Environmental Toxicologic Pathology

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REVIEW ARTICLE

# Environmental Pathology: New Directions and Opportunities*

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## About the Author

Brooke Taylor Mossman, M.S., Ph.D., has been studying the mechanisms of environmental lung disease for approximately 20 yr and has generated over 175 publications. Her interest in the field began with graduate training in the laboratory of Andrew Sivak, Ph.D., at the New York University Institute of Environmental Medicine in Tuxedo, New York, where she worked on the effects of phorbol esters in skin carcinogenesis. She completed her Ph.D. degree in the laboratory of John E. Craighead, M.D., in the Department of Pathology at the University of Vermont; her subject was the mechanisms of tumor promotion in tracheobronchial epithelium. She then pursued postdoctoral research on interactions of asbestos and cigarette smoke in lung tumors with Edward Bresnick, Ph.D., Department of Biochemistry, University of Vermont. She is a past director of the Cell and Molecular Biology Program at the University of Vermont and is now a professor in the Department of Pathology and Director of the Environmental Pathology Program. Her current research, which focuses on cell signaling by asbestos, silica, and oxidant stress in cells of the respiratory tract, is funded by grants from the National Institute of Environmental Health Sciences and the National Heart, Lung and Blood Institute. In addition, she is a coinvestigator on projects with researchers at Wayne State University and the University of Texas at Galveston; this research concerns the roles of antioxidant enzymes and DNA repair, respectively, in fibrogenesis and carcinogenesis by asbestos. The technologies described in this review are powerful tools for her research, which combines *in vitro* approaches with inhalation experiments using rat inhalation models of asbestosis and silicosis and transgenic mouse models.

ABSTRACT

The National Institute of Environmental Health Sciences (NIEHS) supports a number of training programs for predoctoral and postdoctoral (D.V.M., M.D., Ph.D.) fellows in toxicology, epidemiology and biostatistics, and environmental pathology. At the Experimental Biology meeting in April 1997, the American Society of Investigative Pathology (ASIP) sponsored a workshop including directors, trainees, and other interested scientists from several environmental pathology programs in medical and veterinary colleges. This workshop and a related session on "Novel Cell Imaging Techniques for Detection of Cell Injury" revealed advances in molecular and cell imaging approaches as reviewed below that have a wide applicability to toxicologic pathology.

*Keywords.* Cell biology; molecular biology; microscopy; cell imaging; careers; diagnostics

## WHAT IS ENVIRONMENTAL PATHOLOGY?

In the broadest sense, environmental pathology is the study of diseases attributable to environmental factors in the world around us. Classically, it encompasses morbidity and mortality from indoor and outdoor air pollution and exposures to metals, chemicals, inorganic and organic dusts, radiation and electromagnetic injury, and pesticides. The study of environmental diseases often includes occupational diseases, nutritional and alimentary excesses and deficiencies, and maladies acquired from tobacco, alcohol, and drug abuse. Many of these agents may be causative or contributing factors involved in the initiation of disease as well as promoting influences during the progression of disease. Thus, common goals of the environmental or toxicologic pathologist are (a) identification of lesions related to toxic exposures and (b) understanding of the pathogenic mechanisms of toxic agents.

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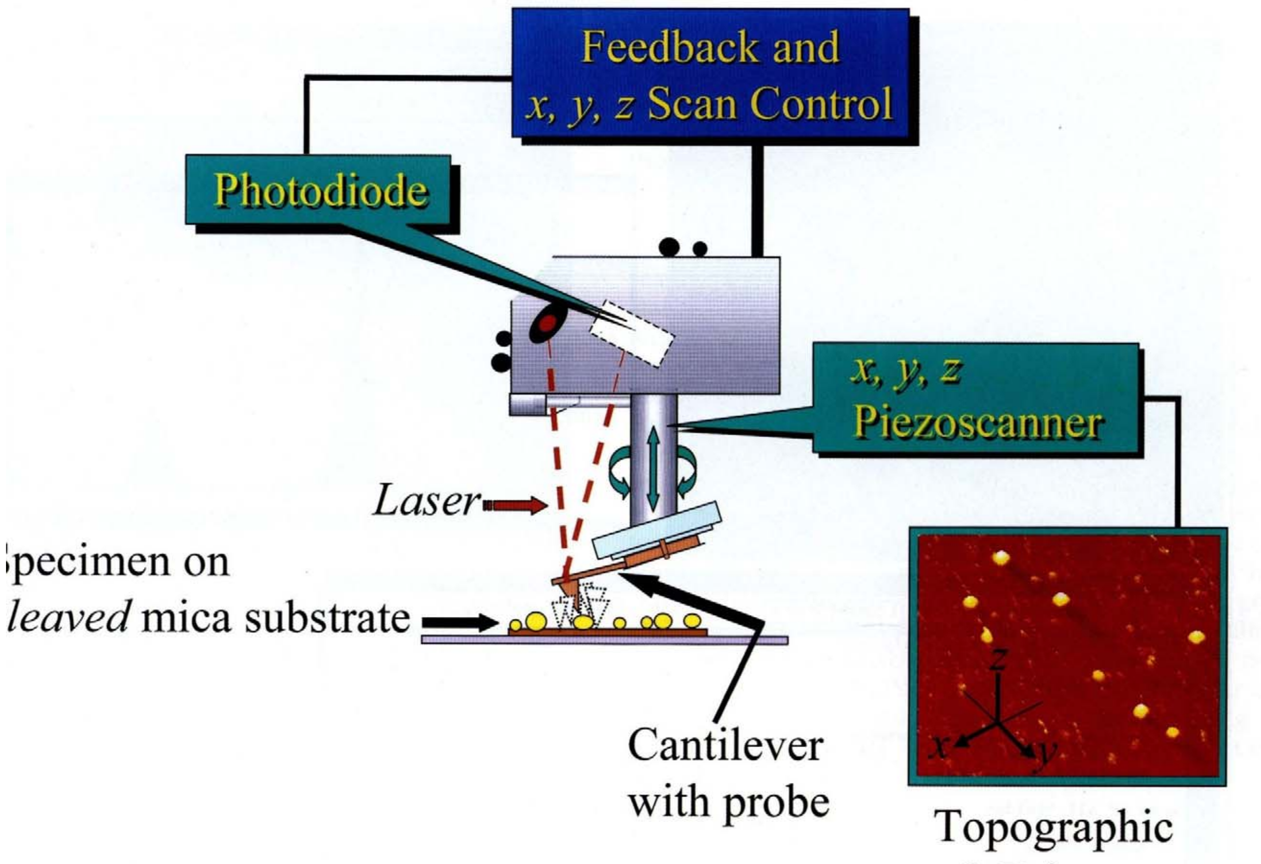


FIG. 1.—Schematic representation of the principle of AFM. (Modified from D. Braunstein, *American Laboratory,* January 1998, pp. 17–18.)

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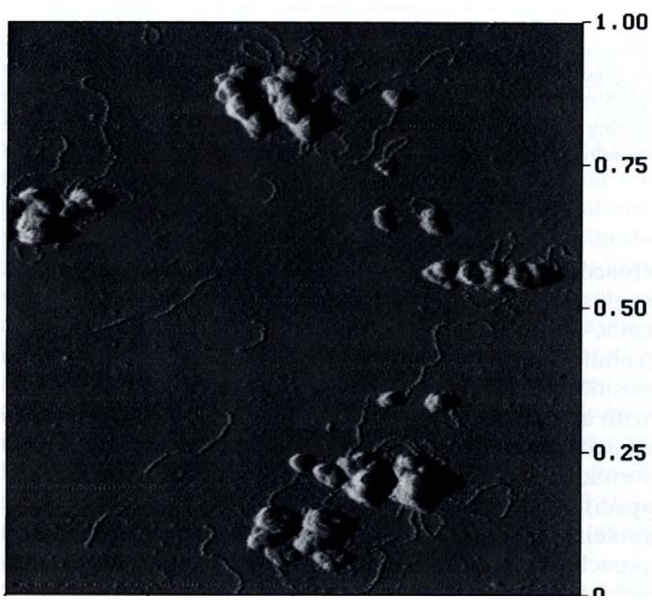



FIG. 2.—Visualization of multimerization of a zinc-finger DNA binding protein on linear DNA fragments. (Courtesy of Bill Montigny, Chris Houchens, and Nicholas Heintz, Department of Pathology, University of Vermont College of Medicine.)

## TOOLS OF THE TRADE AND RETOOLING

There is no doubt that traditional pathology plays a crucial role in the diagnosis of specific environmental diseases, and examination of gross and microscopic lesions is essential to understanding associated tissue alterations and to establishing "hallmarks" for recognition of specific agent-related lesions. In addition to conventional light and electron microscopic techniques, tools such as energy-dispersive x-ray spectrometry (EDAX) allow identification of inorganic substances such as metals, chemicals, and minerals in tissues; they also allow us to determine whether or not agents are modified after their intracellular or extracellular deposition. The development of sophisticated histochemical methods and the burgeoning world of molecular probes and antibody production now enables the pathologist to identify genes, proteins, and other macromolecules that have been altered by exposure to environmental agents or that have been expressed in toxicant-exposed cells. Using these advances, the toxicologic pathologist is poised to exploit new methodologies in cell imaging, as described below.

## ATOMIC FORCE MICROSCOPY

Atomic force microscopy (AFM), a member of the expanding group of scanning probe microscopies, employs a scanning probe tip with the potential and sensitivity to

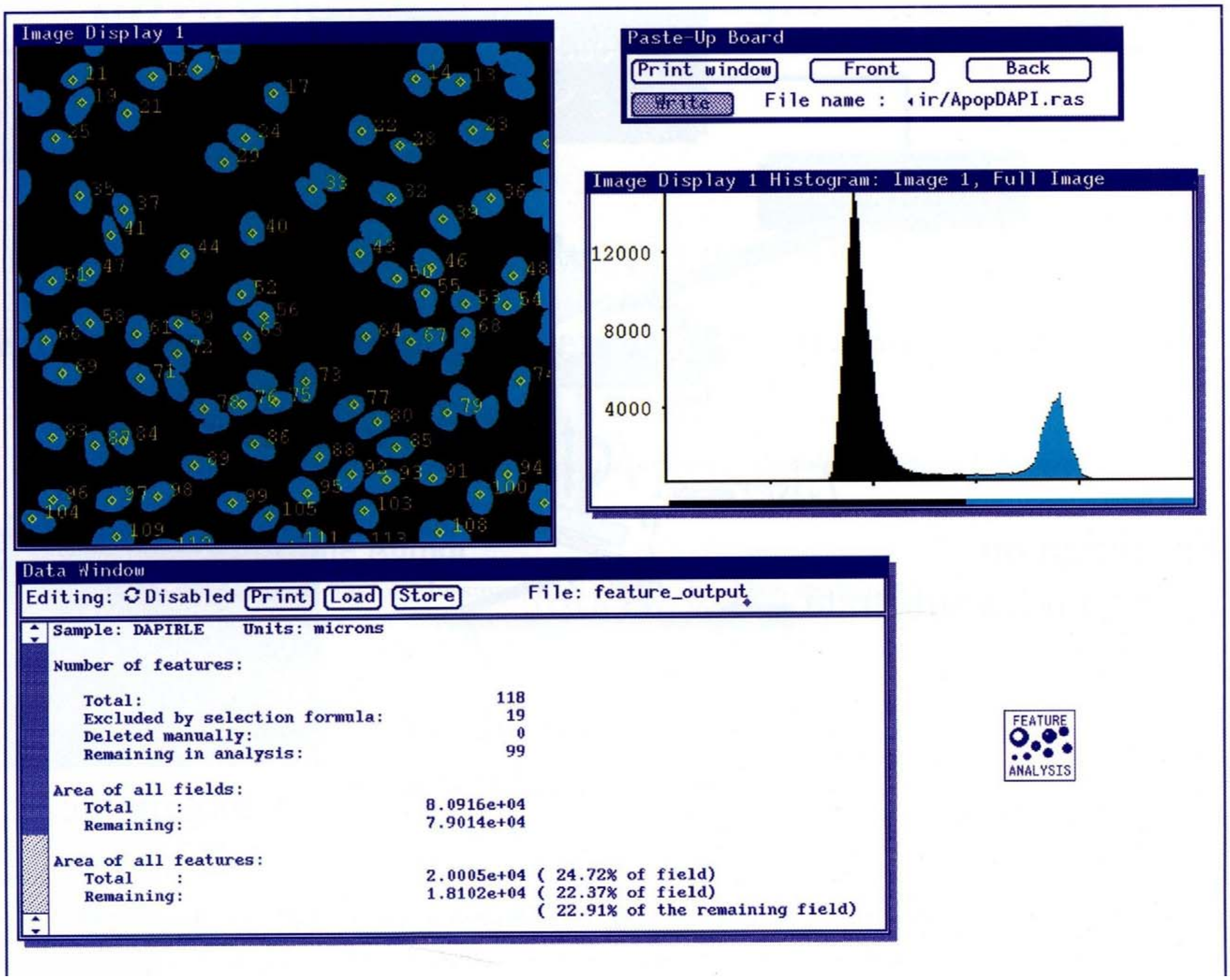



FIG. 3.—Computer-assisted image analysis of apoptosis in cultured cells. Rat pleural mesothelial cells were stained with DAPI and imaged by epifluorescence microscopy. Histograms of digitized gray-scale images were used to convert the images to binary form. The Feature Analysis software module of IMIX (Princeton Gamma Tech, Princeton, NJ), together with our own derived macro, was used to automatically determine the number of apoptotic cells in a given field (5). This example shows panels depicting the binary image, the histogram from the image, and the results of automated analysis. (Courtesy of Douglas Taatjes, Ph.D., Department of Pathology, University of Vermont College of Medicine.)

resolve molecules on the surfaces of cells and other materials, with a magnification potential of $\times 10^6$. An AFM system consists of a flexible cantilever, which contains a tip (probe) on its free end, mounted on a piezoelectric scanning tube (Fig. 1). The tip is raster scanned across a sample surface, and the deflection of the cantilever as it "feels" its way over the surface is monitored by a laser beam focused on the cantilever, thus producing a measure of the local height. By plotting the local sample height versus the horizontal probe position, a 3-dimensional map of the surface is produced and displayed on an image monitor. The entire scanning head assembly can be mounted on top of an inverted light microscope with a specially designed rigid stage. This type of configuration is particularly suited for investigation of biological materials, since areas of interest can be quickly located (using the optical microscope) and then scanned with the AFM. Moreover, the addition of fluorescence capabilities to the optical microscope allows localization of fluorescence-tagged molecules amongst a heterogeneous preparation, or on live cells, followed again by scanning with the AFM. This technique can be used to identify the topography and composition of toxic agents and to determine how they interact with and are taken up by cells. Recently, AFM has enabled direct observation of enzyme activity, as depicted by conformational changes of enzymes during hydrolysis (8), specific interactions between ligands and receptors (3), and protein binding to DNA (Fig. 2). These experimental approaches will be invaluable for investigating alterations in ligand receptor binding by agents such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (9) and mineral fibers (12). AFM may also allow resolution of proteins and other macromolecules bound to toxins after various routes of administration and may help us determine whether or not these interactions persist after deposition, metabolism, and/or excretion.

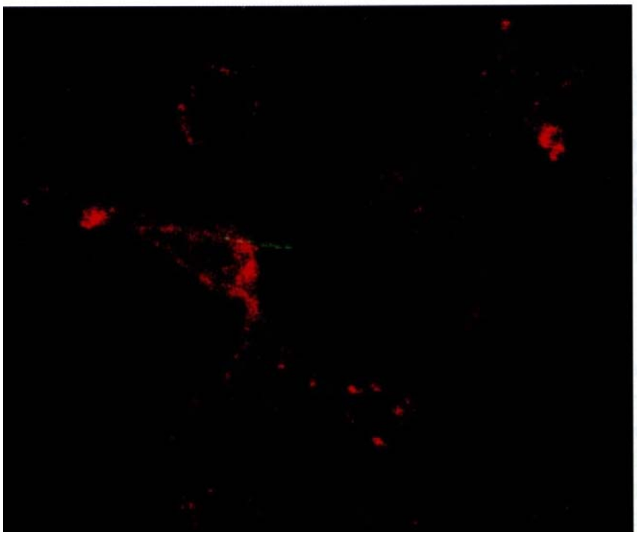


FIG. 4.—Demonstration of EGFR immunoreactivity by CSLM in human mesothelial cells exposed to asbestos fibers. Separate confocal images were collected in fluorescence mode (EGFR) and reflectance mode (asbestos fibers); this was followed by electron merging of the images. In the resultant merged image, the asbestos fibers are depicted in green and the EGFR immunoreactivity in red. (Courtesy of Michael Jung and Jean-Claude Pache, Department of Pathology, University of Vermont College of Medicine.)

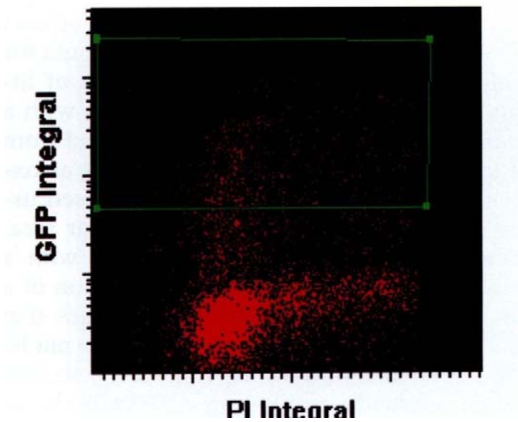
## COMPUTERIZED IMAGE ANALYSIS FOR QUANTITATION OF APOPTOSIS AND PROLIFERATION INDUCED BY ENVIRONMENTAL AGENTS

Programmed cell death (i.e., apoptosis) and increases or decreases in DNA synthesis are phenotypic changes observed in cells after exposure to a number of toxic substances. Cells in S-phase, an indication of proliferation or unscheduled DNA synthesis, can be identified by assessing the incorporation of 5′-bromodeoxyuridine (BrdU), a nonradioactive thymidine analog, into DNA and by use of an anti-BrdU monoclonal antibody. Alternatively, antibodies against proliferating cell nuclear antigen can be used to demonstrate cell-cycle alterations. Several immunocytochemical and biochemical methods, including flow cytometry (after staining with propidium iodide); the poly(ADP-ribose)polymerase cleavage assay for activation of caspases; DNA gel retardation assays (revealing short fragments characteristic of apoptotic DNA); and the transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) technique (which identifies the 3′-OH termini in DNA breaks generated during apoptotic DNA fragmentation), have been developed to demonstrate apoptosis. However, the "gold standard" is positive identification of apoptotic cells using light or transmission electron microscopy.

Identification and quantitation of proliferating and/or apoptotic cells *in situ* can be subjective and time-consuming, but computerized methods coupled with dual-fluorescence microscopy can be used effectively to quantitate both phenotypic end points in cells exposed to environmental agents. These approaches have revealed that agents such as asbestos fibers may have dual effects (i.e., induction of both apoptosis and increased DNA synthesis in mesothelial cells) (5). In brief, a numerical formula for classification of apoptosis can be derived in cells of interest using the TUNEL technique in conjunction with a DAPI counterstain. Images from untreated cells and from those exposed to a known apoptotic stimulus such as oxidative stress can be collected, stored, and processed using appropriate software. Measurements of nuclear area, perimeter, diameter, etc., are then are compiled with a feature-analysis module (Fig. 3), enabling derivation of a statistical formula based on distributions of values that will differentiate between normal and apoptotic nuclei with less than a 1% chance of misclassification. A second dual fluorescence method, employing an antibody to BrdU and nuclear counterstaining with an oxazole yellow homodimer (YOYO-1) dye interacting with DNA, is used on a second coverslip of cells from identical treatment groups. After exposures to low concentrations of asbestos, this approach reveals increases in BrdU-positive cells per total numbers of nuclei (YOYO-positive), an indication that increased numbers of cells are undergoing DNA synthesis. At higher, toxic concentrations of fibers, decreases in both BrdU-positive cells and total cell numbers (numbers of YOYO-positive intact nuclei per area of dish) are observed. These novel cell-imaging approaches are presently being adapted for analyses of cells in tissue sections.

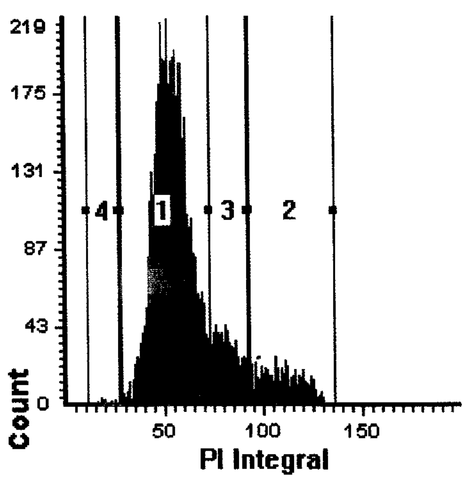
## CONFOCAL SCANNING LASER MICROSCOPY AND MULTIFLUORESCENCE TECHNIQUES

Early response protooncogenes and/or tumor suppressor genes can be expressed in normal or transformed cells after exposure to environmental carcinogens and are possible diagnostic tools in the detection or staging of tumors (1). Moreover, changes in their expression may contribute causally to the induction of apoptosis or cell proliferation. To allow detection of protooncogenes in cells *in vitro* after oxidative stress and to determine their relationship to the development of apoptosis, we developed a multifluorescence labeling method for simultaneous detection of mRNA, nuclear DNA, and apoptosis (2). Sense (control) and antisense digoxigenin-labeled cRNA probes to the protoooncogene c-jun were detected with an anti-digoxigenin monoclonal primary and a fluorophore-conjugated secondary antibody. Cells exhibiting apoptosis were simultaneously identified by the TUNEL method using a streptavidin-conjugated far-red fluorophore and nuclear DNA with YOYO-1. Using confocal scanning laser microscopy (CSLM) and this triple fluorescence labeling technique (combining nonisotopic *in situ* hybridization and the TUNEL method), increased c-jun mRNA expression was observed within the cytoplasm of both TUNEL-positive and nonapoptotic cells exposed to asbestos or hydrogen peroxide. These results, coupled with the recent observation that phosphorylated Jun kinase and Jun are observed preferentially in the nuclei of mitotic cells (10), suggest that activation and nuclear translocation of the Jun protein may be important in the advent of cell proliferation by oxidative stress. This hypothesis is supported by our previously published results, which showed that overexpression of c-jun causes increased cell




PI Integral

| Rgn. # | Count | Pct. | Mean |
|---|---|---|---|
| 1 | 424 | 7.1% | 1.36 |
| Total | 5937 | | 1.26 |

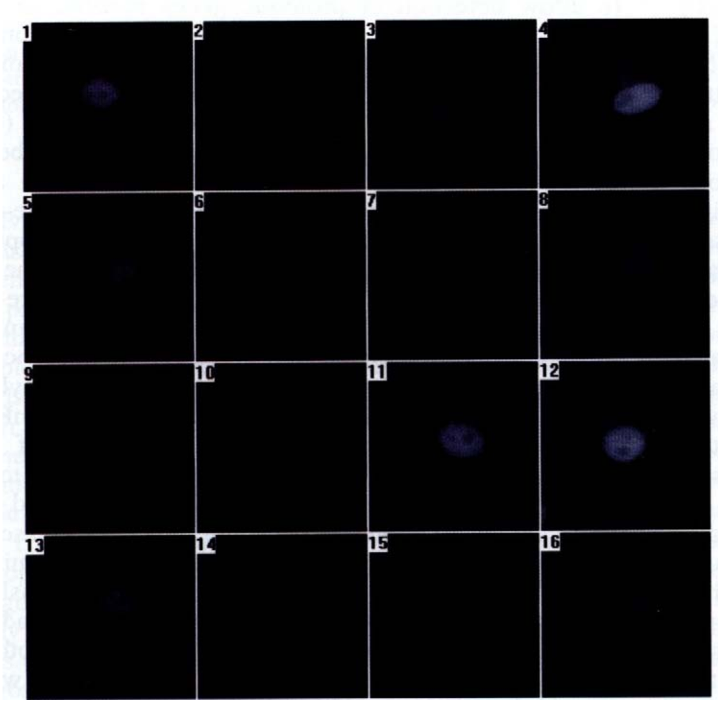



PI Integral: Color # 1

| Rgn. # | Count | Pct. | Mean | FWHM | CV |
|---|---|---|---|---|---|
| 1 | 4601 | 77.5% | 1.09 | 17.5% | 15.9% |
| 2 | 658 | 11.1% | 2.19 | 13.2% | 9.4% |
| 3 | 731 | 12.3% | 1.65 | 9.9% | 7.0% |
| 4 | 31 | 0.5% | 0.43 | 5.7% | 16.3% |
| Total | 5937 | | 1.26 | 14.4% | 32.3% |

PI Integral: Color # 2

| Rgn. # | Count | Pct. | Mean | FWHM | CV |
|---|---|---|---|---|---|
| 1 | 298 | 70.3% | 1.14 | 14.8% | 14.3% |
| 2 | 62 | 14.6% | 2.21 | 10.7% | 9.4% |
| 3 | 62 | 14.6% | 1.66 | 8.7% | 6.7% |
| 4 | 4 | 0.9% | 0.41 | | 15.6% |
| Total | 424 | | 1.36 | 14.1% | 31.5% |




| Cell # | DI Index | Area | X Pos. | Y Pos. | Integral 0 | Max pxl 0 |
|---|---|---|---|---|---|---|
| 1 | 2.03 | 137 | 70005 | 26988 | 613123 | 1858 |
| 2 | 1.31 | 105 | 70384 | 27072 | 396281 | 2333 |
| 3 | 1.08 | 95 | 73000 | 26929 | 324906 | 1385 |
| 4 | 1.20 | 103 | 74627 | 26829 | 363566 | 1529 |
| 5 | 1.62 | 122 | 75145 | 26858 | 490400 | 1872 |
| 6 | 1.33 | 119 | 75042 | 27016 | 402854 | 1271 |
| 7 | 1.27 | 101 | 70458 | 27189 | 383958 | 1432 |
| 8 | 1.18 | 101 | 70525 | 27316 | 357647 | 1607 |
| 9 | 1.02 | 97 | 70953 | 27440 | 309294 | 1154 |
| 10 | 1.66 | 121 | 71217 | 27354 | 500504 | 2440 |
| 11 | 1.55 | 124 | 73535 | 27225 | 467735 | 1529 |
| 12 | 1.63 | 120 | 73565 | 27248 | 491913 | 2308 |
| 13 | 1.53 | 107 | 75281 | 27352 | 461651 | 2394 |
| 14 | 1.21 | 98 | 70787 | 27709 | 364310 | 1808 |
| 15 | 1.28 | 99 | 70864 | 27669 | 388050 | 2096 |
| 16 | 1.75 | 129 | 71226 | 27623 | 527702 | 2256 |

proliferation and morphologic transformation of tracheal epithelial cells (11).

Dual immunofluorescent labeling methods and CSLM have also been valuable in colocalizing DNA repair enzymes (induced by asbestos) to the mitochondria and nucleus (4). Moreover, these approaches have revealed the interaction of asbestos fibers with the epidermal growth factor receptor (EGFR) on the cell surface and with increased expression of EGFR protein in mesothelial cells after exposure to asbestos (Fig. 4) (7).

## Laser Scanning Cytometry

The laser scanning cytometer (LSC) is a powerful new tool that combines cell imaging methods and flow cytometry (6). The LSC (CompuCyte Corporation, Cambridge, MA) is a modular unit consisting of an upright light microscope with a computer-controlled x,y stepper motorized stage; a scanning module containing lasers, photomultiplier tubes, and electronics; and a personal computer, which drives the system. Fluorescently tagged cells or tissue sections are excited by a collimated argon laser (488 nm line) or a helium/neon laser (643 nm line), which is directed through a computer-controlled scanning mirror that produces a sawtooth motion in the form of a scan line at a rate of 350 Hz. The stage stepper motor moves the specimen under the laser, thus forming a series of scan strips. Emitted fluorescence energy passes back through the same optical pathway, through a series of dichroic mirrors and optical interference filters, and finally passes to 1 of up to 4 photomultiplier tubes (allowing simultaneous multifluorescence). A solid-state forward-scatter detector is located beneath the stage to gather scatter information. Two ccd cameras are mounted with phototubes to the top of the microscope; 1 camera is used to monitor the scanning of the slide while the other is used for image capture.

LSC should complement the use of flow cytometry for many types of investigation, and in addition, it offers the following advantages: (a) cell position as a measurement feature, allowing cell relocation based on a variety of criteria; (b) specimens can be restained with additional fluorophores or dyes and can be reexamined using the relocation function; (c) preparations are imaged on slides, allowing for analysis of touch preparations, smears, or tissue sections without requiring dispersal or clump size reduction; (d) no centrifugation steps are required during specimen preparation, resulting in less sample loss; and (e) far fewer cells are required for analysis.

Using this methodology, tissues, suspensions of cells, and cells adherent to slides can be "mapped" on a single-cell basis for localization of plasma membrane, cytoplasmic, and nuclear proteins or fluorescent *in situ* hybridization probes. By measuring the fluorescence and calculating the 2-dimensional morphometric features of cells, protein or DNA expression can be correlated with the cell cycle (Fig. 5). In addition, the position of individual cells on a slide is recorded by a computer so that the cells can be restained, relocated, or reexamined.

Although this technique is in its infancy, its potential application to the field of environmental pathology is limitless. For example, many toxic agents perturb the cell cycle and may cause alterations in expression and/or distribution of proteins causally related to cell cycle regulation. Cells exposed to putative cell cycle–disruptive agents could be stained with fluorescein isothiocyanate–conjugated antibodies to specific proteins and propidium iodide for DNA content to determine cytoplasmic and nuclear distributions at various stages of the cell cycle in comparison with control cells. These studies might reveal dysregulation of key genes and proteins, which is important in the origin or control of DNA replication.

## Conclusions

The goal of environmental pathology programs is to produce scientists capable of functioning within multidisciplinary research teams. While some programs place more emphasis on anatomic pathology, others emphasize training in diagnostic and histopathologic skills. Curricula also provide laboratory and lecture series on principles of molecular biology and their application toward studying mechanisms and biomarkers of environmental disease. Required courses in most programs include a basic toxicology or pharmacology course, an ethics course that addresses current issues and controversies in scientific research, and biostatistics.

Placement of fellows after training in various environmental pathology programs is exceptional. An informal survey of Ph.D and D.V.M. trainees at the ASIP meeting indicated that most aspire to research careers in academia or industry. However, several past trainees have entered positions in government and regulatory agencies or have joined private environmental consulting firms. A clear advantage of environmental pathology training is that most individuals are trained in anatomic pathology, histopathology, and many of the novel cell-imaging techniques described above. This background and a motivation for unraveling the mechanisms of disease should engender new advances in diagnostics, prevention, and therapy of occupational and environmental disease.

## Acknowledgments

Douglas Taatjes, Ph.D., the Director of the Cell Imaging Facility at the University of Vermont, provided

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FIG. 5.—Example of data obtained by the CompuCyte LSC. Rat lung epithelial cells grown on glass coverslips were transfected with green fluorescent protein (GFP), fixed with paraformaldehyde, and stained with propidium iodide. Cells were scanned for GFP expression and cell cycle analysis with the LSC. Top panel shows scattergram of GFP integral versus propidium iodide integral; gating (green rectangle) based upon cell relocation (see bottom panel) shows 7.1% GFP positive cells. Middle panel shows cell cycle analysis of entire cell population; cells in green represent gated region in top panel. Region 1, $G_0/G_1$; Region 2, $G_2/M$; Region 3, S-phase; Region 4, apoptotic. Bottom panel shows a gallery of 16 cells representative of cells from the gated region, with accompanying cell statistics. (Courtesy of Jan Schwarz and Douglas Taatjes, Department of Pathology, University of Vermont College of Medicine.)

valuable input on methodologies and illustrations. Current NIEHS-supported environmental pathology programs and contact information include the following:

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