. Olympus America Inc., Melville, NY, 11747, USA; (516) 844-5039.
4. Carl Zeiss MicroImaging Inc., Thornwood, NY 10594, USA; (914) 747-1800.

2.4. Spot-Scanning Confocal Microscope Vendors

1. Leica Microsystems Inc., Exton, PA 19341, USA; (610) 321-0460.
2. Nikon Inc., Melville, NY, USA; (516) 547-8500.
3. Olympus America Inc., Melville, NY, 11747, USA; (516) 844-5039.
4. Carl Zeiss MicroImaging Inc., Thornwood, NY 10594, USA; (914) 747-1800.

2.5. Multiphoton Microscope Vendors

1. Leica Microsystems Inc., Exton, PA 19341, USA; (610) 321-0460.
2. Carl Zeiss MicroImaging Inc., Thornwood, NY 10594, USA; (914) 747-1800.

2.6. Microscopy Chemicals and Supplies Vendors

1. Electron Microscopy Sciences, Fort Washington, PA 19034, USA; (215) 646-1566.
2. Energy Beam Sciences Inc., Agawam, MA 01001, USA; (413) 786-9322.
3. Polysciences Inc., Warrington, PA 18976, USA; (215) 343-6484.
4. Ted Pella Inc., Redding, CA 96049, USA; (530) 243-2200.
5. Structure Probe Inc., West Chester, PA 19381, USA; (610) 436-5400.

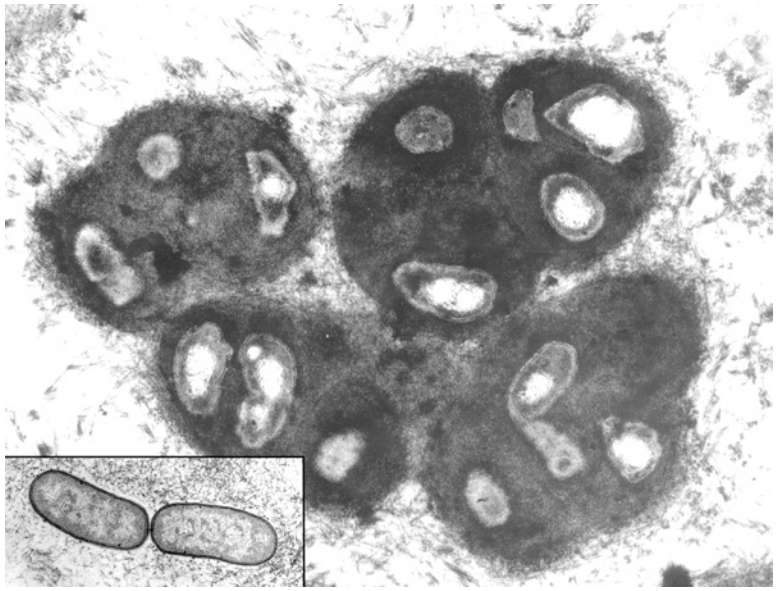

Fig. 1. Transmission electron microscope image of *Pseudomonas* bacteria in sputum from a CF patient. The main image was fixed using OsO_4 dissolved in perfluorocarbon (PFC), dehydrated in 100% ethanol and embedded in Eponate 12 resin, avoiding all aqueous solutions in order to preserve the biofilm matrix surrounding the cells. This technique also works well in preserving the mucus layer on epithelia. The inset shows the same sample processed using a conventional aqueous method, with fixation in gluteraldehyde and OsO_4. Note the loss of the matrix integrity compared to the PFC-processed sample. Inset bacteria are 0.5 μm in diameter.

3. Methods

For much of the past two decades, we have been involved in research to identify the specific gene, gene product, and cell structure that is compromised in CF patients and to develop an efficacious gene therapy treatment. Significant effort has been expended to identify effective viral and other vectors for transfer of the normal gene construct into defective airway cells. In addition to CF, we are also involved with investigations for genetic therapy of hemophilia, hypertension, neurological problems, cancer, and skin disorders. Light and electron microscopy has been instrumental to the advancement of these projects.

Cystic fibrosis is the most common lethal genetic disease in Caucasians, with a carrier rate of 5% in the population. It is caused by mutations in a cell membrane chloride channel called the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) *(2)*. This gene is expressed in several epithelia, including airway, sweat glands, and the pancreas. Pathology associated with the lung is the major reason for

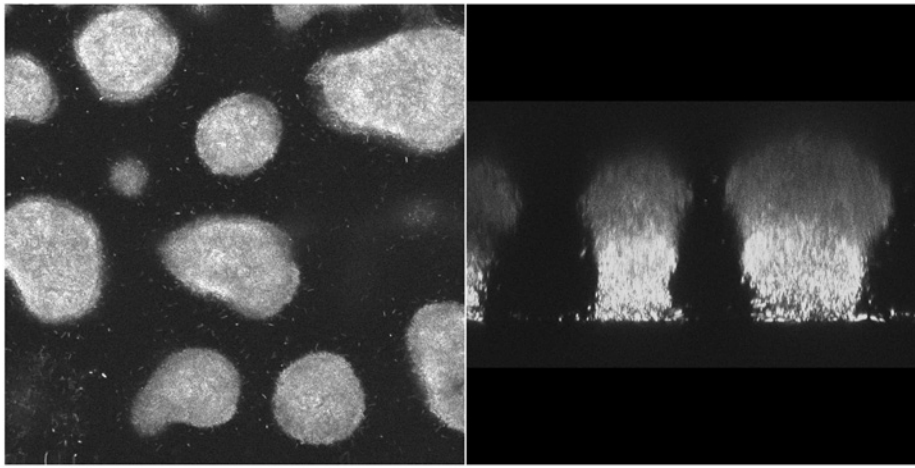

Fig. 2. Confocal images of a green fluorescent protein (GFP) expressing *P. aeruginosa* PAO1 bacterial biofilm grown in a chamber designed to facilitate observation in a confocal microscope. The image on the left is an X-Y optical section through the middle of a field of biofilm columns. The image on the right is an X-Z vertical section through the biofilm columns. The columns are approx 200 µm in height.

the morbidity caused by this disease. These defective ion channels result in high concentrations of chloride in the surface airway liquid, which becomes thick and difficult to expectorate. Consequently, naturally occurring antimicrobial agents are inactivated resulting in high concentrations of bacteria, most commonly *Pseudomonas aeruginosa* and *Staphylococcus aureus* ***(3)*** (*see* **Fig. 1**). Once one of these bacteria has colonized a CF patient's lung, that clone can be detected in sputum for up to a decade ***(4)***. It is believed that these bacteria are able to persist because of the formation of biofilms, that are extremely resistant to antimicrobial treatment (**Fig. 2; *5***). This infection and the resulting inflammation cause chronic damage and scaring to the lung ultimately resulting in loss of function and death. In addition to lung airway epithelia being affected, the pancreas, intestine, sweat glands, and other parts of the body can be compromised, although with less severe consequences. Treatment of CF patients involves antibiotics, enzyme supplements, dietary intervention, chest massage, and even organ transplants.

The gene for CFTR was isolated in 1989 ***(6)***. cDNA was administered to human nasal epithelial, with the result that the CF defect was temporarily corrected as measured by cAMP-stimulated, amiloride-sensitive voltage ***(1)***.

Since 1993, various vector systems have been investigated to more naturally introduce the normal CF gene into human airway epithelial cells. Most of these investigations were carried out using a novel primary human airway cell culture

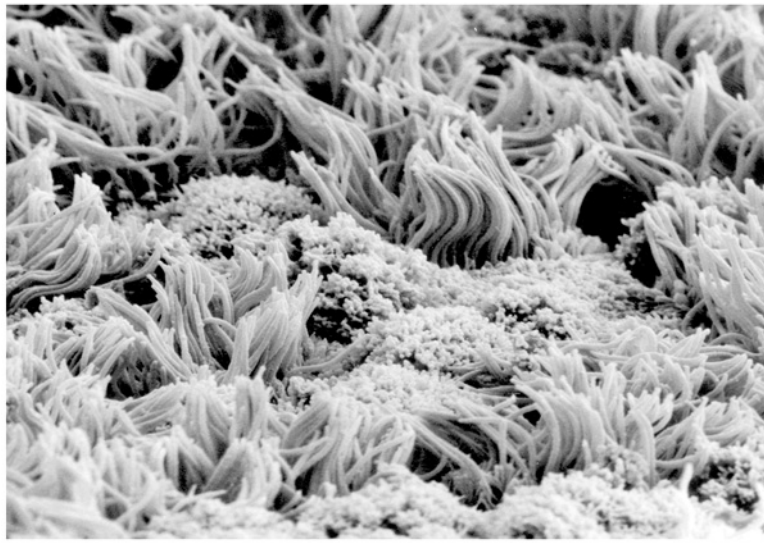

Fig. 3. Scanning electron microscope image of the surface of a 3-wk-old primary human airway culture. The sample was prepared using conventional aqueous methods, thereby removing the mucus covering the culture and allowing the cell surface to be viewed. Note the ciliated cells and the group of microvilli-covered goblet cells in the center of the field. The cilia extend 10 µm from the surface of the cells.

model ***(7)***. In this model airway, cells from either CF or non-CF donor lungs are cultured on filter membrane inserts and grown with an air interface on the apical surface. Over the course of 2 wk, the cells differentiate into pseudostratified columnar epithelia containing basal, goblet, and ciliated cells covered by a mucus blanket (*see* **Figs. 3** and **4**). Under sterile conditions, these cultures can be maintained for months.

Initially, CFTR cDNA was complexed with different cationic lipids and polymers (*see* **Figs. 5** and **6**). This combination was effective in stimulating uptake of the DNA and temporarily corrected the CF defect ***(8–11)***. However, effective concentrations of these lipids and polymers tended to be toxic to the cells and the correction was short-lived.

Viral vectors were also considered as a means of transfecting cells. Initial experiments involved adenovirus (*see* **Fig. 7**), which is about 80 nm in diameter, has a double-stranded DNA genome of 36,000 bases, and is able to infect human airway cells. In 1993 an adenovirus expressing CFTR transiently corrected the chloride defect in the nasal epithelium of CF patients ***(12)***. Unfortunately, adenovirus infects many cell types at very low levels and is prone to eliciting an immune response. Protocols were developed that involved mixing adenovirus with lipids (cholesterol and phosphotidyl inositol based),

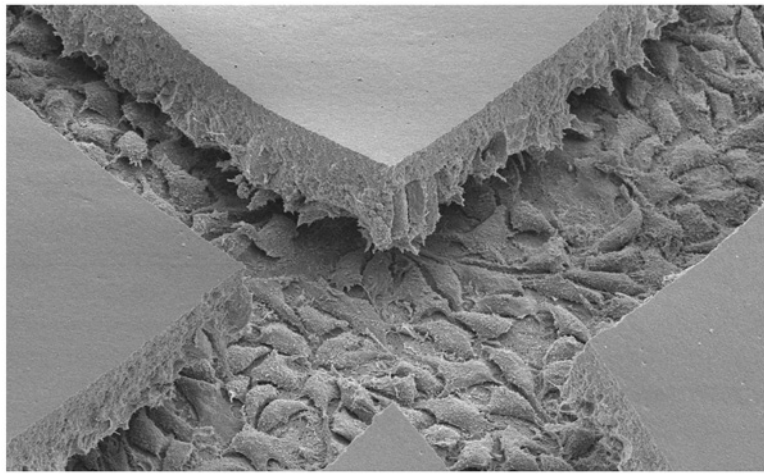

Fig. 4. Scanning electron microscope image of a 3-wk-old primary human airway culture. In contrast to **Fig. 3**, this sample was prepared using the nonaqueous OsO_4/perfluorocarbon method, thereby retaining the thick mucus blanket on the surface of the culture. Note that in this fractured area, the basal cells remained attached to the filter while the goblet and ciliated cells remained with the mucus layer. Total thickness of the cell and mucus layers is 45 μm.

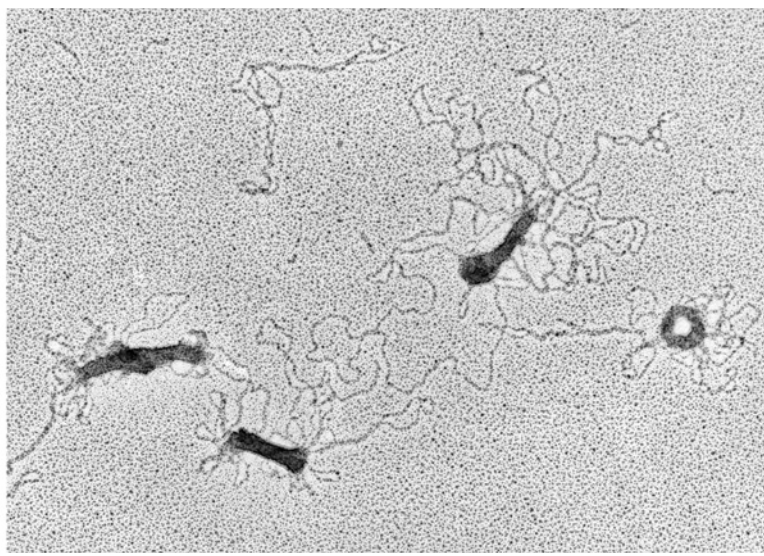

Fig. 5. Combination 1% uranyl acetate negative stain and platinum rotary shadowing of DNA/Poly-L-lysine transfection complexes displaying the typical rod and torus morphologies as well as uncomplexed DNA. The torus on the right is approx 40 nm in diameter.

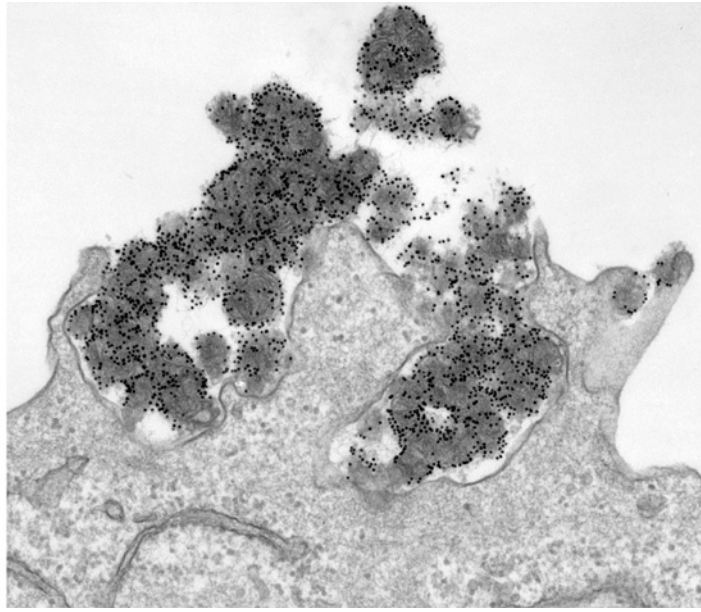

Fig. 6. Transmission electron microscope image of DNA/cationic lipid complexes being engulfed by a COS-7 cell. The DNA has been labeled with 10 nm of gold to facilitate visualization of the fate of the complexes.

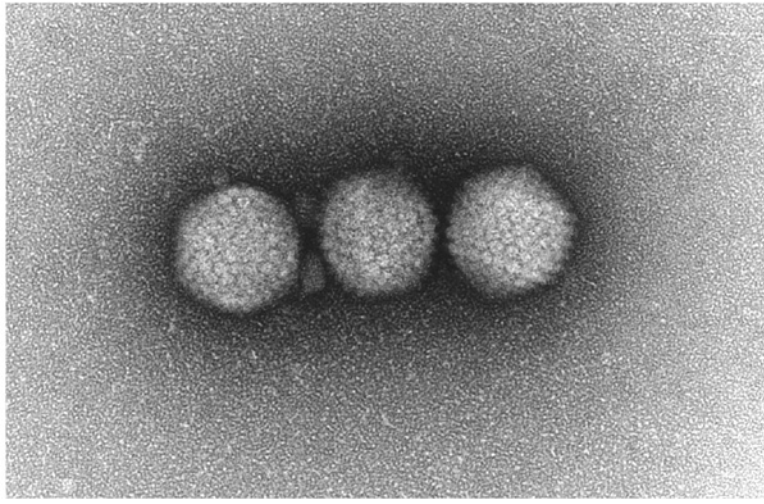

Fig. 7. Negative stain of adenovirus using 1% ammonium molybdate. The particles are 80 nm in diameter.

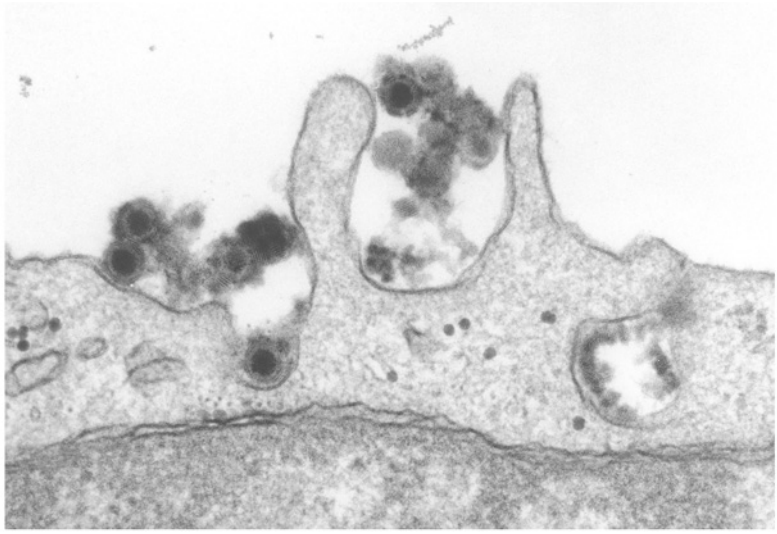

Fig. 8. Transmission electron microscope image of adenovirus/lipid complexes being engulfed by a COS-1 cell.

polymers (poly-L-lysine, dendrimers, polyethylene glycol), or calcium phosphate. These techniques resulted in significant increases in the amounts of virus that were taken into the airway epithelial cells. Analysis indicated that the majority of the viral complexes were taken in by bulk phagocytosis as opposed to a receptor-mediated mechanism (*see* **Fig. 8**). This resulted in significant viral particles being destroyed by lytic activity. Also, as with naked DNA complexes, at effective concentrations the lipids and polymers tended to be toxic. In addition, because adenovirus does not integrate into the cells genome, any correction of the CF defect was transient, necessitating subsequent reapplications. In human subjects, this results in an ever-increasing immune reaction to the adenovirus.

The lentiviruses have several advantages for gene therapy as compared to adenovirus. Prime among these attributes is the ability to transfect differentiated and stem cells. This is a critical advantage because the outer layer in the airway epithelia is made up of differentiated ciliated and mucus-secreting cells that are destined to die and be replaced from offspring of the stem or progenitor cells. Feline immunodeficiency virus (FIV) is 20 nm in size, has a capacity of 10,000 bases, and is able to transfect both differentiated and stem cells. Little if any immunoreaction occurs to FIV. Transfection of airway epithelia with FIV results in a permanent correction of the gene defect. Unfortunately, the apical surface of airway epithelium is naturally very resistant to infection by this virus. Treatment of the airway epithelium with a hypotonic buffer containing EGTA

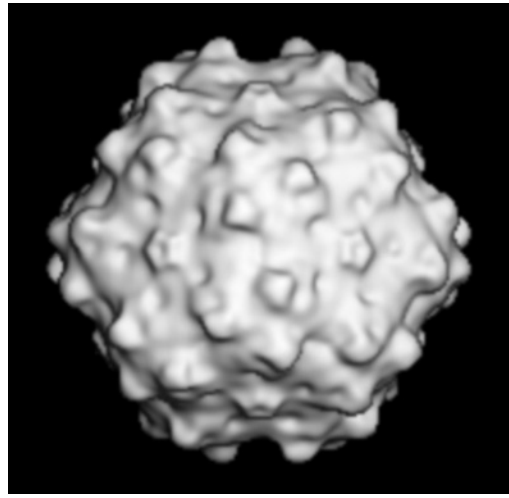

Fig. 9. Adeno-associated virus serotype 5 (AAV5). The 16-Å resolution three-dimentional reconstruction of AAV5 viewed along the twofold axis calculated from cryo-transmission electron microscope images of vitreous-ice-embedded samples.

transiently opens the tight junctions between the cells and allows FIV to transfect the basally occurring stem cells ***(13)***. Alteration of the apical surface of the airway epithelial cells by enzymatic treatment has not resulted in increased uptake of virus into the differentiated cells.

Another promising vector is adeno-associated virus (AAV). AAV is a member of the Parvoviridae family, genus *Dependovirus*, and is 20 nm in size (*see* **Fig. 9**). It has a single-stranded DNA genome and has been shown to be effective in muscle, brain, retina, and liver. It also is able to transduce nondividing cells. Significantly, there is no known pathology associated with the various serotypes of AAV, minimizing the deleterious effects of inflammation. One shortcoming of AAV as a gene therapy vector is the limited length of DNA that is about 4900 bp. Full-length CFTR cDNA (including the promoter, the 5′ untranslated region, the ploy-A-tail, and internal terminal repeats) is 5435 bp. One way past this roadblock is to determine what parts of the cDNA can be deleted and still allow production of a functional channel ***(14)***. Another problem is that recombinant AAV can be difficult to prepare and purify.

Current efforts also involve evaluation of pseudotype virus that incorporates characteristics of two or more types of virus. As a result, vectors can be tailor-made to receptors on specific cells. Proposed experiments involve FIV incorporating glycoproteins from Ross River virus. Alternative approaches involve chemically modifying cell surface receptors, isolating tissue-specific stem cells to transfect and reintroduce into the host, as well as other nongenetic

therapies. It should be emphasized that each genetic disease will most likely involve specific and unique approaches to treatment. As in the example of CF, the airway epithelium is naturally resistant to transfection by virus and other vectors. Conversely, the transfection of hemopoetic cells does not present such a resistant barrier.

Because of the nature of this overview, specific techniques and protocols have not been presented. The reader is invited to refer to other chapters in this manual and to the references at the end of the chapter for specific protocols. Inquiries to the authors are also welcome.

4. Note

A wealth of related information is available on the internet. Microscopy Society of America, http://www.msa.microscopy.com/; Microscopy Vendors database, http://www.kaker.com/mvd/vendors.html; and Molecular Expressions Virtual Microscopy, http://micro.magnet.fsu.edu/primer/virtual/virtual.html

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