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EXTENDED ABSTRACTS

Probing Individual Cells: Applications to Signaling, Structure and Function

Bethesda, Maryland, March 12–14, 2001

The extended abstracts that follow provide a summary of the proceedings of the Workshop ''Probing Individual Cells: Applications to Signaling, Structure and Function'', held in Bethesda, MD, on March 12–14, 2001, which was jointly organized by the Chromosome Alterations Branch of the National Cancer Institute's Division of Cancer Biology and the Columbia University Radiological Research Accelerator Facility.

The Workshop was motivated by the recent rapid development of new technologies to study biological responses in individual cells, as well as new technologies (such as single-particle/single-cell microbeams) for inserting perturbations into individual cells or parts of cells.

The Workshop provided a forum to assess how such tools may enrich ''conventional'' structural biology studies of multi-component signaling and DNA repair complexes and to discuss future directions, both technological and biological, in these fields. The meeting brought together individuals from a number of very different fields, and much cross-fertilization of ideas and technologies in this emerging area of basic cancer research took place.

Roughly 55 scientists attended the workshop. A list of attendees can be obtained from David Brenner.[1]

Support for this workshop from the Chromosome Alterations Branch, Division of Cancer Biology, National Cancer Institute is gratefully acknowledged.

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Single-Cell Gene Expression Analysis

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Cell physiology is a consequence of the genes that are expressed, so it should eventually be possible to predict function from the profile of genes expressed in a given cell. To approach such a goal, expression profiling methods which detect the expression of functionally relevant genes in individual cells need to be developed. The ever-increasing amounts of genomic information which are becoming available suggest that it will be possible to determine most of the genes expressed in any given cell type, provided that the expression of large numbers of genes can be assessed simultaneously. Recently, a few methods have been developed, in particular those derived from the laboratories of Eberwine and Rossier which permit the expression of many genes to be assessed in samples derived from single cells.

In our laboratory, we have developed a global gene expression profiling technology that is capable of detecting the expression of all the genes from any given genome in samples derived from single cells. This technique is based on the PCR-mediated amplification of all mRNA sequences in a given sample, followed by gene-specific PCR for the detection of individual gene sequences (*1*). It was important to demonstrate that the profile of gene expression that was detected reflected the physiology of the cells under investigation. Accordingly, we chose to study the cholinergic neurons of the mammalian striatum, which constitute approximately 2% of the total population of neurons in this area of the brain. Thus we could control for contamination of the samples under consideration.

Cytoplasm from individual cells was harvested from living brain slices using a patch-clamp electrode under infrared optics, which permitted visual control of the harvesting operation. The neurons were identified according to their electrophysiological, morphological, biochemical and pharmacological properties. Approximately 40% of the somatic cytoplasm was routinely harvested from these cells, and the electrode was withdrawn from the cell. Contamination of the electrode contents was minimized by using a nucleated patch to seal the electrode.

We assessed the expression of mRNAs encoding a variety of transmitter receptors, including the mRNAs for receptors responding to sulfonylureas, growth factors, adenosine, glutamate, tachykinins and histamine. In each case, the corresponding expression of functional receptors or protein was demonstrated. This technique is therefore suitable for the analysis of gene expression in samples from single cells.

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The Columbia University Microbeam Facility

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An ion microprobe facility has been constructed for the study of the effects of radiation on mammalian cells growing in culture (*1*). Hydrogen or helium ions are collimated using a 5-μm-diameter aperture. Individual cells are placed one by one over the exit aperture by an integrated video analysis and stage control system, and an exact number of ions are allowed to strike the cell or a chosen subcellular component. The system was originally designed to deliver single α particles through the center of the nucleus of each cell to model the effects of low-level radon exposures. In response to the interests of our collaborating radiobiologists, we have added other capabilities to the control system, for example, delivering the ions to the cytoplasm of the cells or within the nucleus but off center. Other protocols were designed to study the so-called bystander effect whereby only a fraction of the cells are irradiated and a response is observed among the unirradiated cells. A variety of irradiation protocols are available, and others could be implemented. Planned facility developments include replacement of the collimation system with electrostatic lenses and the addition of heavy ion capabilities.

Acknowledgments

This work was supported by NIH grant RR-11623.

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Probing the Origins of Radiation-Induced Biological Damage in Normal Human Cells with a Charged-Particle Microbeam

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The selective exposure of parts of intact individual cells to high-energy radiation was recognized many years ago by Zirkle and Bloom (*1*) as having the potential to provide information about the mechanisms whereby radiation produces such ''strikingly injurious effects in living systems'' and to ''aid in analyzing the normal functions of the various cell parts by selectively altering them''. Accelerator-based microbeams and collimated or microneedle coated isotopic α-particle sources helped to establish one of the basic paradigms of radiation biology: that it is the hit cell nucleus from which deleterious effects originate. In 1962, a symposium on partial and microbeam irradiation, reported on by Smith (*2*), was held at which ''[a]ll participants were persons who are actively interested or working in this rapidly growing field''. It now appears that the growth of the field stalled for a number of years, with resurgence in interest developing in part from concern about the effects of single α particles and from low-dose studies with isotopic α-particle sources. The results of these latter studies challenged the basic paradigm of the action of radiation. Low fluences of α particles were delivered to mammalian cells such that only a small fraction of cells were likely to have had their nuclei hit by an α particle. Significantly more cells showed changes in sister chromatid exchange frequencies, in chromosomes, and in gene expression than were traversed by α particles; hence some responding cells were bystanders of hit cells. In addition, new cellular/molecular end points with relevance to the risks associated with exposure to ionizing radiation became available in the early 1960s, facilitating studies at the level of a single cell. The development of pertinent risk and mechanistically oriented assays may perhaps be considered to be the greatest incentive for the burgeoning current interest in microbeams.

Studies with broad-beam α-particle sources, either isotopic or accelerator-based, cannot of course readily distinguish between cells known to be hit and cells known to be bystanders, nor can the relative contributions of hits to the nucleus, of hits to the cytoplasm, or even of hits to the medium to the final biological response be ascertained. The ability to establish the origin of the biological responses individual cells is, however, attainable with a microbeam, consistent with the original expectations of Zirkle and Bloom (*1*).

We have used the Columbia University RARAF microbeam and have devised specific protocols to target the nuclei of individual cells or the cytoplasm of individual cells and protocols to miss cells completely, hitting only the intercellular medium. Using these protocols, all sites or a known proportion of sites can be irradiated with a precisely known number of α particles. That is, some cells can be hit cells, while the remaining proportion will be bystander cells.

Alternatively, cells can be labeled with two vital dyes and then plated in known proportions and only one cell population irradiated. After irradiation, the hit cells can be discriminated from the bystander cells, with the relative positions of each cell at the time of irradiation being recorded. End points examined include frequencies of micronuclei, cell growth by monitoring cell numbers (all cells on a dish are examined), progression of cells through the cell cycle by monitoring bromodeoxyuridine uptake, and the expression of the stress-related genes, *TP53* and *CDKN1A* (also known as *p21, WAF1/Cip1*). Gene expression is measured in cells *in situ* using immunofluorescence, and also by single-cell RT-PCR performed after the removal of individual cells of known radiation history with a micromanipulator.

The conclusions from an extensive series of studies with normal human fibroblasts using RARAF microbeam protocols are as follows:

1. Deliberately missing the cells and irradiating the culture medium between them produces no detectable response.
2. Irradiating the cell cytoplasm (including the medium surrounding the cell) produces no detectable increase in the frequency of micronuclei, but it does induce some cell cycle delay in synchronized G_0/G_1-phase cells. No delay is seen in cycling cells.
3. Irradiating the cell nuclei (including surrounding cell cytoplasm and medium) results in fluence-dependent increases in micronuclei, cell cycle delays, and increases in gene expression. This response specifically includes exactly one α particle.
4. Irradiating 50% of cells through their nuclei produces a response in known non-hit bystander cells which is not dependent on the number of particles through hit cells.
5. Reducing the proportion of cells with hit nuclei results in a proportional lessening of the response in bystander cells.
6. Irradiating hit cells through the cytoplasm does not produce a response in bystander cells. The expression of a bystander effect in non-hit cells originates from insult to the nuclei of hit cells.

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Measuring Molecular Flux in the Diffusionary Boundary Layer of Cells and Tissues

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The relationship between a cell and its environment is a dynamic balance of different transporting mechanisms, both internally and across the plasma membrane. Much has been learned about cellular function, disease and homeostasis by the careful dissection of the transporters that sustain this relationship. These studies will accelerate as we explore the possibilities of manipulating the cell through genomics. Key to progress in linking genomics to cellular properties is the availability of tools for analyzing function. One of the most powerful approaches has been to compare the intracellular environment with conditions present in the bulk medium supporting the cell. However, these studies do not provide access to the complex ionic and molecular environments which surround actively transporting cells, environments which can vary significantly from the bulk conditions. There is therefore a need for tools that can ''microsample'' the boundary layers surrounding cells, making quantitative measurements of transport phenomena and comparing these values with conditions both in the bulk medium and within the cell. It is in this area of research that externally located electrochemical sensors offer particular promise and form a focus of instrument development at the BioCurrents Research Center (www.mbl.edu/BioCurrents).

For simplicity, we can divide the boundary conditions around a cell into two broad categories—those confined to within the Debye length of the plasma membrane (gray area in Fig. 1) and those forming diffusionary boundary conditions resulting from net differences in molecular transport (stippled area in Fig. 1). The conditions set up by surface voltages and charge accumulation remain a matter of debate and extensive modeling, but will have an impact only on the bulk concentrations within angstroms of the cell surface, and are not within the scope of the measurement techniques being reported here (*1*). This presentation focuses on the sensitive measurement of differential concentrations within the diffusionary boundary layer and exploits well-established principles of electrochemistry, operating in a noninvasive, self-referencing mode.

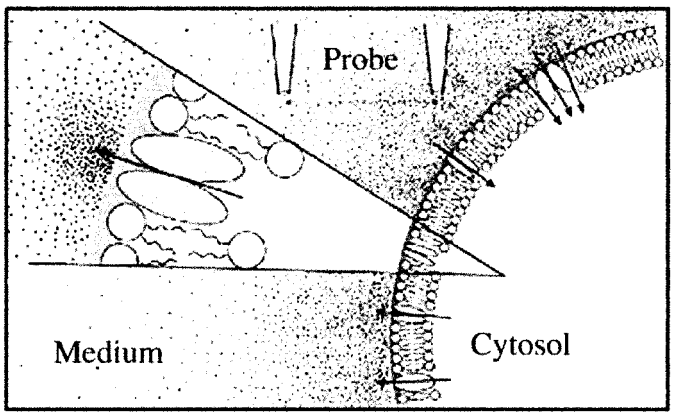

FIG. 1.

Self-referencing is a technique for making differential measurements in voltage or chemical gradients over micrometer distances. The strength of the approach is in minimizing the impact of drift on signal extraction. The approach uses the controlled motion of a single sensor (illustrated diagrammatically, and not to scale, in Fig. 1). The technique is now well established; it was originally developed for the detection of voltage fields (*2*), but more recently has been diversified to yield a quantitative and chemically selective methodology. Flux measurements of several ion species are now being made on a routine basis using the self-referencing approach, by employing commercially available liquid membranes. This ion-selective technique is a voltametric procedure based on the Nernstian characteristics of the sensors (*1, 3*). Recent applications of amperometric sensors have broadened the approach markedly, with boundary detection of several other molecules related to cellular metabolism and signaling. The first application used a self-referencing oxygen sensor to follow consumption at the single cell level (*4, 5*); the approach was subsequently diversified to cover nitric oxide (*6, 7*) and ascorbate (Pepperell *et al.*, unpublished results). Theoretically, any molecule with a characteristic redox potential can be examined by this approach.

As with ion-selective techniques, those based on redox potentials are limited. The scope of molecules open to detection can be expanded, however, by incorporating an enzyme-based reaction whereby electrically inert substances, such as glucose or lactate, can be reacted by virtue of an enzymic process to produce electrically active products. Glucose oxidase, for example, converts glucose to gluconic acid and hydrogen peroxide, with the latter being oxidized at +600 mV compared to a Ag/AgCl reference electrode. The current flow resulting from this process is

an indirect measure of the original glucose concentration. This approach has recently been developed successfully (*8*) and offers new horizons for analyzing the diffusionary boundary conditions of metabolites around single cells.

To date, self-referencing probes located outside a cell have not been used extensively while the intracellular or membrane conditions are being directly monitored. Some notable exceptions exist; for example, whole cell currents from clamped microglia have been correlated with transmembrane flux measurement deduced by self-referencing ion-selective electrodes (*9*). Hydrogen ion flux, based on the activity of the vacuolar-type ATPase, has been correlated indirectly with protein trafficking by pharmacological dissection and antibody binding (*10*), but no examples of simultaneous optical measurements based on fluorescent reporters and flux measurements have been published. Exploring the potential of hybrid sensors, combining intracellular reporting with extracellular flux measurements, to understand cellular signaling, structure and function, is now a major emphasis in the BioCurrents laboratory. We have successfully produced the prototypes of two electro-optical probe designs that are simultaneously capable of the localized imaging of the intracellular compartment and of collecting data on molecular flux. In both cases, these sensors require the delivery of excitation light through a single-mode optical fiber such that fluorescent reporters can be excited directly beneath the membrane area over which a flux signal is recorded. The first of these devices requires the optical fiber to be placed through the ion-selective liquid membrane held in a glass micropipette. The second requires that an amperometric sensor be constructed directly on the surface of the optical fiber. Both prototypes have been used successfully in recording internal calcium activity while measuring either calcium flux or oxygen consumption at the single cell level (work in progress).

In conclusion, electrochemical sensor designs offer a tremendous opportunity to follow the transport activities of single cells more closely. When used in a self-referencing mode, these sensors deliver excellent sensitivity and selectivity. Combined with optical measurements, particularly if extended to include cage compound release, reciprocal emission collection, and near-field scanning capability, self-referencing principles of boundary detection offer novel and unique approaches to the examination of cell signaling, structure and function.

Acknowledgments

The BioCurrents Research Center is a national resource of the NIH (NCRR) and encourages applications to visit and make use of the several techniques discussed above. Please contact the first author (psmith@mbl.edu). This work is funded by P41 RR01395 (NIH:NCRR to PJSS) and the German Science Foundation (AH).

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Optical Nanosensors for Single-Cell Analysis

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Fiber-optic chemical sensors and biosensors offer important advantages for *in situ* monitoring applications due to the optical nature of the detection signal. Our laboratory has been involved in the development of a variety of fiber-optic chemical sensors and biosensors (*1–6*). Recent advances in nanotechnology leading to the development of optical fibers with submicrometer-sized dimensions have opened new horizons for intracellular measurements. The application of submicrometer fiber-optic chemical probes has been pioneered by Kopelman and coworkers, who have developed probes for monitoring pH (*7*) and nitric oxide (*8*). The use of submicrometer tapered optical fibers has also been demonstrated and used to investigate the possible spatial resolution achievable using near-field scanning optical microscopy (*9*).

We have recently reported the development of nanosensors and *in situ* intracellular measurements of single cells using antibody-based nanoprobes (*3–6*). Biosensor/bioprobe technology has been at the forefront of analytical instrumentation research. The nanoprobe described in this work has antibody-based receptors targeted against benzopyrene tetrol (BPT). We have selected BPT as the analyte model system, because this species has been used as a biomarker of human exposure to benzo[a]pyrene (BaP), a polycyclic aromatic hydrocarbon (PAH) of great environmental and toxicological interest because of its mutagenic and carcinogenic properties and its ubiquitous presence in the environment.

A challenging aspect of this project involved the fabrication of reproducible nanoprobes for *in vivo* studies. Since optical fibers with submicrometer-size diameter cores are not available commercially, we have fabricated them by pulling larger silica optical fibers using a laser-based micropipette pulling device (Sutter Industries; Model P-2000) that has been optimized for pulling silica fibers. Optimization of this fiber-pulling device involved varying the following user input parameters: heat, velocity, delay and pull strength. Using this pulling method, fibers with submicrometer-diameter tips were produced. Figure 1 shows a scanning election micrograph of an example of a fiber probe fabricated using our fiber-pulling procedure. The scale on the photograph indicates that the distal end of the fiber shown is approximately 50 nm (Fig. lA). To prevent leakage of the excitation light on the tapered side of the fiber, the outside wall of the tapered end was coated with a thin layer of silver (100–300 nm), leaving the distal end of the fiber free. In this example, a 200–250-nm silver layer was deposited around the fiber. With the metallic coating, typical diameters of metal-covered bioprobes are less than 1 μm, of the order of 250–300 nm (Fig. 1B). The next step in the preparation of the bioprobes for the present work involved covalent immobilization of anti-BPT antibodies onto the fiber tip.

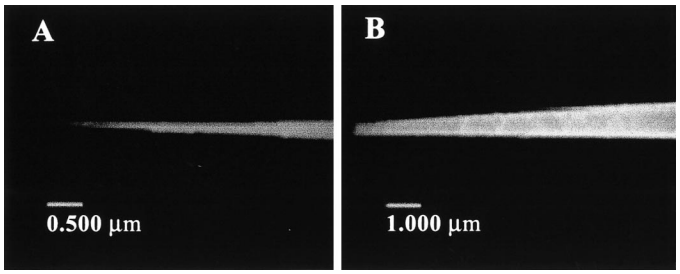

FIG. 1. Photographs of antibody-based nanoprobes. The small size of the probes allowed manipulation of the nanoprobes to sample specific locations within each Clone 9 cell.

To demonstrate the proof of concept of single-cell measurements with antibody-based nanoprobes, experiments were performed using cells of the rat liver epithelial Clone 9 line as the model cell system. The cells were incubated with BPT prior to the measurements. Interrogation of single cells for the presence of BPT was then carried out using antibody nanoprobes for excitation and a photometric system for fluorescence signal detection. Figure 2 shows a photograph of an antibody-based nanoprobe used to measure the presence of BPT inside a single cell. The small size of the nanoprobe allowed it to be manipulated to specific locations within the Clone 9 cells.

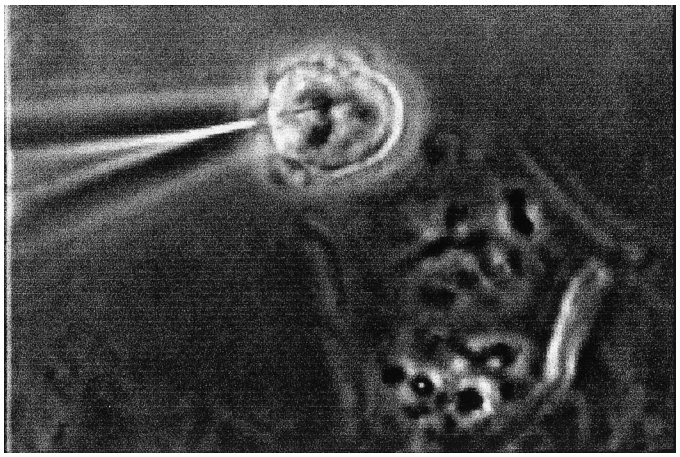

FIG. 2. Photograph of single-cell sensing using the nanoprobes.

The nanoprobes in this work are single-use bioprobes, because they were used to obtain only one measurement at a specific time and could not be reused due to the strong association constant of the antibody-antigen binding process. Multiple (e.g. five) recordings of the fluorescence signals could be taken with each measurement using a specific nanoprobe. We made a series of calibration measurements of solutions containing different BPT concentrations to obtain quantitative estimates of the amounts of BPT molecules detected. For these calibration measurements, the fibers were placed in Petri dishes containing solutions of BPT with concentrations ranging from 1.56×10^{-10} *M* to 1.56×10^{-8} *M*. By plotting the increase in fluorescence from one as a function of the concentration of BPT, and fitting these data with an exponential function to simulate a saturated condition, a concentration of $(9.6 \pm 0.2) \times 10^{-11}$ *M* has been determined for BPT in the individual cell investigated.

This study demonstrates the first application of antibody-based nanoprobes for measurements of chemicals inside single cells. Further studies will be conducted to improve the sensitivity and applicability of the technique. Such nanoprobes open new horizons to a host of applications in biotechnology, molecular biology research, and medical diagnostics. For instance, different antibodies as well as other bioreceptors such as DNA probes targeted to chemical species of biological and medical interest could be used in nanoprobe arrays to provide highly multiplexed probes for high-throughput drug discovery. In addition, this technique could be extended to nonfluorescent antigen species by using a second, fluorescently tagged antibody in a sandwich-type immunoassay, or by performing a competitive binding assay with a fluorescently labeled antigen. These nanodevices could also be used to develop advanced biosensing systems for studying *in situ* intracellular signaling processes and investigating gene expression inside single cells.

Acknowledgments

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Computational Dosimetry for Electron Microbeams: Monte Carlo Track Simulation Combined with Confocal Microscopy

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Both *in vitro* and *in vivo* experiments show that cells that do not receive energy directly from the radiation field (bystanders) respond to radiation exposure. This effect is demonstrated most easily with radiation fields composed of particles with high linear energy transfer (LET) that traverse only a few cells before they are stopped. Even at a moderate fluence of high-LET radiation, only a small fraction of cells in the irradiated population are hit; hence many bystanders are present. Low-LET radiation tends to generate a homogeneous distribution of dose at the cellular level, so that identifying bystanders is much more difficult than in experiments with the same fluence of high-LET radiation.

Experiments are under way at several laboratories to characterize bystander responses induced by low-LET radiation. At the Pacific Northwest National Laboratory, experiments of this type are being carried

out with an electron microbeam. A cell selected to receive energy directly from the radiation source is placed over a hole in a mask that covers an electron gun. Monte Carlo simulations by Miller *et al.* (*1*) suggested that individual mammalian cells in a confluent monolayer could be irradiated by 25 to 100 keV electrons with minimal dose leakage to their neighbors. These calculations were based on a simple model of the cellular monolayer in which cells were assumed to be cylindrically symmetrical with concentric cytoplasm and nucleus. Radial profiles, the lateral extent of cytoplasm and nucleus as a function of depth into a cell, were obtained from confocal microscopy of HeLa cell monolayers.

To obtain a better model of cellular monolayers, we have applied unstructured grids to define a coordinate system in the reconstructed image of a monolayer derived from confocal microscopy. Figure 1 shows grids on two adjacent cells in the same image of HeLa cells used by Miller *et al.* (*1*) to derive radial profiles. The cells are not overlapping; however, a part of the dark gray cell lies behind the light gray cell when viewed from this perspective.

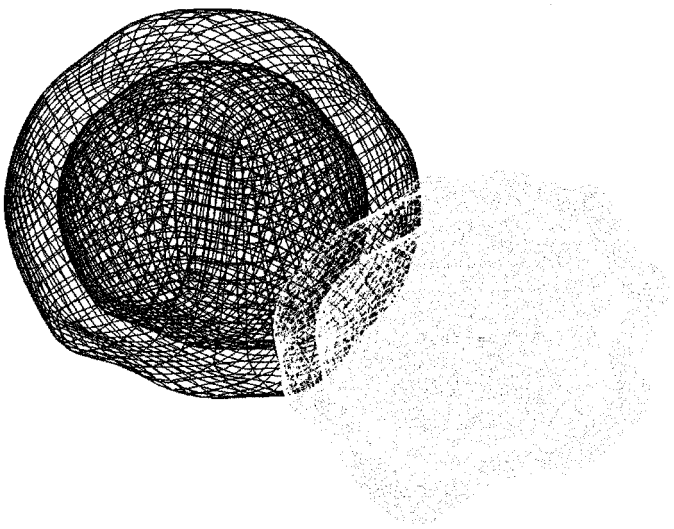

FIG. 1. Unstructured grids on images reconstructed from confocal microscopy of HeLa cells.

Cell morphologies quantified by these grids can be used to score virtual electron tracks generated by Monte Carlo methods. In principle, this approach could yield the probability that a specified amount of energy will be deposited in each volume element defined by the grid; however, in general, it is more meaningful to group all of the volume elements that define an organelle, such as the nucleus. The amount of cellular substructure that can be included by this method is limited only by the experimental capabilities of confocal microscopy.

Qualitative information concerning the feasibility of single-cell irradiation of a given cell type at various beam energies can be obtained with only a few confocal images that capture the typical morphology of the cells under investigation. Grids on these images allow the data from confocal microscopy to be merged with spatial patterns of energy deposition in a homogeneous water medium to provide a qualitative picture of the microdistribution of dose expected when a particular cell type is used in experiments with microbeam irradiation.

The spatial dependence of the mean energy deposited by 25 keV electrons injected into a uniform water medium has been published by Wilson *et al.* (*2*). Figure 2 shows these results superimposed on the images of HeLa cells shown in Fig. 1. Contours of equal dose in centigrays are shown in a view that is a vertical slice through the center of the cell on the left, which was selected for irradiation. A part of the cell on the right in Fig. 1 can be seen in this slice. Note that the dose to this cell, which was not targeted for irradiation by the electron microbeam, is predicted to be negligible. The dose averaged over the entire volume of the targeted cell is only 0.1 cGy; however, much higher doses are delivered near the beam entry point.

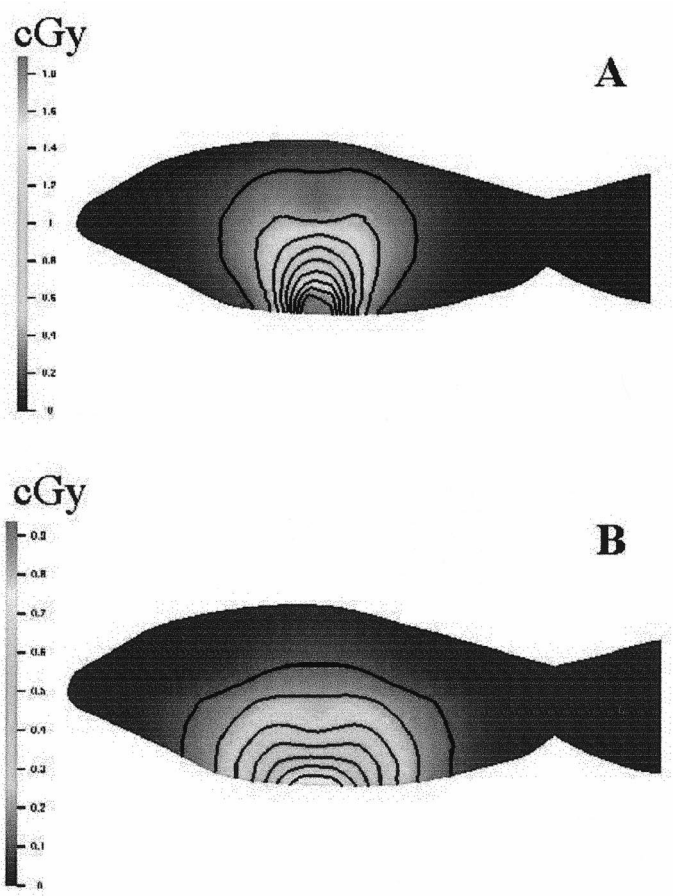

FIG. 2. Distribution of dose calculated for a HeLa cell irradiated by a 25 keV electron microbeam: (A) electrons injected at a single point; (B) electrons injected through a 2-μm-diameter hole.

Computational efficiency is a major advantage of the approach illustrated in Fig. 2. After a grid has been placed on the cell image, scenarios with different beam entry points, hole sizes, window thicknesses, and beam energies can be explored easily. Panels A and B of Fig. 2 illustrate the effect of the finite size of mask holes on dose distributions. Figure 2A shows results for 25 keV electrons injected at a single point. Figure 2B illustrates the more diffuse dose distribution expected when the microbeam has a diameter of 2 μm. In both cases, the window thickness is equivalent to 2.76 mm of water.

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Charged-Particle and Focused Soft X-Ray Microbeams for Investigating Individual and Collective Radiation Responses of Cells

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Until a few years ago, almost all research into the effects of radiation on cells and tissues employed conventional sources of radiation. This

meant that, at the cellular level, the passage of radiation tracks was random and unknown. Many of the effects of radiation occur very close to the track, and consequently conventional irradiation methods give information only about responses induced in a population of cells, each of which has been irradiated differently. When more specific information is needed, for example about the locations of sensitive targets, it is preferable to use a microbeam to irradiate preselected regions of the cell. The microbeam approach is of particular importance in studies related to radiation risk. This is because it is possible to determine the actions of single particle tracks and thereby mimic *in vitro* the exposure conditions that generally apply at doses of concern in radiation protection, especially those involving medium- to high-LET radiations.

In recent years, a number of groups in Europe, the U.S. and Japan have developed techniques for microbeam irradiation of cells. These systems take advantage of modern developments in particle delivery, focusing and detection, image processing and recognition, and computer control. Developments from other fields have contributed, for example, microbeam elemental analysis techniques (e.g. PIXE) and single-event upset testing of semiconductor devices. Also, in radiation biology, there have been important advances in the development of individual-cell assays, which allow a wide range of end points to be studied with improved accuracy at low doses.

We have developed two microbeam systems for cell irradiation. One uses charged particles (generally protons and helium ions) and provides a targeting accuracy of ± 2 μm. It also allows us to deliver precisely counted numbers of charged particles to each cell (0, 1, 2, etc.). Cell recognition and the imaging of cellular targets, as well as programmed irradiation of large numbers of cells, all take place automatically under computer control. The other system is generally similar to the charged-particle microbeam, but uses a finely focused beam of soft X rays which are brought to a focus using a zone plate diffraction device operating in first order. The zone plate images a spot source of 278 eV carbon K X rays produced by electron bombardment of a carbon target. The targeting accuracy of this device is within ± 0.25 μm, and it delivers up to about 20,000 carbon K photons per second, corresponding to a dose rate of ~ 2 Gy per second (expressed as an average throughout the nucleus of a cell). An outline of the system was given together with examples of data obtained in microbeam experiments, including effects of direct irradiation on cells and bystander-induced damage in neighboring unirradiated cells.

Overall, microbeam methods provide a new dimension in the study of the mechanisms of radiation effects at the cellular level.

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Induction of Bystander Mutagenesis by Single Alpha Particles

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Recent evidence indicated that extranuclear or extracellular targets might be important in mediating the genotoxic effect of ionizing radiation. It was found, for example, that very low doses of α particles induced clastogenic responses (principally sister chromatid exchanges) in both CHO and human fibroblast cultures at levels significantly higher than expected based on the number of cells estimated to have been traversed by a particle (*1, 2*). In other words, either cytoplasmic sites or an extracellular component may modulate the observed genotoxic response. The observation that cytoplasmic irradiation can result in gene mutations (*3*) provides circumstantial support of a mechanistic basis for the bystander effect. Using the Columbia University charged-particle microbeam and A_L human–hamster hybrid cells, which are sensitive in detecting mutagens that induce predominately multilocus deletions, we showed previously that in a population in which only 20% of the cells were irradiated with a near lethal dose of α particles, the resultant mutant yield was threefold higher than the expected yield, assuming no bystander effect (*4*). In other words, irradiated cells clearly induced a bystander mutagenic response in neighboring cells that were not directly traversed by α particles.

To ascertain whether the mutagenic response in bystander cells could be demonstrated with lower doses of α particles, we irradiated a selected cell population with a single α particle through their nuclei. We showed that irradiation of 5–20% of the cells, selected at random, with a single particle each resulted in mutant fractions that were significantly higher than expected assuming no bystander modulating effect. It is of interest to note that there is very little difference in the mutagenic yield when the fraction of irradiated cells varied from 10 to 100%. Furthermore, pretreatment of cells with the intracellular radical scavenger *N*-acetyl cysteine (10 m*M*) resulted in only a modest reduction in the incidence of the bystander mutagenic response. In contrast, A_L cells transfected with a dominant negative connexin 43 vector that shut down gap junction communication showed little or no bystander mutagenic response. Our studies provide clear evidence that a radiation dose as low as a single α particle can induce a large bystander mutagenic response in neighboring cells that are not directly traversed by α particles, and that gap junction cell–cell communication plays a critical role in mediating such phenomena. The results of our current studies address some of the fundamental issues regarding both the actual target and the radiation dose effect, and they are likely to have an impact on our understanding and assessment of radiation risk.

Acknowledgments

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Comets and Complexes as Complementary Indicators of DNA Repair in Individual Cells

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Survival of tumor cells exposed to DNA-damaging agents is generally dictated by the most resistant cells within the population. This makes analysis of population averages a poor indicator of response. The comet assay, a single-cell gel electrophoresis method, is able to measure initial DNA damage (strand breaks, crosslinks or base damage) in individual

cells; it can also provide some information on how cells handle that damage (rate and extent of ''repair'' of lesions, development of apoptosis/necrosis) (*1, 2*). Although it has found widespread applications in genetic toxicology and oncology, often the most useful feature of this method, the response of individual cells, is not reported. Heterogeneity in response can be used to identify hypoxic cells, noncycling cells, drug-resistant populations, and the response of cells with different DNA contents (*3*). Adaptations of this method may be useful in probing chromatin structure (DNA matrix attachment sites) and in identifying damaged genes (Comet-FISH) (*4, 5*). It is also possible to combine the comet assay with antibody detection of specific DNA adducts (*6, 7*).

The comet assay measures DNA damage and rejoining of breaks but provides little insight into fidelity of repair. It may be possible to obtain relevant information on repair fidelity by measuring the number of cells with DNA repair complexes or the number of complexes per nucleus as a function of time after exposure to genotoxic agents. We have recently begun examining the development of complexes containing replication protein A (RPA) (*8*). RPA is a single-strand DNA-binding protein that is essential for DNA replication, but also plays an important role in nucleotide excision repair, mismatch repair, and recombinational repair (*9–11*). Our recent results indicate that RPA foci form in S-phase cells in response to radiation doses as low as 50 cGy, and the number of foci/nucleus is linearly related to dose up to 50 Gy. The maximum number of cells with foci occurs 4–8 h after exposure to 4 Gy and subsequently declines. However, the number of RPA foci per nucleus (in those cells with foci) reaches a maximum after 2–4 h. RPA complexes do not form in G_1-phase cells until they enter S phase, and RPA foci are actively sequestered in micronuclei. The percentage of cells that retain complexes about 24 h after treatment may be an indication of which cells are likely to die. An interesting observation is that genomic instability may also trigger RPA complex formation, since RPA foci can reappear in the progeny of irradiated cells up to 10 generations after irradiation.

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Radiation-Inducible Clusterin (CLU): A Molecular Switch between Life and Death

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Stress-inducible gene products that interact with DNA double-strand break repair proteins, or that are secreted by damaged cells and subsequently interact with bystander cells, will be important to identify and functionally elucidate if we are to understand the overall responses of tissues and tumors to ionizing radiation. Using yeast two-hybrid analyses along with other molecular and cellular biology techniques, we cloned transcripts/proteins that are involved in, or that are presumed to affect, nonhomologous DNA double-strand break end-joining (NHEJ) repair mediated by the DNA-PK complex. Using G22P1 (also known as KU70) as bait, we isolated a number of KU-binding proteins. X-ray-inducible transcript/protein 8 [i.e. clusterin (CLU)] was shown to be associated with DNA-PK using confocal microscopy, co-immunoprecipitation and GST pulldown assays. A nuclear form of CLU (nCLU) prevented DNA-PK-mediated end joining and stimulated cell death in response to relatively low doses ($\geq$1.0 Gy) of radiation, or when it was overexpressed in the absence of radiation. Structure–function analyses using molecular and cellular (i.e. green fluorescence-tagged protein trafficking) biology techniques revealed that the ~47-kDa nCLU protein was inactive and resided in the cytoplasm of epithelial cells. After irradiation, CLU levels increased and an undefined post-translational modification(s) altered the protein, exposing nuclear localization sequences and coiled-coil domains. The modified, ~55-kDa nCLU protein translocated to the nucleus and triggered cell death, presumably through its interaction with G22P1. In dose–response experiments, radiation induced nCLU in direct proportion to lethality in human breast, colon and prostate cancer cells. An understanding of nCLU responses may be used to improve the anti-tumor efficacy of radiation, as well as chemotherapeutic agents (e.g. paclitaxel).

Interestingly, another radiation-inducible form of CLU was also noted. The secretory form of clusterin (sCLU) was observed to increase about twofold at 24–72 h after low doses ($\geq$0.02 Gy) of radiation in human breast or colon cancer cells. In dose–response studies, radiation induced dramatic levels of sCLU at doses that did not cause lethality. We speculate that sCLU may be a cytoprotective protein. Unlike nCLU, the sCLU protein does not interact with G22P1. Recent data strongly suggest that induction of sCLU after irradiation is negatively regulated by TP53, since expression of E6 in MCF-7 cells or somatically knocking out TP53 in HCT116 human colon cancer cells caused greater induction at earlier times than that seen in wild-type cells. *Cis*- and *trans*-regulatory elements within the CLU promoter are being elucidated. Since sCLU and nCLU originate from the same mRNA, we are interested in the stress-inducible molecular switch that regulates expression of this interesting protein. sCLU is known to interact with the TGFB receptor. We are currently exploring the possibility that this interaction leads to cytoprotective signaling responses that up-regulate anti-apoptosis proteins such as BCL2. Since fairly dramatic sCLU levels have been found in the medium of MCF-7 human breast cancer cell cultures after high doses of radiation, we are exploring the potential bystander effects of this protein at lower doses of radiation. Induction of CLU appears to be an important molecular switch within cells after irradiation.

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Antibody to γ-H2A-S$^{(4C)}$Q Detects DNA Double-Strand Breaks

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The introduction of double-strand breaks into the DNA of eukaryotic cells or organisms induces the immediate and extensive phosphorylation of a member of the histone H2A family on a serine situated four residues from its C-terminus. This serine residue is part of a consensus motif, ASQ(E,D)(I,L,Y,F)*, where ''*'' denotes the C-terminus (*1*). Due to the confusing state of histone terminology below the family level, the relevant histone H2A species have unrelated names in different organisms. The term H2A-S$^{(4C)}$Q is used here to include all the relevant H2A species and γ-H2A-S$^{(4C)}$Q for the phosphorylated forms.

In mammalian cells, half-maximal phosphorylation is reached by 1 min after exposure of cultures to ionizing radiation. About 0.03% of the H2A-S$^{(4C)}$Q molecules are phosphorylated per DNA double-strand break, a value corresponding to chromatin containing about 2×10^6 bp DNA. Thus large regions of chromatin and thousands of γ-H2A-S$^{(4C)}$Q molecules are associated with each DNA double-strand break. An antibody raised to γ-H2A-S$^{(4C)}$Q detects foci in nuclei after introduction of DNA double-strand breaks (*2*). The number of foci is consistent with the number of predicted DNA double-stranded breaks after various treatments. Individual DNA double-strand breaks are detectable.

To determine whether each γ-H2AX focus identifies the position of a DNA double-strand break, advantage was taken of the finding that γ-H2A-S$^{(4C)}$Q was formed when DNA double-strand breaks were introduced into cells by the BrdU dye–UVA light procedure of Limoli and Ward (*3*). A UVA pulsed laser (390 nm) commonly used in LaserScissors™ devices can be substituted for the UVA fluorescent light source (365 nm), and has the advantage of permitting the illumination of specific partial nuclear volumes as small as 0.5 μm in diameter. When MCF7 cells with DNA containing BrdU were exposed to the laser in the presence of the dye, those nuclear regions traversed by the laser bound the antibody to γ-H2A-S$^{(4C)}$Q. Formation of γ-H2A-S$^{(4C)}$Q was heavily dependent on BrdU, with 1% relative laser power being sufficient for the formation of γ-H2A-S$^{(4C)}$Q foci in the presence of BrdU while 30% was necessary in its absence. This latter result indicates that the laser microbeam technique is not limited to cycling cells but can be used, with higher energies, on cells that are unable to incorporate BrdU.

γ-H2A-S$^{(4C)}$Q foci form at the sites of DNA double-strand breaks. Formation of γ-H2A-S$^{(4C)}$Q is a rapid and sensitive cellular response to the presence of DNA double-strand breaks, a response that may be useful as a biodosimeter as well as providing insight into higher-order chromatin structures.

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Biosensing with Live Cells Using a High-Density Optical Fiber Array

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Imaging optical fibers are composed of thousands of individual and identical single optical fibers, each with a diameter of a few micrometers, coherently bundled together. An array of thousands of microwells can be fabricated on the tip of these imaging fibers. These microwells are fabricated by selectively etching the individual fiber cores on the distal tip of the imaging fiber. Single cells can be loaded into the resulting microwells. The microwells range in size from 3–25 μm such that the array can be used to accommodate different types of cells. The imaging fiber bundles can be used to analyze individual cells based on the ability of each individual fiber to carry its own light signal from one end of the bundle to the other. Thus, by containing a single cell in each individual microwell, the responses of all the cells in the array can be monitored simultaneously. Because the cells are distributed randomly into the array, each cell must be encoded with a unique dye or fluorescent protein to enable it to be identified. Each microwell, containing a single living cell, can be used to monitor several physiological and genetic responses simultaneously. A CCD detector is used to monitor and spatially resolve the fluorescence signals obtained from each cell.

Gene expression in single bacteria cells was monitored by fusing the *lacZ* reporter genes to different bacterial promoters. The green, yellow, red and cyan fluorescence reporter proteins were expressed in single cells and were used to encode the bacterial cells to identify their locations in the array. Several yeast strains were encoded using different fluorescent dyes. Other fluorescent dyes and fluorescent indicator nanobeads were immobilized to the yeast cell surface to measure O_2 and pH changes in the yeast microenvironment.

The use of imaging fibers for the rapid and repetitive optical analysis of large cell populations provides a powerful tool for examining the responses of single cells. The array can simultaneously measure responses of different cell strains in the same array and observe the unique multiple responses of each strain to temperature, pH, medium composition, and potential drug candidates. The resulting cell array can be employed for both screening and biosensing.

Computer-Assisted Reconstructions of Live, Crawling Cells (Including Nuclei and Pseudopods) during Metastasis, Chemotaxis and Embryogenesis

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We have developed both software and hardware that allow us to reconstruct live, crawling cells in 2D and 3D (*1–3*). The 2D software (2D-DIAS) automatically outlines cells every thirtieth of a second. Edge detection is automatic. The method used for automatic edge detection depends on the level of edge contrast. A ''pixel complexity measurement'' is effective in detecting the edges of cells imaged with differential interference contrast microscopy (*3*). The power of the 2D system is that it provides 40 computed parameters of motility and dynamic morphology every thirtieth of a second for up to 50 cells in parallel, and does it automatically and in near-real time. Its application thus far has been mostly in the analysis of cytoskeletal and regulatory mutants of *Dictyostelium* (*4, 5*), white blood cell motility and chemotaxis (*6, 7*), and larval behavior (*8*). Because it uses differential interference contrast optics, it also provides measurements of nuclear, pseudopod and vesicle dynamics. We have also developed a 3D system (3D-DIAS) that uses customized hardware and

software to section a cell optically in 1 or 2 s (30 to 60 optical sections through 10 to 30 μm in the *z* axis), and to reconstruct the cell, nucleus and pseudopods in 3D. This process is repeated at intervals as short as 1 s, leading to a 3D movie that must be visualized with polarizing glasses on a stereoworkstation. 3D-DIAS provides over 100 parameters of motility and dynamic morphology every second. 3D-DIAS has been used to characterize not only *Dictyostelium* mutants, but also differences between a carcinoma cell line that exhibits low levels of invasiveness and cells of the same line transformed with the vimentin gene, which exhibits high levels of invasiveness (*9*). The 3D computer analysis revealed a major role in nuclear orientation and localization in metastatic cell migration (Daniels *et al.*, manuscript in preparation). The cell line expressing keratin but not vimentin and exhibiting low invasiveness showed no polarity, a relaxed nucleus in the cell center, no dominant anterior pseudopod, and no persistent translocation along a substrate (Fig. 1A). Cells of this same cell line that were transfected so they express both keratin and vimentin were polar, had an unrelaxed nucleus that tracked the dominant pseudopod, and translocated along the substratum (Fig. 1B). Finally, we have recently developed a loosely distributed JAVA space cluster with customized parallel processing software that allows us to reconstruct and analyze the motion of every cell, nucleus, mitosis and pseudopod in a single live, developing embryo over time, and to reconstruct and track single cells in tissue. The participants in the workshop were provided with blue and red glasses to review examples of these dynamic reconstructions in 3D. In the future, researchers studying problems related to cancer should consider applying these systems to the general processes of mitosis, chromosome separation, and cancer cell migration and division, and to the specific effects that microbeams have on these processes.

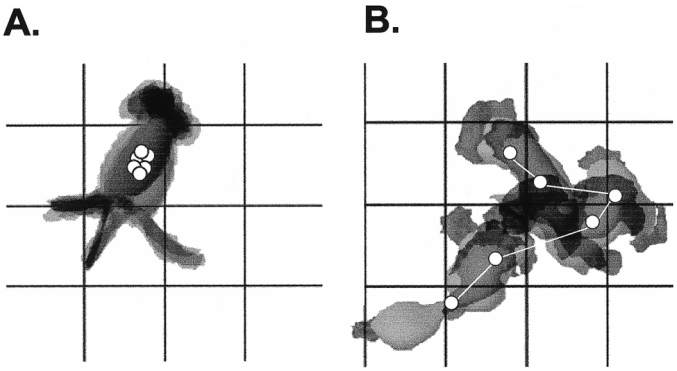

FIG. 1. Panel A: Human breast cancer cell line that expresses keratin. This cancer is nonmotile and relatively noninvasive. Panel B: The same cell line transfected so that it expresses vimentin. This cancer cell is motile and invasive. Cells were reconstructed in 3D at 20-min intervals. Dark gray regions, nonparticulate pseudopodial regions. White dots, centroids of cells.

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Quantitative Single-Cell Molecular Biology: mRNA to Protein—Results from Basic Science Studies and the Analysis of Human Disease

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Even in the simplest of diseases, where an individual protein may bear mutations that initiate the disease process, the coordinated dysregulation of multiple downstream genes and proteins usually results in the disease phenotype. Methods for analysis and comparison of the expression of multiple genes and proteins from normal and disease tissue are required to establish the set of genes that are coordinately regulated in the disease process. An added complication to this analysis is that individual diseased cells are mixed in a heterogeneous population of cell types, making it difficult to analyze the diseased cells independently of other cells. A further complication in the molecular analysis of human disease is the limited amount of well-characterized human tissue available for study. To address these issues, over the last several years, we have developed methods that allow the analysis of complex mRNA populations from discrete amounts of tissue, down to single cells and subregions of single cells. Additionally, these methods can be applied to fixed human tissue specimens, making it possible to analyze the mRNA complement of pathological tissue samples. In our clinically related studies, we have, for example, determined preliminary molecular fingerprints of tangle-bearing neurons in Alzheimer's disease, entorhinal cortex neurons in schizophrenia, and individual striatal neurons as a function of Huntington's disease. Since it is possible to characterize all of the changes in mRNA abundance in even a single cell, the real challenge before us is the interpretation of these complex mRNA abundance data sets and sorting through the data to find the differences in mRNA abundance that are diagnostic for the disease. Indeed, it is hoped that such changes will suggest potential therapeutic targets that can be manipulated pharmacologically to alter the course of disease pathology.

In addition to our continuing expression-profiling efforts, we have developed a novel proteomics methodology (IDAT) that now permits the detection and quantification of multiple proteins from protein lysate isolated from a single cell. This is an important area for technology development, since changes in mRNA abundance often do not reflect changes in protein levels and cannot predict the post-translational modifications that proteins are subject to. Using this IDAT methodology, we can also detect post-translational modifications of proteins. Further,

we have reduced the output of this assay to a fluorescence-based system that permits the quantification of protein amounts in approximately 15 min. We are currently robotizing the procedure so that we will be able to detect and quantify thousands of proteins simultaneously in approximately 15 min. We are actively applying this methodology in our studies of the neuronal dendrite in the hope that we will be able to generate both an mRNA and a proteomic analysis of dendritic function.

The Single-Cell Proteome Project

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Now that the sequence of the human genome has been published, the next task facing the bioanalytical community is the understanding of gene expression and function. While mRNA studies have been valuable in understanding changes in gene transcription in response to environmental changes, mRNA expression is not necessarily well correlated with protein expression. Most importantly, mRNA expression is silent on post-translational modification of proteins, such as proteolysis, phosphorylation and glycosylation, which often determines the protein's activity and function.

Proteomic research has taken on an added sense of urgency with the annotation of the human genome. The human genome contains only twice as many genes as are found in much simpler invertebrates such as *C. elegans* and *D. melanogaster*. To create the complex physiology of humans and other vertebrates, it is likely that many genes generate a number of protein products. These products may result from alternative splicing of introns or from extensive post-translational modification. Study of the proteome is the most straightforward means of identifying these products.

The tools available for proteome research today resemble the tools used for genomic analysis two decades ago. Cumbersome two-dimensional gels require extensive manual manipulation. Once the electrophoresis is completed, the gels must be stained and the resulting spots quantified, usually through visual inspection. Spots of interest are excised, proteins are extracted and digested, and MALDI or electrospray mass spectrometry is used for identification. Proteomics is as much an art as a science. Analysis is handcrafted, and proteomic research is essentially a cottage industry.

Proteomic research is inherently more difficult than genomic research. Unlike DNA, which can be amplified by either cloning or PCR, proteomic research must be performed on the material obtained from an organism. As a result, sensitivity is a fundamental issue in proteomic research. Classic two-dimensional gels require the protein extracted from millions of cells. All protein expression studies are averaged across the cell population.

Modern Instrumentation for Proteomic Analysis

This research group has been developing technology for proteomic research, and this technology is directed along four paths to address the major limitations of proteomic research. First, we are developing ultrasensitive laser-induced fluorescence technology to provide proteome analysis from a single somatic cell. Second, we are developing automated capillary electrophoresis instruments to perform two-dimensional protein analysis without the need for operator intervention. Third, we are developing multiple capillary instruments that will allow the simultaneous analysis of large numbers of samples; like the commercial DNA sequencers that have been so successful in genomic research, these multiple capillary protein analyzers would analyze 96 samples simultaneously. Last, we are creating green fluorescent protein hybrids with low-expression-level regulatory genes; these products, their post-translational modifications, and their complexes with other proteins can be determined at the single copy level.

Single-Cell Proteomics

We have generated one-dimensional capillary electrophoresis data from single cells obtained from a cancer cell line. We work with HT29 human adenocarcinoma cells. In our single-cell proteomic technology, a single cell is subjected to the following steps: (1) cell characterization; (2) cell injection into the capillary; (3) cell lysis; (4) fluorescent labeling of the resulting proteins; (5) apillary electrophoresis on the proteins; and (6) detection by laser-induced fluorescence.

Briefly, we characterize the cells by determination of DNA ploidy. The cells are treated with the DNA-intercalating dye Hoechst 33342. The treated cells are inspected with an epi-illumination fluorescence microscope, and a photomultiplier tube is used to measure the fluorescence intensity, which is used to estimate the amount of DNA within the cell, and hence to determine the phase of the cell in the cell cycle.

A cell is injected into the capillary by first using micromanipulators to center the capillary over a cell of interest. Then a computer-controlled pulse of vacuum is applied to the distal end of the capillary, which draws the cell reproducibly and automatically $\sim$300 μm into the capillary.

The capillary is filled with electrophoresis buffer, which usually contains a surfactant, such as SDS. This surfactant diffuses into contact with the cell, causing the cell to lyse within 30 s of injection into the capillary.

Proteins are fluorescently labeled with the fluorogenic reagent 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ). This reagent is nonfluorescent until it reacts with a primary amine in the presence of a nucleophile, which causes a ring-closure reaction and a dramatic increase in absorbance cross section and fluorescent quantum yield. This reaction is rapid at elevated temperatures, and we typically perform the reaction at a temperature from 50 to 95°C. The reaction is performed within the capillary. Once the cell has been injected, a plug of the derivatizing reagent is injected into the capillary and allowed to mix with the proteins. The reaction requires 30 s to 5 min to complete.

We have several forms of electrophoresis available for single-cell analysis. Initially, we used free-solution electrophoresis in the presence of a submicellar surfactant concentration. This buffer system eliminated the band broadening associated with incomplete labeling of proteins with derivatizing reagent. More recently, we have used a form of capillary SDS-PAGE, in which proteins are separated in a semi-viscous polymer solution. This material provides a rapid protein separation based on the molecular weight of the protein.

Detection is by laser-induced fluorescence performed in a sheath-flow cuvette. This instrumentation, borrowed from the flow cytometry community, produces exquisite sensitivity when used as a detector for capillary electrophoresis, has allowed the detection of single phycoerythrin molecules separated by free-solution electrophoresis, and was the enabling technology used to sequence the human genome.

Using this instrumentation, we have obtained one-dimensional capillary electropherograms from a single human cancer cell and from an embryo of *C. elegans* at the single-cell stage. These experiments reveal significant cell-to-cell variations in protein expression. Much of this variation is correlated with the different positions of the cells in the cell cycle, but it is likely that additional cell-to-cell variations are due to variation in the confluence of the cultures and other differences in the handling of the cells.

Automated Protein Analysis

Identification of proteins separated by capillary electrophoresis remains a significant issue. We have resorted to performing two-dimensional gel electrophoresis on cell extracts, excising spots, using classic protein extraction, digestion, and mass spectrometric analysis on the majority of the protein, and using a small fraction of the extracted protein to spike a sample prepared for capillary electrophoresis. This technology is unacceptably cumbersome, and we are working with Ruedi Aebersold of

the Institute for Systems Biology to interface our capillary electrophoresis instruments with mass spectrometer for online protein identification.

We are also developing automated two-dimensional electrophoresis instruments based on successive separation of proteins in two capillaries. In principle, the first capillary can perform isoelectric focusing on a protein sample. A single fraction can be eluted from the first capillary and transferred to a second capillary, where another electrophoresis technique can be employed, such as SDS-PAGE. Once the second-dimension separation is complete, another fraction can be eluted from the first capillary and subjected to the second-dimension separation. We have achieved a proof-of-principle separation based on this technology. Submicellar electrophoresis was performed in both dimensions with different pH buffers. We are in the process of incorporating both SDS-polymer and isoelectric focusing in the first dimension, with free-solution electrophoresis in the second dimension.

Multiple Capillary Protein Analysis

Our research group has developed a number of multiple capillary electrophoresis instruments for high-throughput DNA sequencing. These instruments represent a powerful tool for large-scale protein analysis, wherein a large number of samples may be analyzed in parallel. To put this technology into perspective, a 96-sample instrument, when combined with our two-dimensional protein analysis technology, could perform nearly 1,000 two-dimensional electrophoresis runs per day. This fully automated instrument would likely have the same throughput as all of the two-dimensional gels being run in the world today, and such instrumentation would revolutionize proteomic analysis, just as multiple capillary DNA sequencers revolutionized genomic analysis.

We also believe that this instrument could be applied to single-cell analysis, wherein 96 cells are analyzed in parallel. Sample injection will be by means of an array of hybridization pads formed on a microscope slide. The pads would be coated with antibodies to a specific cell surface marker and would hold the cells in a regular array, which would mate with a corresponding array of capillaries.

Single-Copy Detection

All proteomic tools analyze the most highly expressed proteins. Issues of sensitivity and selectivity plague attempts to monitor low-level regulatory proteins. These proteins do not generate sufficiently large signals to be detected above the background, and if their signal could be detected, it would likely be buried under the signal of another, more highly expressed, protein. We have begun a project to detect selected proteins at the single-copy level, to monitor their post-translational modifications, and to detect their complexes with other proteins. In this technology, GFP is fused to the protein of interest using integrative plasmids, so that the expression of the hybrid is under the control of the selected gene's promoter. Single-cell capillary electrophoresis is performed with an ultrasensitive laser-induced fluorescence detector. This instrumentation can detect and count each copy of the GFP hybrid. Capillary electrophoresis is vital for two reasons. First, it allows us to distinguish between GFP-based fluorescence and autofluorescence, which is particularly problematic in *S. cerevisiae.* Second, it allows us to observe different GFP-containing species. For example, in the worst-case scenario, proteolysis may degrade the target protein but leave GFP intact; in this case, fluorescence would be unrelated to the expression of the protein of interest. Also, capillary electrophoresis allows us to monitor more subtle post-translational modifications of the target proteins. Most interestingly, our preliminary results demonstrate that capillary electrophoresis is sufficiently gentle that protein–protein complexes may be preserved and monitored during electrophoresis.

Conclusions

Several research projects are under way in our laboratory to develop proteomic tools with six orders of magnitude higher sensitivity and three orders of magnitude higher throughput than current tools. These tools, when successfully developed and disseminated, will revolutionize proteomic research, much as modern bioanalytical instrumentation has revolutionized genomics research.

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