invited review

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Multimodal optical imaging

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[1]*National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709;* [2]*Department of Environmental and Occupational Health, University of Pittsburgh Graduate School of Public Health, Pittsburgh 15260; and* [3]*Department of Cell Biology and Physiology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261*

Lawler, Cindy, William A. Suk, Bruce R. Pitt, Claudette M. St. Croix, and Simon C. Watkins. Multimodal optical imaging. *Am J Physiol Lung Cell Mol Physiol* 285: L269–L280, 2003; 10.1152/ajplung.00424.2002.—The recent resurgence of interest in the use of intravital microscopy in lung research is a manifestation of extraordinary progress in visual imaging and optical microscopy. This review evaluates the tools and instrumentation available for a number of imaging modalities, with particular attention to recent technological advances, and addresses recent progress in use of optical imaging techniques in basic pulmonary research.[1] Limitations of existing methods and anticipated future developments are also identified. Although there have also been major advances made in the use of magnetic resonance imaging, positron emission tomography, and X-ray and computed tomography to image intact lungs and while these technologies have been instrumental in advancing the diagnosis and treatment of patients, the purpose of this review is to outline developing optical methods that can be evaluated for use in basic research in pulmonary biology.

fluorescent probes; green fluorescent protein; fluorescence resonance energy transfer; electron paramagnetic resonance; electron microscopy

SIGNIFICANT PROGRESS in the visualization of biological processes has been achieved within the past 5 yr. Dramatic advances in imaging technologies have shaped our understanding of a variety of spatially regulated intracellular events, including mitogenesis, differentiation, receptor trafficking, apoptosis, and DNA damage recognition. High-performance computers and novel image analysis tools have been harnessed to provide insights into the three-dimensional structure of complex cellular organelles. Nanoscale biosensors have been developed that provide optical readout of various aspects of cellular physiological state. Development and refinement of technologies to visualize events in three-dimensional living tissues and organisms is well under way. Indeed, such technological advances have underscored a recent series of applications using intravital microscopy to assess microvascular events in the proximal (23) and distal (19, 43) lungs of intact rodents.

Successful application requires integration of the imaging platform, appropriate reporters, and detectors. A recent workshop at National Institutes of Environmental Health Science[1] considered advances in all three aspects of such integration. This review was inspired by discussions held at the workshop on optical imaging including light and electron microscopy, nanoscale biosensors, and imaging with electron paramagnetic resonance spectroscopy (EPR). The first section of this review will consider instrumentation for light microscopy (platforms, illumination system, detectors, and computer-aided data collection) and developments in fluorochromes. The second section will consider application of these developments for drug discovery and high-throughput screening and intravital microscopy (including advances in multiphoton microscopy). We will then consider other advances in optical imaging, including nanoscale optical biosensors, EPR imaging, and electron microscopy, including tomography and fluorescence photooxidation. In the last section, applications in pulmonary biology will be reviewed.

INSTRUMENTATION FOR LIGHT MICROSCOPY: CURRENT STATUS AND FUTURE PROSPECTS

Imaging of biological specimens has traditionally relied on light microscopy. This favored status is likely to continue as improvements in illumination and detection systems, optical and digital deblurring methods, and image processing tools provide enhanced ca-

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[1]This review was inspired by the proceedings of a workshop on emerging visualization methodologies in toxicological and pulmonary science held at the National Institute of Environmental Health Sciences in Research Triangle Park, NC, on July 14, 2000.

pabilities. The central technique in this field is fluorescence imaging, discussed briefly as follows.

The principle of fluorescence microscopy depends on the inherent property of a fluorophore to emit a photon of a known energy (or wavelength) after appropriate excitation. This phenomenon is a quantum event and depends on excitation of the fluorophore by quanta of specific energy that move electrons, generally in double bonds or pi orbitals, from the ground state to a higher energy singlet state. Different electrons have different rotational and vibrational energies. Moving to singlet state demands change to an equivalent vibrational or rotational energy at a higher electrical state. If the ground state changes, then the energy (of the photon) to get to a singlet state may also change. The energy required depends on the electrical, rotational, and vibrational energy needed to move electrons between specific states. A simplified example of a theoretical excitation is shown in Fig. 1 as a Jablonski diagram. As can be seen, energy is lost, generally through thermal decay, after which a lower energy photon is emitted. The wavelength difference between the excitation and emission light is known as the Stokes shift. This is the essential principle of all fluorescence methods. The following sections describe changes in the design of microscopes over the last few years as well define the tremendous increase in the utility of fluorescent probes.

Microscope Platforms and Lenses

All current microscope platforms are optimized to allow high-quality images to be collected from tissues and cells over extended periods of time. Thus little substantive change is expected in conventional microscope platform design. The repertoire of lenses currently available is adapted and optimized for illuminating wavelength and chromatic and spherical aberration as well as maximizing light throughput and collection efficiency. However, considerable opportunities for light imaging exist if one thinks outside the rubric of a conventional microscope. For example, if one were to build a microscope that could produce focused images yet remain handheld, one would be able to image structures that are not immediately amenable to conventional microscopy (such as intravital imaging in large animals).

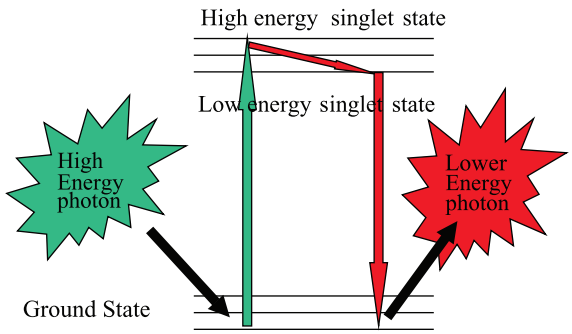

Fig. 1. A highly simplified Jablonski diagram.

Illumination Systems

To use a fluorescence microscope, it is essential to excite with one wavelength and collect another spectrally separate signal. This depends on a constant illumination and on filters to separate the excitation and emitted signal. A conventional fluorescence imaging system is shown in Fig. 2. The most commonly used illumination system employs an arc lamp illumination system with changing glass filters. There have been considerable advances in the spectral reliability of glass filters and mirrors over the last few years as manufacturers move from simple glass to dielectric coatings to filter light. Although lasers offer a polarized, high-intensity source at a specific wavelength, their use is restricted primarily to confocal microscopy. To select wavelengths in fluorescence microscopy (in order of increasing speed) the primary barrier filter can be changed manually, using wheels holding several filters, or using a monochromator. Intensity can be modulated using acoustooptical filters. A similar situation exists for the barrier filters, and wavelengths may be selected using the same optical systems. Currently, the speed-limiting component of the microscope is changing the dichroic filter. Since all filters have to be perfectly aligned, changes using stepping motor-driven changers generally take at least 200 ms. However, specialized developments in the mechanical handling of dichroics will continue to improve the speed of these systems.

Detection Systems

Detectors have advanced from film through scan tubes (such as the Uvicon tube) to charge-coupled devices (CCD). Cooled or uncooled CCD cameras exhibit high resolution, large dynamic range, and low noise. Lowered costs for these systems have been accompanied by marked improvements in speed and sensitivity [in general, a cooled CCD purchased today will have 10× the speed and twice the quantum efficiency (QE) of a camera available 7 yr ago, see Fig. 3]. Furthermore, the spectral cross section for cameras now stretches far into the infrared range with high QE and, accordingly, the flexibility of the standard cooled CCD is impressive. However, there is still room for devices such as intensified CCD (ICCD) cameras that may offer advantages in speed over standard cooled CCD. ICCDs, however, are still only eight-bit devices and thus have a limited dynamic range, and they are still quite expensive, since one must purchase both the intensifier and a standard CCD device. It is expected that, as the speed and flexibility of cooled CCDs continue to grow over the next few years, ICCDs will fall out of favor. On the other hand, small (and low-cost) complementary metal-oxide image sensors (CMOS) are now available that display high resolution and are quite versatile. Although current models have a relatively poor signal-to-noise ratio, there is continual and significant improvement in this area, and it is expected that these less expensive CMOS cameras will become increasingly useful over the next few years. Point source

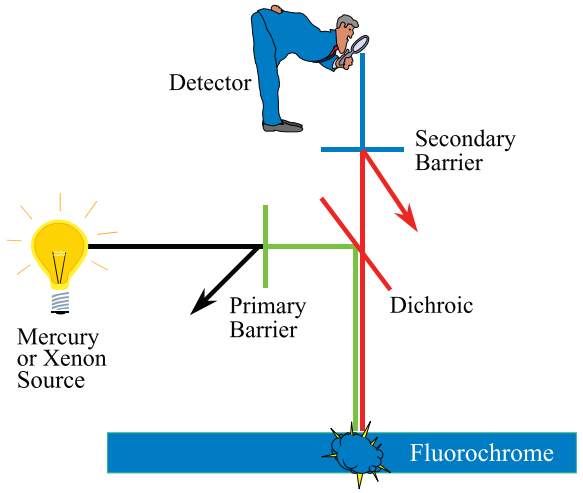

Fig. 2. Principle of immunofluorescence.

detectors are central to confocal microscopy and use photomultiplier tubes. These devices are very reliable with a high signal-to-noise ratio and, over the last few years, have achieved improved QEs (40%). Perhaps most excitingly in this area, avalanche photodiodes have become available recently that exhibit very high QE (85%). Finally, a number of hybrid systems are now available, including electron-bombarded CCD. These hybrids provide high quality imaging but limited gain and a relatively low QE. Over the next few years, it is expected that video and tube systems will no longer have a place in modern imaging as rapid advances in speed, bandwidth, and signal-to-noise ratio occur in solid state digital devices.

Computer-Aided Methods

While growth in imaging technologies depends greatly on improvements in microscope, filter, and detector design and advances in fluorescent proteins, perhaps the most significant contribution to the imaging field is the advent of low-cost, high-power computers. Several commonly used imaging methods are practically impossible without computers (for example, confocal microscopy) to scan the specimen, collect point source data, and integrate them into a two- or three-dimensional data set. Currently, data sets collected by microscopes controlled entirely by computers may now extend into the gigabyte range and include temporal, spatial, and physical attributes for multiple molecular species within cells throughout tissues or in culture. Rapid improvements in image processing have made specialized boards obsolete so that most calculations are now accomplished in the host computer and thus there is a growing need for uniformity in software and handling of large data sets. For example, optical deblurring or deconvolution is a commonly used alternative to optical sectioning or confocal imaging. Its use is limited by its extraordinary computer requirements (sometimes taking days to treat data sets using a high-power graphics workstation).

FLUOROCHROMES

Although the mechanics of fluorochrome function have been described above, the importance and relevance of the rapid development of modern fluorescent proteins and dyes cannot be understated. The following describes probable changes and development in this area over the next few years.

Fluorescence Labeling With Traditional Reagents

Traditionally, a limited repertoire of molecules, such as fluorescein or rhodamine, was used by investigators to report the localization of molecules using antibodies in a fluorescent microscope. Currently, however, a diverse collection of fluorescent labeling reagents is available. These reagents can be covalently bound to proteins and other biomolecules to allow fluorescence imaging of pertinent cellular molecules. The principal spectral range for these dyes is between 500 and 670 nm, thereby staying within the visible range of the human eye. However, as detectors become increasingly useable in the near infrared region, we are now starting to use dyes that emit in this range (and that cannot be seen by the naked eye). The fundamental flexibility of these dyes is that they allow many fluorochromes to be used at the same time, e.g., cells may be labeled with blue, green, orange, red, and near infrared dyes, with ease. There are also noncovalent probes available for fluorescence imaging. These include DNA intercalators or lipid probes and, in each case, there are generally several different dyes with different excitation and emission spectra available for each probe. Recently, triple-color immunofluorescent imaging of lung sections was useful in determining the effectiveness and specificity of lipid-mediated delivery of oligonucleotide to pulmonary endothelium (Fig. 4, Ref. 49).

A further considerable and ongoing development is in the area of physiological indicators. In the past,

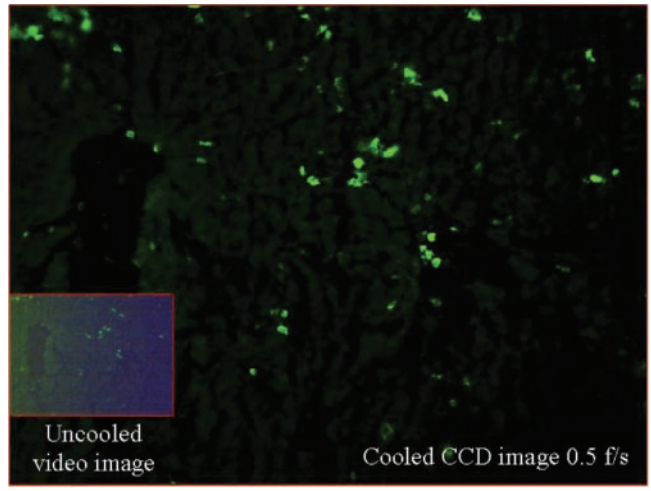

Fig. 3. Decreased noise and increased sensitivity of slow scan, cooled charge-coupled device (CCD) image compared with video rate image. Rat liver was labeled with antibody to inducible nitric oxide synthase with Kuppfer cells staining positive. The large image was obtained with a photometrics cooled CCD. *Inset*: same field obtained with a Sony pal video camera (30 frames/s).

this was limited to a few reporters, such as fura (calcium) or fluoroscein (pH). Currently, this latter category includes fluorescent indicators suitable for measurement of pH, calcium, and redox status. Ionic dyes that exhibit differential distribution across charged membranes can function as fluorescent indicators of membrane potential. Dyes are available that report specific cellular compartments, such as mitochondria, Golgi apparatus, or lysosomes. Finally, fluorophores have been incorporated into many enzyme substrates to allow monitoring of enzyme activity. There are now innumerable fluorescent probes suitable for use in biomedical research. Many of these fluorophores have been developed and marketed by Molecular Probes, and they publish an extensive list of products and applications on their website (www.probes.com).

It is important to recognize that sensitivity or specificity of fluorescent indicators may be reduced by a variety of factors. Aggregation of proteins can limit the access of probes and/or serve to quench fluorescence. Problems of photobleaching, autofluorescence, scattering, and absorption are encountered commonly. Cross talk between fluorophores can be problematic with the use of multiple probes. The potential perturbation of cellular function that may be associated with introduction of the fluorescent molecule into the biological system of interest is another potential drawback.

Genetically Encoded Optical Probes

Approaches based on green fluorescent protein. Genetically encoded probes are one of the most important developments in optical methods over the last few years. Most of these are based on green fluorescent protein (GFP) (39). GFP is a spontaneously fluorescent protein originally isolated from the Pacific jellyfish, aequorea victoria. The fluorophore in GFP is derived from three consecutive amino acids (aa): Ser-65, Tyr-66, and Gly-67. After synthesis of GFP, these three aa undergo autocatalytic cyclization and oxidation to form the functional fluorophore. The fluorophore contains a series of conjugated double bonds that result in the fluorescent properties of GFP.

Several aspects of the GFP molecule have made it particularly attractive for use in optical imaging. First, GFP consists of only 238 aa and can be fused to a variety of cellular proteins at either the COOH or NH_2 termini without affecting native function or localization. Second, GFP is highly stable. Therefore, in addition to being imaged in living cells, it can also be used in fixed or frozen cells and tissue. Finally, mutations within the three aa fluorophore have produced a variety of GFP variants with differing spectral characteristics, thus increasing the number of potential uses for GFP. For example, a double mutation of Phe-64 to Leu and Ser-65 to Thr yields enhanced GFP (EGFP), the most commonly used GFP variant. This variant exhibits a red-shifted excitation maximum from 395 nm (wild type) to 488 nm, allowing for the use of standard FITC optics, and it is more photostable and sixfold brighter than wild-type GFP.

GFP fusions have been used to measure translocation and activation of a huge number of proteins. The following describes some of the more current uses of the fluorescent proteins in biological research.

GFP-BASED BIOSENSORS OF CELLULAR STATE. Recent studies have demonstrated the utility of pH-sensitive GFP fusion proteins for elucidating events controlling mito-

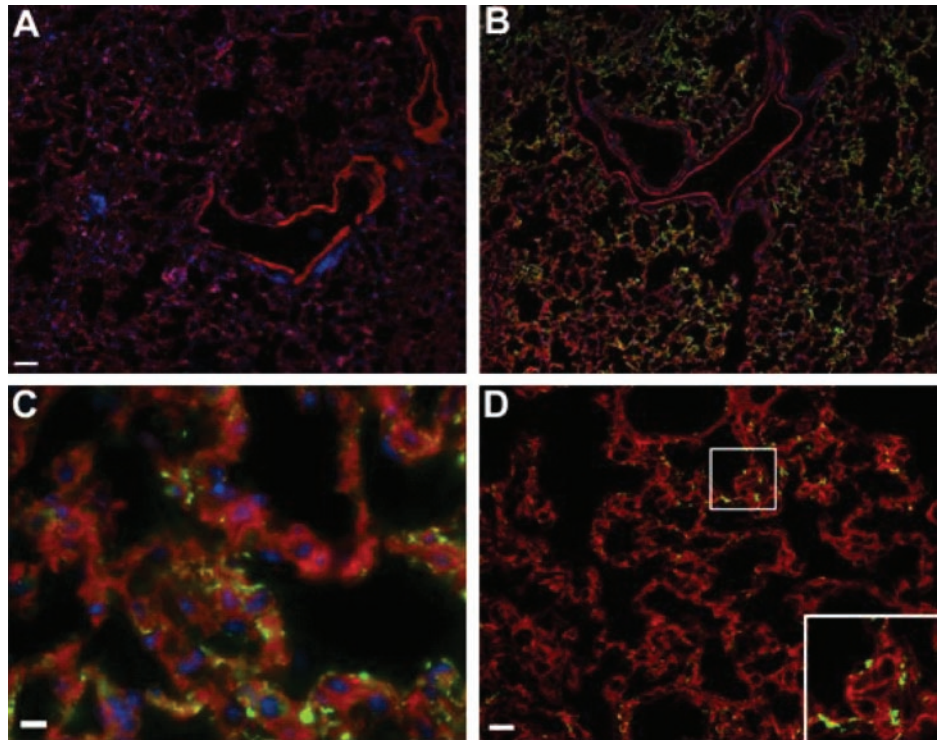

Fig. 4. A lipidic vector composed of 1,2-dioleoyl-3-trimethylammonium-propane liposomes and protamine effectively delivered antisense oligodeoxynucleotides (ODN) to the pulmonary endothelium. *A*: control lung from mouse that received unlabeled ODN. Nuclei were visualized with Hoescht (blue). Rhodamine-phallodian labeled the actin cytoskeleton (red). *B*, *C*, and *D*: images of lung sections from mice that received bromodeoxyuridine (BrdU)-labeled ODN detected with biotin-conjugated anti-BrdU and streptavidin Alexa 488 (Molecular Probes, Eugene, OR; green). Scale bars in *A*, *C*, and *D* are all 50 μm.

chondria-mediated apoptosis (26). The ability to monitor pH changes in subcellular compartments over time with a pH-GFP mitofusion protein revealed that mitochondrial alkalinization is mediated by the Bax death signal in both yeast and mammalian cells. Moreover, alkalinization of mitochondria was found to coincide with cytosolic acidification and to precede cytochrome *c* release, caspase activation, and loss of mitochondrial membrane potential. This pattern of results suggests that efflux of protons from mitochondria is an early critical event in mitochondrial-associated cell death.

GFP-FLUORESCENCE RESONANCE ENERGY TRANSFER MEASUREMENTS OF INTER- AND INTRAMOLECULAR INTERACTIONS. Fluorescence resonance energy transfer (FRET) can occur between two fluorophores (i.e., donor and acceptor pair) that have appropriate spectral properties and become closely apposed (10–40 Å). Several GFP variants are suitable as FRET candidates (e.g., cyan and yellow). The phenomenon of FRET occurs when an overlap in "donor" emission spectra and "acceptor" absorption spectra allows the transfer of excitation energy. This transfer results in a detectable alteration in fluorescent emission. The FRET effect decreases with the sixth power of the distance between donor and acceptor fluorophores. This relationship allows the use of FRET to measure physical interactions among proteins or among domains within a single protein. Two general strategies have evolved for GFP-FRET (34). The first involves the incorporation of GFP donor and acceptor fluorophores at opposite ends of a conformationally active protein. Alterations in protein conformation change the relative positions of the two fluorophores, and this is reflected in alterations in FRET. The second strategy involves the encoding of GFP donor and acceptor fluorophores on distinct proteins. In this case, FRET between the two fluorophores provides direct evidence for a physical interaction between the two proteins.

GFP-FRET methodologies were recently used to elucidate the role of metallothioneins (MT) in nitric oxide signaling (29, 36). Previous data had suggested that metal ion homeostasis is regulated in part by a linkage between MT and cellular redox status via the interaction of MT with the biologically important free radical nitric oxide (NO). Evidence for a close interaction between NO and MT was obtained by construction of a chimera reporter fusion (Fig. 5*A*) protein consisting of MT sandwiched between enhanced cyan fluorescent protein (ECFP) and enhanced yellow fluorescent protein (EYFP). The partial overlap of excitation and emission spectra of ECFP and EYFP allowed the use of FRET to monitor conformational alterations in the core MT protein. Expression of FRET-MT in live sheep pulmonary endothelial cells revealed that alterations in the FRET-MT signal, indicative of metal release, were produced by exposure to agents that elevate intracellular Ca^{2+} (Fig. 5*B*). Moreover, this effect was mediated by changes in NO production by endothelial nitric oxide synthase. GFP-FRET methodology has also been used to study the ligand-dependent association of nuclear hormone receptors and coactivators (24). These studies revealed a rapid agonist-induced increase in the interaction of nuclear receptors and coactivators. Moreover, results indicated that estrogen receptors, unlike retinoic acid receptors, are partially associated with coactivators even before agonist binding. Low-affinity estrogen antagonism could be reversed, whereas high-affinity agonism or antagonism was irreversible. Static and homomeric preassociations, as occur between Fas and Fas receptor, are detectable using photobleaching calibrations of FRET.

GFP-FRET CAMELEONS. A novel means for exploiting the properties of GFP fusion proteins has been described recently with the creation of GFP-based cameleons. The first cameleon created was a fusion protein consisting of two GFP variants, CFP and YFP, separated

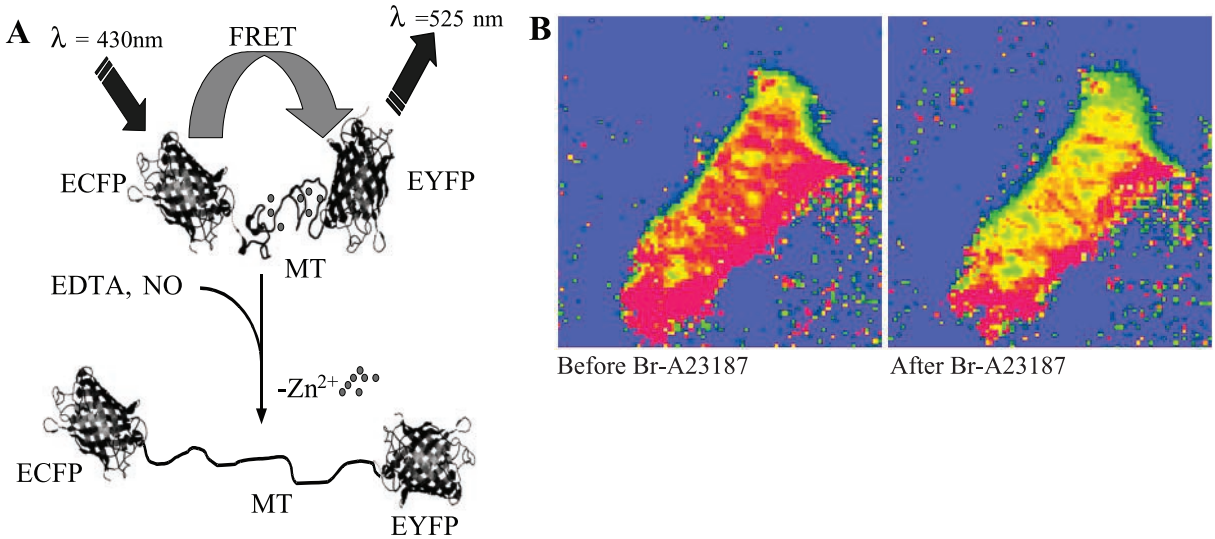

Fig. 5. *A*: fluorescence resonance energy transfer (FRET) reporter cDNA construct of human type IIa metallothionein flanked by enhanced cyan fluorescent protein (ECFP) and enhanced yellow fluorescent protein (EYFP). *B*: pseudocolored image of transfected pulmonary endothelial cells shows the decrease in the emission intensity ratio (535 nm/480 nm) after addition of the calcium ionophore Br-A23187 (29). NO, nitric oxide; MT, metallothioneins.

by calmodulin (CaM) and CaM-binding peptide (28). The binding of CaM and its peptide ligand is Ca^{2+} dependent and elicits a conformational alteration that increases proximity between CFP and YFP, positioned at opposite ends of the fusion protein. This alteration in relative position of CFP and YFP allows the use of FRET between these two GFPs as a surrogate indicator of Ca^{2+}.

FRET between GFP mutants offers a general mechanism to build genetically encoded indicators and to monitor dynamic protein-protein interactions. Ca^{2+} imaging with cameleons can monitor excitable cell firing and explore mutant phenotypes in intact *Caenorhabditis elegans* (17). Use of cameleons in transgenic mice may help elucidation of mutant phenotypes and imaging of whole tissues and specific cell types. First-generation transfectable indicators for cAMP (48) and cGMP (15) have been developed.

Red fluorescent protein. A new family of fluorescent proteins has been recently cloned from anthozoan corals (27). A red variant (dsRed) from the Discosoma coral is of particular interest. In principle, the ability to genetically encode red fluorescence should provide maximal contrast with GFP and be helpful for minimizing autofluorescence and scattering problems. The primary limitation with dsRed is the lengthy time required for development of the full red color. It is likely that mutagenesis may lead to novel red fluorescent variants with improved ripening characteristics.

Fluorescent arsenical hairpin binder. FlAsH (fluorescent arsenical hairpin binder) represents a novel approach to fluorescent labeling of molecules in living cells (13, 14). This approach involves the covalent interaction between a pair of molecular components. The receptor domain consists of a tetracysteine motif of six amino acids that can be genetically encoded within a protein structure. The second component involves a synthetic, membrane-permeant, small (<700 Da) ligand that is a biarsenical fluorescein derivative. This ligand is nonfluorescent until it binds to its receptor. The basis for molecular recognition between the binding pair is the formation of covalent bonds between arsenic centers in the ligand and thiol pairs in the peptide domain. This approach affords significant advantages relative to GFP. The small size of the receptor domain (net gain of <20 amino acids vs. 238 for GFP) makes it less likely to alter native protein structure or function. Moreover, it is easier to incorporate the recognition domain into a recombinant protein, either at COOH or NH_2 termini, or in an existing α-helix or β-hairpin. Finally, derivatives of the FlAsH ligand can be synthesized with greater structural diversity than GFP to allow expression of a number of unique physical properties that can be exploited for visualization. This methodology can be easily adapted for fluorescence photooxidation (BrAsH) to allow visualization of samples with both light (LM) and electron (EM) microscopy.

It is envisioned that novel FlAsH analogs will be developed for a wide variety of applications, including the labeling of proteins that are perturbed by GFP fusions. The creation of analogs with longer wavelengths of excitation and emission (e.g., red or infrared) or with longer lifetimes to enable phosphorescence or luminescence should be possible as well. FlAsH analogs might be designed to reveal segmental flexibility of local domains by virtue of their rigidity of attachment to α-helix. The generation of singlet oxygen or hydroxyl radicals by FlAsH analogs may enable electron microscopic staining or cause localized toxicity either to the host protein or to the surrounding cell. The ability to carry other analyte-binding groups could confer useful properties as well. For example, the incorporation of chelating centers could be used to report the local environment (e.g., Ca^{2+} concentrations), whereas heavy atoms could be used to aid X-ray crystallographic structure determination. Similarly, the incorporation of paramagnetic atoms or molecules could enable magnetic resonance spectroscopy or imaging. The ability to bias protein conformations toward α-helical configurations or to enable binding to a chromatographic support to aid protein purification should be achievable with appropriate structural modifications of FlAsH. Finally, FlAsH dimers (4 arsenics) might reversibly cross-link proteins in situ to modulate their function; such complexes should be visible by fluorescence imaging.

β-Lactamase reporters. Novel substrates recently developed for the bacterial enzyme β-lactamase have been exploited to enable its use as a sensitive reporter gene in individual living mammalian cells (50). The enzyme amplification afforded by this system renders it much more sensitive than detection with GFP. This methodology makes use of a cephalosporin-based substrate flanked by two fluorophores that can serve as a FRET donor and acceptor pair. β-Lactamase acts to cleave the cephalosporin substrate and thus disrupts FRET between the two fluorophores. When 6-chloro-7-hydroxycoumarin and fluorescin are used as the donor and acceptor, respectively, and the substrate is excited at 409 nm, FRET transfer to the fluorescin acceptor can be observed as green emission (520 nm). Cleavage of the substrate, indicative of β-lactamase-driven gene expression, disrupts FRET and produces a shift to the blue wavelength (447 nm) emission of the donor. This color change can be used to select cells that selectively express the β-lactamase-driven gene(s) of interest. The noninvasive nature of the reporter system means that cells remain alive and subclones with homogeneous responses can be selected readily. Future modifications of substrate properties could enable the use of positron emission tomography to image β-lactamase-coupled gene expression in whole animals.

Organelle-targeted fluorescent probes. A variety of chimera plasmids have been developed recently that, when transfected into cells in culture, encode proteins that localize to selected cellular organelles and that can be used in conjunction with fluorescent dyes or GFP to measure organelle function. Fluorescent probes have been genetically targeted to the plasma membrane (and even to specific regions of the plasma membrane), recycling endosomes, endoplasmic retic-

ulum, Golgi, trans-Golgi network, secretory granules, nucleus, peroxisomes, and mitochondria, and these chimeras can then be used in conjunction with digital imaging microscopy to measure pH and its regulation in these compartments. For example, preopiomelanocortin-avidin targets to secretory granules, and addition of a membrane-permeant, fluorescent, pH-sensitive biotin to the extracellular medium allows for uptake and binding of the fluorescent dye only to this organelle for studying its pH properties (44). Similarly, sialyltransferase-EGFP targets to the Golgi, and the pH sensitivity of GFP can be used to measure pH regulation in this compartment (5).

There is compelling support for the importance of organelle physiology to toxicology. In some cases, toxins use organelles to their own ends (e.g., a protein from papilloma virus that causes cancer alkalinizes the Golgi). Many agents trigger rapid alkalinization of mitochondria as a precursor to apoptosis. Chlamydia and some other pathogenic bacteria survive inside cells by entering and then alkalinizing endosomes, thereby evading the immune system. Cholera toxin and *Pseudomonas* exotoxin both enter cells through the endosome and then enter the cytosol in an acid-dependent step; viruses enter cells using a pH-dependent step.

The development and application of organelle-targeted optical probes will inform our understanding of the effects of environmental toxins on the functions of all organelles in intact cells. Studies of the effects of environmental toxins, including bacteria and viruses, on organelle (including plasma membrane and cytosol) function in cells in culture and in intact tissues and animals are now possible. In combination with gene chip technology, as well as the β-lactamase gene reporter method of Zlokarnik and colleagues (50), studies of the effects of environmental toxins on organelle and cellular function could lead to understanding of the potential feedback that may occur between organelles and gene expression, e.g., as occurs in endoplasmic reticulum control of cholesterol biosynthetic enzymes. Use of organelle probes for high-throughput screens to determine the identities and also effects of environmental toxins is feasible as well. Finally, the genetic approach for measuring organelle function now allows measurements in tissues in intact animals, including yeast, flies, worms, fish, and mice.

OPTICAL IMAGING FOR DRUG DISCOVERY

Recent advances in combinatorial chemistry have enabled development of novel small molecule libraries that can be exploited for the identification of useful biochemical reagents and potential lead drug structures. Within this arena, imaging technologies enable measurement of a variety of spatially regulated subcellular cellular events, including mitogenesis, intercellular communication, differentiation, apoptosis, and DNA damage recognition. For example, subcellular movements control the cell cycle; thus agents that alter sequestration of key components (e.g., cdc25c) of this signaling pathway can be identified on the basis of changes in spatial location. Mammalian MAP kinase pathways provide another illustrative example. A defining feature of these pathways is that extracellular stimuli (e.g., growth factors, cytokines) induce a chain of events that produces nuclear localization of extracellular signal-regulated kinases. Likewise, binding of insulin to cell surface receptors alters subcellular distribution of activated Raf-1/Ras G protein complexes. This ligand-mediated internalization of Raf-1 can be visualized by creation of a GFP-Raf-1 fusion protein (32).

A number of instrumentation challenges exist for image-based screening. Cameras are limited by exposure times, resolution, and transfer speed. Peripheral device speed may create problems as well. The use of air objectives for light throughput requires lower numerical apertures and may present problems when using multiple probes. Scan times increase significantly with increases in the number of probes, site, objective magnification, and autofocus. For example, a low-magnification scan of a 96-well plate may require 2–4 min. Medium magnification of this same sample format with multiple probes may require 8–10 min, whereas high magnification with multiple probes may require 30–45 min. Data analysis, review, and management can present significant challenges as well.

Despite these drawbacks, the use of imaging for screening is becoming more feasible. Standard epifluorescence microscopes can be used as a base for an acquisition system. This represents significant cost savings and allows the microscope to be used for other imaging tasks. The disadvantage is a loss of speed. Dedicated systems are more expensive and offer higher performance. They also can be used for other camera-based applications. Recent improvements in system operation, viewing, and automated analysis have significantly increased the ease of use of image-based screening systems.

It is likely that imaging will become increasingly important for screening applications in the industrial sector. It is also anticipated that this technology will become popular as a tool for cell biologists. Improvements in camera speed and QE are likely to occur as well as development of larger format chips. Scanning methods are likely to remain expensive, and limitations in usable excitation wavelengths will be difficult to overcome. A proliferation of assays and probes will make biotechnology more accessible. Higher density plates, chip technology, or other novel substrates offer the possibility of higher throughput. This, in turn, will present challenges for sample preparation and handling. In the area of analysis and informatics, automated feature recognition will become increasingly important, and this will require the development of novel tools. Proliferation of analysis routines is expected. In the arena of informatics, mining imaging data, correlation of data from a variety of sources, and open architectures are envisioned.

INTRAVITAL IMAGING

Future studies in intravital imaging are likely to be greatly facilitated by advances in somatic gene transfer of fluorescence-sensitive reporter molecules. The proof of principle for this is provided by recent demonstrations of particle-mediated "gene gun" delivery into a variety of organs, including fragile tissues such as brain (33). Furthermore, as discussed in the Introduction, it will be critical to move beyond the traditional microscopic platform to a more flexible microscope that can be used directly on animals. Ideally, these images will be collected at high resolution and speed, hopefully using near-infrared or two-photon microscopy to improve depth penetration into tissue as described below.

Two-photon imaging. Progress in intravital imaging technologies will require increases in our ability to visualize events in three dimensions. The recent advances in multiphoton imaging hold great promise for this application (35). The powerful advantages of two-photon excitation in laser-scanning microscopy derive from the localization of excitation to the focal point of the microscope. This produces minimal photobleaching outside the plane of illumination, with attendant decreases in cell damage. Moreover, the long wavelength photons penetrate deeply into tissues and cells, allowing imaging of very thick sections (e.g., hundreds of micrometers) that would not be possible with other methods. For example, multiphoton laser-scanning microscopy was used to examine zinc homeostasis within the pulmonary endothelium in an isolated perfused mouse lung preparation (Fig. 6). Furthermore, in other applications, the simultaneous excitation of multiple color fluorophores coupled to distinct targets allows multiparametric analyses.

Two-photon excitation microscopy has been applied successfully to the study of a variety of systems, including organ slices, embryos, and primary cell cultures. Likewise, two-photon microscopy can be harnessed to study transdermal transport of biochemicals with the use of lipophilic probes, such as 1,1′dioctadecyl-5,5′-diphenyl-3,3,3′,3′-tetramethyl indocarbocyanine chloride. Transport can be estimated by comparing the fluorescence intensity as a function of depth in the presence and absence of test compounds (47). Video rate two-photon scanning can be used for viewing a variety of ex vivo preparations. For example, collagen-elastin fiber structure is readily visualized in ex vivo dermal preparations. Two-photon imaging can be used for detecting cell death within tissue masses, where the three-dimensional resolved detection allows for the extraction of the cell viability spatial distribution within the tissue mass. A dorsal skin fold chamber preparation allows two-photon imaging to be used for applications in cancer biology, such as measurement of angiogenesis (6).

Despite its many attractive features, there are significant disadvantages associated with the use of multiphoton microscopy. The most important is the high expense associated with the need for a confocal microscope and a Ti-sapphire laser. Another consideration is the complexities of laser operation that present challenges for multiuser environments. A final consideration is that multicolor experiments may be difficult due to complex multiphoton excitation profile of dyes.

NANOSCALE OPTICAL BIOSENSORS

A variety of nanometer-scale, minimally invasive optical probes have been developed for real-time imaging of cellular functions in the visible spectrum (8, 9). These probes are an outgrowth of fiberoptic nanosensors and consist of fiber tips with adherent polymer. Polymer matrices that exhibit excellent chemical stability and biocompatibility can be employed in probe construction. Use of different polymers (e.g., polyacrylamide gel, decyl methacrylate, sol gel, and polyvinyl chloride) allows control of hydrophobicity, and this

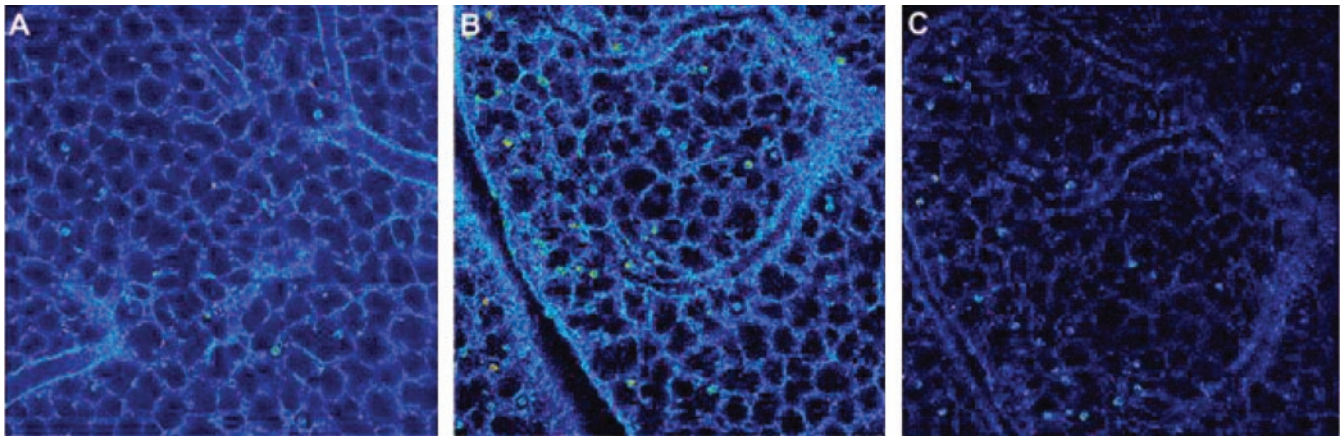

Fig. 6. Imaging of the zinc-specific fluorophore, Zinquin (Toronto Research Chemicals), in isolated, perfused mouse lung using multiphoton laser scanning microscopy. Ventilation was stopped during imaging, with the lungs statically inflated. The dual-pulsed laser was tuned to 840 nM, and images were collected using a ×20 infrared (IR)-corrected oil-immersion optic at 512 × 512 pixels with a scan time of a second (4 ms dwell time). Baseline Zinquin fluorescence (*A*) was significantly increased 10 min after the addition of 200 μM $ZnCl_2$ (in the presence of zinc ionophore pyrithione) to the perfusate (*B*) and attenuated by the zinc chelator *N,N,N′,N′*-tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN; *C*).

property can be exploited for biological studies. The polymer matrix serves to embed the sensing element. The first generation of nanoscale optical chemical sensors (NCOS) was termed probes encapsulated by biologically localized embedding (PEBBLEs). The simplest PEBBLE consists of a polymer tip embedded with a single-ionophore species. A variety of calcium and pH PEBBLEs have been developed. These PEBBLEs have been shown to be easily calibrated and to retain excellent linear ranges. PEBBLE delivery has been achieved successfully by several means, including liposomal and phagosomal mechanisms.

The second generation of NCOS is smart sensors that can incorporate molecular recognition elements targeted against specific extracellular or intracellular components. A number of advantages can be identified. First, NCOS permit spatial, temporal, and spectral resolution of ions and small molecules. They are biologically inert, enabling the use of sensors that contain toxic or lethal components. They allow rapid sensor-response time, ranging from microseconds to milliseconds. Hierarchical modular design of NCOS is possible. This allows incorporation of several distinct recognition and sensing elements to permit simultaneous analysis of multiple small molecules and ions. Finally, the superior biological and chemical stability of the embedding matrix enables probe use in the harshest environments. Currently, these sensors have been applied to the measurement of an array of chemically induced alterations in the cellular environments through measurement of pH, Ca^{2+}, Cl, K^{+}, O_2, nitric oxide, and glucose (10, 37). Perhaps the most exciting applications of NCOS will be in intact organisms. Noninvasive delivery to embryo through yolk sac is possible by incorporation into vitelline vasculature.

EPR IMAGING

EPR measurements have been used for the quantitation of free radical distribution, metabolism, and tissue oxygenation in a variety of tissues and organs. This methodology relies on the reaction of unstable free radicals with suitable spin trap probes and the subsequent detection of alterations in EPR spectra that reflect local concentrations of the paramagnetic species. Recent developments now allow noninvasive spatial-spectral imaging of free radicals in intact biological tissues for mapping of tissue oxygenation under a variety of conditions, including disease models.

One example of the rapid pace of progress in the refinement of spatial-spectral EPR is provided by the recent description of a method that avoids the influence of local spin probe density on the obtained images. Separate spatial maps of local spin density and maximum EPR spectral line amplitude are first created. This information allows computation of local EPR spectral linewidth images that correspond directly to local oxygenation. The successful application of this method to map arteriovenous oxygenation in a rat tail in vivo has been demonstrated (40).

Application of EPR to Cardiopulmonary Pathobiology

Work by Kuppusamy et al. (20) used the EPR signal of nitroxide as a surrogate marker of local oxygen consumption. Three-dimensional nitroxide imaging of the isolated rodent heart with 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) enabled visualization of hypoxia under ischemic conditions. Temporal resolution was limited by the rapid decay of the nitroxide EPR signal by its reduction to a hydroxylamine EPR-silent species. Subsequent work has demonstrated improved resolution of EPR images by use of TEMPOL and polynitroxyl albumin, the latter functioning to reoxidize the bioreduced nitroxide hydroxylamine (22). This allowed maintenance of signal intensity for an extended 2.5-h period of global ischemia. Submillimeter resolution of cardiac structure was achieved that allowed comparison of TEMPOL intensities in coronary arteries and myocardium.

Recent improvements in spatial-spectral EPR imaging demonstrate the rapid pace of progress, in adaptation of these methodologies, to use in living organs and tissues (51). Imaging of the beating heart required development of a synchronized pulsing and timing system to allow gated acquisition of images. Perfusion of isolated rat hearts with a nitroxide spin label allowed time-resolved measurement of free radical distribution during systolic and diastolic phases of the cardiac cycle.

Application of EPR in Tumor Biology

Spatial-spectral EPR has been applied to examine the distribution and lifetimes of nitroxides in tumor and normal tissues (12). Tumor cells implanted in mice were allowed to grow to 10–15 mm, and then the intravenous nitroxide 3-carbomoylproxyl was infused to allow noninvasive in vivo EPR spatial-spectral imaging. Three-dimensional images showed significant heterogeneity of 3-carbomoylproxyl distribution in tumors compared with normal tissues and a significantly higher rate of clearance (i.e., nitroxide reduction). Spin label oximetry showed lower P_{O_2} levels in tumor cells. This study demonstrates the ability of EPR imaging to provide anatomical and functional information relevant to cellular metabolism in normal and tumor tissues.

EPR Limitations

A significant limitation of EPR has been the lack of ideal single-line radical probes. Nitroxides in common use exhibit multiple lines that introduce artifacts in image reconstructions. A novel triarylmethyl paramagnetic probe has been described that exhibits a sharp single-line EPR spectrum and that is relatively stable in tissue. Utility of this label was demonstrated by use of spiral phantoms and isolated perfused rat kidney in which >100 μm resolution could be achieved (21).

Under optimal circumstances, the detection limit of EPR imaging is good, with 10- to 100-nM concentrations detectable. Limitations of this methodology in-

clude the small range of labels available. Improvements in sensitivity and resolution are needed as well. Development of novel probes to measure additional cellular or molecular processes would be desirable. Speed of image acquisition is problematic, with existing protocols requiring up to an hour. Thus far, EPR has not been applied to microscopy, although such applications are feasible with the use of higher frequencies.

ELECTRON MICROSCOPY: NEW TOOLS AND IMPROVED ACCESS VIA WEB-BASED TECHNOLOGIES

Electron tomography. Recent advances in EM instrumentation and analysis tools have been essential to the development of electron tomography, a method for reconstructing the interior of an object from its projections. High-voltage electron microscopes are used to acquire data from thick (2.5–4 μm) biological specimens. Typically, a series of single-axis tilt images is obtained by rotating the specimen in increments of 1–2° over a range of ±60–70°. The success of three-dimensional reconstruction with this method depends critically on obtaining sufficient resolution with tomographic image analysis and reconstruction and the use of appropriate tools to enable segmentation, classification, and visualization of large complex structures. The iterative methods required for reconstruction are computationally intensive and require access to high-performance computers.

Electron tomography has been applied to study the three-dimensional aspects of a variety of cellular components, including mitochondria, Golgi apparatus, and endoplasmic reticulum. An elegant demonstration of the utility of this methodology is provided by recent studies of mitochondria (30, 31). In contrast to the traditional Palade model of "mito-baffles" found in current textbooks, tomographic reconstruction of mitochondria revealed an invagination of the inner mitochondrial membrane to form cristae through small tubular cristae junctions of uniform size. An additional novel insight was gleaned from analysis of contact sites between the inner and outer membrane and cristae junctions. The location of contact sites revealed was found to be random with respect to cristae junctions, a finding contrary to established dogma.

Web-based access for EM. Limited access to specialized imaging instruments, such as the intermediate high-voltage electron microscopes required for three-dimensional tomographic reconstruction of biological specimens, provides a significant impediment to the widespread application of these promising technologies. Availability of computing platforms sufficient to enable use of image-processing tools represents another large obstacle. To address these needs, a number of projects are under way to provide web-based remote access to centralized imaging facilities and high-performance computers. These programs capitalize on recent developments in web interfaces, high-speed networks, and high-performance computing platforms to provide remote access for data acquisition, processing, analysis, and advanced visualization, including linkage to large-scale data bases. An ancillary advantage of web-based telemicroscopy is that it encourages interaction among researchers located at different sites. Preliminary results support the feasibility of these programs.

Fluorescence photooxidation: bridging the gap between LM and EM. Another area of recent progress is the development of the first class of tools to bridge the gap between EM and LM. Traditionally, EM is used as an end point to solve protein structure. Biological samples subjected to EM must tolerate significant radiation to allow acquisition of many images that can be processed for reconstruction. In contrast, LM can be used for localization of labels to different living organelles and to understand the dynamic interaction of intracellular molecules with stressors. Fluorescence photooxidation is a promising new method that enables the same sample to be used with both EM and LM instruments (11). This method uses the reactive oxygen generated by a fluorescent molecule to oxidize diaminobenzidine into a reaction product that can be visualized with an electron microscope. The small size of the oxidizing agent allows for excellent three-dimensional labeling with high spatial resolution. Fluorescence photooxidation has been applied recently to study the distribution of nicotinic acetylcholine receptors (nAChRs) at the neuromuscular junction. The conjugation of eosin to bungarotoxin enabled fluorescent labeling and subsequent photoconversion for visualization of the α-subunit of nAChRs with both LM and EM (25).

OPTICAL IMAGING TECHNOLOGIES IN PULMONARY RESEARCH

In situ imaging has always been at the forefront of contemporary pulmonary research since videomicroscopy was used to determine pulmonary capillary transit time by measuring the time required for fluorescent dye to pass from an arteriole to a venule on the surface of an intact dog lung (16, 42). More recently, the pulmonary microcirculation of the intact, perfused rat lung has been examined using confocal luminescence microscopy and high-speed video analysis to study the kinetics of stimulated leukocytes (3, 45).

Two independent groups have utilized intravital fluorescent microscopy techniques to observe endothelial cell function in situ in isolated and perfused rat lung. In a series of elegant studies, Ying and colleagues (46) used this model to describe the complexities of Ca^{2+} regulation in the pulmonary capillary and to demonstrate that adjacent epithelial cells and endothelial cells of the alveolar-capillary junction communicate Ca^{2+} signals through a receptor-mediated mechanism initiated in the epithelium (18). The ability to conduct these experiments while preserving the architecture and function of intact tissue was fundamental to these investigations of intercellular communications. In a similar fashion, Al-Mehdi and coworkers (1, 2) used intravital epifluorescent microscopy to study mecha-

nisms for ischemia-reperfusion injury of lung tissue. With the use of a variety of fluorescent indicators, they showed that, within the initial several minutes after ischemia, lung endothelium responds with plasma membrane depolarization (2), generation of reactive oxygen species via a membrane-localized NADPH oxidase (1), and an increase in intracellular Ca^{2+} (38).

Further examples of the use of intravital microscopy in pulmonary research include the use of pleural surface fluorescence methods to measure transalveolar transport of water, protons, and solutes in intact perfused lungs (4, 7). With this technique, the air space is filled with fluid containing a membrane-impermeant fluorophore, and the pulmonary artery is perfused with solutions of specified osmolalities. The surface fluorescence signal is directly proportional to the air space fluorophore concentration. This methodology can also be used to study H^+, Na^+, and Cl^- transport with suitable membrane-impermeant fluorescent indicators added to the air space fluid (41).

SUMMARY

Within the past 5 yr, the increasing versatility of imaging probes, coupled with dramatic improvements in instrumentation and analysis tools, have provided important insights into myriad aspects of biological function at the subcellular, cellular, tissue, and oganismic level. The primary challenges ahead involve the translation of these new tools and the knowledge base they have spawned to the understanding of biological responses to environmental variables. Additionally, with the rapidly developing advances in computer-driven technologies, the unique challenges presented by ventilation and perfusion of the intact lung can now be revisited. However, the ability to develop and refine new imaging tools depends on the active participation of scientists from a variety of disciplines, including computer science, physics, and biomedical engineering. In addition to increasing effective collaborations among disciplines, there is a need for increased multidisciplinary training of individual scientists and a corresponding shift in traditional thinking concerning the value of such research. Together, the predicted improvements in microscope systems are likely to be reflected in more use of core imaging facilities and screening facilities. In addition, an increasing sophistication of imaging methodologies (FRET, fluorescence recovery after photobleaching, photo-activated fluorescence, total internal reflectance fluorescence microscopy, etc.) is envisioned. These advancements will require the increased involvement of physicists and chemists in the development of microscope-based tools and the total dependence of microscopy on computers.

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