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Microarray Image Scanning

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Summary

Of the technologies available for measuring gene expression, microarrays using cDNA targets is one of the most common and well-developed high-throughput techniques. With this technique, the expression levels of thousands of genes are measured simultaneously. DNA probes are immobilized on solid surfaces, either membrane-based or chemically coated glass surfaces. On glass arrays, the probes are hybridized with fluorescent-labeled target samples. Fluorescence intensities, which reflect gene expression levels, are detected by imaging the array using a laser or white-light source and capturing the image using photomultiplier tube detection or a charge-coupled device camera. Different laser-based scanners are used in laboratories to scan microarray images. This chapter discusses the imaging process and the protocols being developed.

Key Words: Genomics; microarray; laser scanners; imaging; gene expression; fluorescence.

1. Introduction

From data derived from completion of the Human Genome Project, we know that there are approx 40,000 genes in human cells and that hundreds and thousands of these genes interact to perform diverse cellular activities. Microarray or DNAchip technology is a recently developed technique that allows biologists to obtain a bird's-eye view of the expression of thousands of genes in a single experiment. This technique has recently been used to address many biological issues, such as discovery of genes involved in certain diseases and molecular classification of cancers *(1–5)*. The two platforms used with this technique are the membrane-based (porous surfaces like nylon) and chemically coated glass arrays. In both cases, thousands of DNA probes are immobilized robotically and hybridized with either ^{32}P- or ^{33}P-labeled targets in the case of membrane arrays or fluorescent-labeled cDNA targets in the case of glass arrays. With the glass microarrays, where fluorescent-labeled samples are hybridized, the signal

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intensities are measured with fluorescence laser scanners. Detection of signals in glass microarray images is accomplished by measuring fluorescence intensities with scanners that allow simultaneous determination of the relative expression levels of all the genes represented on the array. This technique is the focus in this chapter.

The imaging technique requires light of a specific wavelength to excite the fluorophores in the sample, and scanners can accomplish this by using one of two techniques. Most scanners use laser sources to excite each fluorescent probe, and emitted light is detected by a photomultiplier tube (PMT). This method uses a laser-based scanning system that involves the rapid movement of small points of laser light across the sample, from which the image is then reconstructed. The second imaging technique is based on a charge-coupled device (CCD). Scanners that use this method have a white-light arc lamp as their illumination source. Because white light encompasses all wavelengths in the visible spectrum, it is possible to select the wavelength of choice. Fluorescent light emitted from the sample is collected and imaged using CCD cameras.

In this chapter, the protocol for scanning will be elaborated for the laser scanner using PMT detection.

2. Materials

A total of 2304 known sequence-verified human cDNAs were prepared by polymerase chain reaction (PCR) from the cDNA clone library (Research Genetics, Huntsville, AL) using two primers on the vector. The DNA clones, in 384-well plates, were spotted onto poly-L-lysine-coated microscope slides using an arrayer (Genomic Solutions, Ann Arbor, MI). All clones except those of the controls were duplicated on the array. After printing, the slides were dried and crosslinked by ultraviolet radiation at 650 J/cm^2. The slides were then washed with water, dried, and stored for later use.

Cyanine-dye-labeled reverse-transcribed target cDNA sample in 130 μL of total volume of Express hyb solution (Clontech, Palo Alto, CA) with a mixture of blocking reagents was hybridized to the glass microarray slides. After hybridization, the slides were preprocessed for scanning. Laser scanners with appropriate laser sources for scanning (532-nm excitation for Cy3 and 695-nm excitation for Cy5) were used for scanning the microarray image. Quantification of the spots was carried out using an image analysis software product, ArrayVision (Imaging Research, Inc. Ontario, Canada).

3. Methods

The most frequently used method of labeling gene products or mRNA is to incorporate cyanine (Cy)-labeled nucleotides into cDNA during reverse

transcription of the mRNA. The most common fluorophores are Cy3 (green-excitation light at 532 or 545 nm) and Cy5 (red-excitation light at 635 nm). Microarray scanners capture the Cy3 and Cy5 signals on each spot of the microarray, using either the laser light of the specific wavelength to excite the fluorophores or white light, for which the choice of wavelength is flexible. Superimposing the two images from Cy3 and Cy5 channels provides a composite image that allows straightforward visual estimation. If we color the Cy5 image red and the Cy3 image green, an intense green or red spot will signify a higher level of that particular gene in one of the two samples. A yellow spot will indicate similar expression of the gene in the two samples.

Several microarray scanners are available and appropriate for use in this technique, including a DNA microarray scanner (Agilent Technologies, Palo Alto, CA), ArrayWorx (Applied Precision, Issaquah, WA), ChipReader (Virtek Vision Corporation, Ontario, Canada), GenePix 4000B (Axon Instruments Inc., Foster City, CA), GeneTac LSIV and UC-4 (Genomic Solutions Inc., Ann Arbor, MI), and ScanArray (Packard Bioscience, Billerica, MA). The specifications for these scanners are listed in **Table 1**. Each scanner has unique features, and several factors should be considered when choosing one ***(6)***.

3.1. Signal Detection

3.1.1. Light Source

Laser light is a common light source in which a small point of light moves through the entire microarray slide and reconstructs the image from this point measurement. The laser source is preselected on the basis of excitation and the emission wavelength of the fluorescent probes. Another type of light source is broad-spectrum white light, which is used in scanners such as the ArrayWorx scanner. The advantage of this type of scanner is its flexibility: it offers selection from 330 to 700 nm for excitation and from 380 to 800 nm emission wavelengths via filter selection.

3.1.2. Photon Detection

Two main optical modes are used to detect signals from the microarray slides. Most laser-based scanners use a PMT detector and a scanning process that involves several steps:

$$\text{Dye} \xrightarrow[\text{Excitation}]{\text{Laser}} \text{Photons} \xrightarrow[\text{Amplification}]{\text{PMT}} \text{Electrons} \xrightarrow[\text{Filtration}]{\text{Converter}} \text{Signal}$$

The classic photomultiplier contains a light-sensitive photocathode that generates electrons when exposed to light. These electrons strike a charged electrode, called a dynode, and produce additional electrons. PMTs contain several

Table 1
Specifications for Six Microarray Scanners

	Agilent scanner	ArrayWorx	GenePix 4000B	Gene TAC LSIV	GeneTac UC-4	ScanArray Express	ChipReader
Light source	Laser (for Cy3 and Cy5)	White light	Laser (532 and 635 nm)	Laser (488, 494, 532, and 635 nm)	Laser (532 and 635 nm)	Laser	Laser
Detection	PMT	CCD	PMT and voltage adjustment	PMT	PMT	PMT and laser power adjustment	PMT
Imaging	Simultaneous	Stitch-by-position	Simultaneous	Dark-field, sequential	Dark-field, sequential	Confocal, sequential	Simultaneous
Field focus (μ*M*)	Dynamic autofocus		60 (thick specimen)	100	100	Autofocus	10 (small depth)
Pixel resolution	5, 10 μ*M*	3–26 μ*M*	5–100 μ*M*	1–100 μ*M*	1–100 μ*M*	5, 10, 20, 30 and 50 μ*M*	5, 10, 20, 30 μ*M*
Scan speed	~8 min/slide	>10 min/slide	~5 min/slide	~5 min/slide	~5 min/slide	~5 min/slide	~5 min/slide
Dimensions $l \times d \times h$ (in.)	24 × 36 × 24	31 × 23 × 23	13.5 × 8 × 17.5	36 × 24 × 22	24 × 16 × 12	30 × 16.5 × 14	11 × 9 × 12
Type of autoloader	48-Slide carousell	40-Slide carrier	Single slide	24-Slide carrier	4-Slide carrier	Single slide	Single slide

dynodes, so the electrons emitted are amplified at each dynode, thus amplifying the signal. The electrons are collected at the anode and output as a current that is proportional to the intensity of light that strikes the photocathode. Thus, the PMT amplifies and measures low levels of light. Some scanners, such as the GeneTac LSIV and UC-4 scanners, increase the efficiency of the PMT detector by using a side-window PMT detector to minimize photobleaching and dark-current noise.

Some laser/PMT-based systems, such as the ScanArray scanners, use confocal optics to sharpen the focus of the laser beam. This type of scanner focuses light at both the excitation end on the substrate and at the emission end on the PMT detector. The laser light, focused through a pinhole to restrict the focal length and reduce imaging artifacts, induces fluorescence. The emitted light is then collected by an objective lens and converged through another pinhole to the detector. However, if the substrate surface has large variations in thickness, the intensity measurements can differ depending on the focal plane.

The second method for collecting fluorescent light from a sample is by using a high-performance CCD camera. This is the technology used in the ArrayWorx scanner. A white-light beam is passed through an excitation filter, yielding monochromatic light of the appropriate wavelength to excite the molecules on the array slide. The emitted light is focused onto a CCD camera via emission filters. In the ArrayWorx scanner, the excitation beam is passed through a specifically configured fiberoptic bundle delivering highly stable light onto the sample. The CCD camera collects light from small panels on the slide, and these panels are tiled to reconstruct the image. This method is often referred to as "stitch-by-position." The disadvantage of this system is that fewer emission photons are generated than with the PMT, in which the signal is amplified. To overcome this weakness, CCD systems integrate the signals over a period of time, thus prolonging the scan time. This long exposure time increases the dark current, which is the generation of electrons from heat at a constant rate. Because dark-current electrons are indistinguishable from electrons generated by photons, more noise is introduced. To minimize this noise, the CCD system must be maintained at a low temperature. The above-discussed optics are shown in **Fig. 1**.

3.2. Scanning

3.2.1. Simultaneous and Sequential Scanning

Among the laser-based scanning systems available, some scan two dyes simultaneously, whereas others (sequential scanners) scan one dye at a time. Simultaneous scanning is fast and requires no image alignment after the scanning is completed, whereas sequential scanning acquires one image at a time and builds a composite image. Crosstalk between the two optical channels is

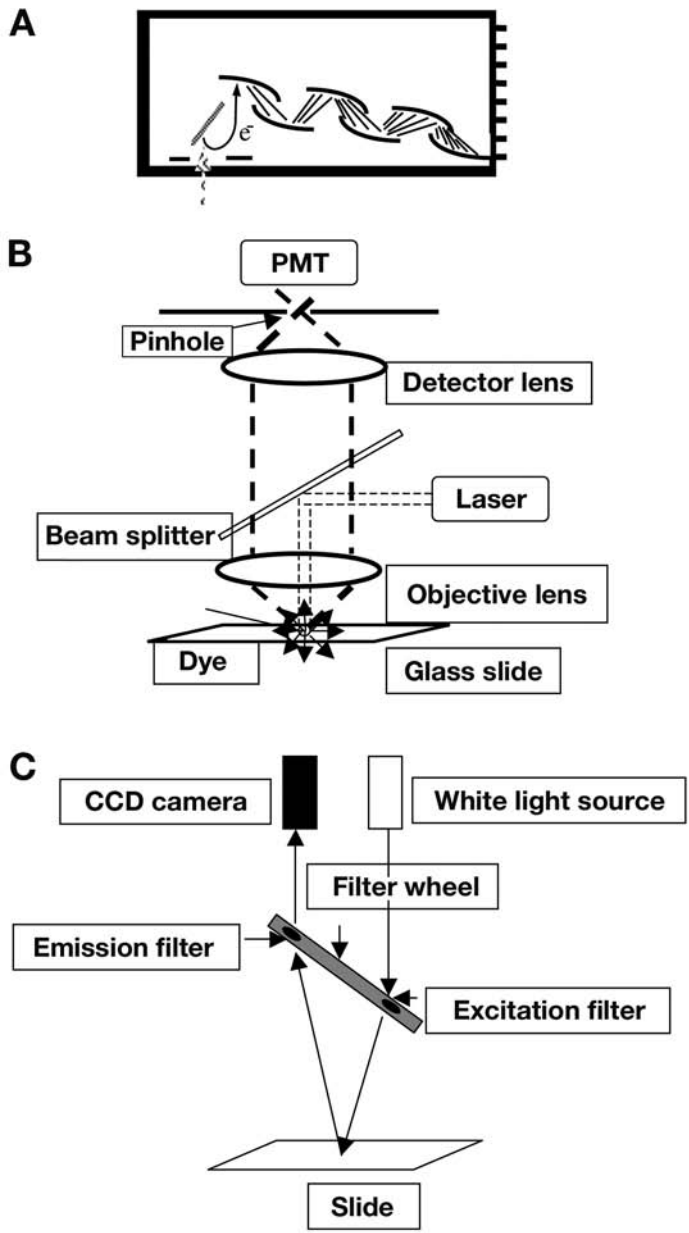

Fig.1. **(A)** The PMT amplifies electrons through dynodes, using the side window to minimize photobleaching, as in the Genomic Solutions GeneTAC UC-4 system. **(B)** The optical path in a confocal imaging technique showing the objective lens and the detector lens, and the convergence of the incident and the emission light through pinholes. **(C)** In the optical path of a CCD system, a white-light beam is passed through an excitation filter, and the chosen wavelength of light illuminates the sample on a glass slide. The light emitted through the emission filter is then focused onto a CCD camera.

not a concern for sequential scanning but could cause problems with simultaneous scanning. To overcome this problem, simultaneous-scanning devices use a laser wavelength of 532 nm instead of 545 nm to excite Cy3 and, thus, allow better spectral separation between Cy3 and Cy5, whose excitation wavelength is 635 nm.

3.2.2. Features of Scanning Devices

Two other scanning features are the stitch-by-position technology in CCD camera detection and the confocal scanning in PMT detection. Dark-field imaging and laser-beam scanning with PMT detection are combined in the GeneTAC LSIV and UC-4 scanners. In the dark-field approach, the laser beam is angled so that the backscattered light cannot reach the PMT, which improves the contrast and the signal sensitivity. As a drawback, these technologies yield images that are dimmer than those projected from other scanners, so the slides require longer exposure to the laser. However, the improved side-window PMT system compensates by preventing photobleaching. In addition, the system has an increased focal depth of approx 100 μm. Thus, there is no variability resulting from uneven slide surfaces.

3.3. Signal-to-Noise Ratio

The goal of scanning is to produce a high-quality image from which gene expression information can be acquired. The quality of an image is judged by its signal-to-noise ratio. Common sources of noise are thermal emissions or dark current, both of which occur in the PMT and CCD camera. The GeneTAC LSIV and UC-4 scanners use side-window PMT detection to reduce this noise, the GenePix 4000B scanner reduces the time the laser spends on each pixel, the ScanArray scanner uses a small depth of focus, and the ArrayWorx scanner uses a thermoelectrically cooled camera to reduce the dark current. Another kind of noise, called shot noise, is caused by the particle nature of light. This is the most dominant type of noise affecting the signal-to-noise ratio. The absolute magnitude of this noise increases with increasing signal intensity; however, the noise only increases by the square root of the signal intensity; thus, the signal-to-noise ratio decreases as the signal intensity increases.

3.4. Comparison of Scanners

The whole process of microarray technology involves many different steps, which is why many of the current protocols are standardized to minimize experimental variations. Standardized procedures are essential for compatible data production, quality control, and analysis. However, reliability of the data from experiment to experiment and comparison of data from laboratory to laboratory or instrument to instrument has not been extensively evaluated. A commonly accepted standard with which to compare the performances of various scanners is presently lacking.

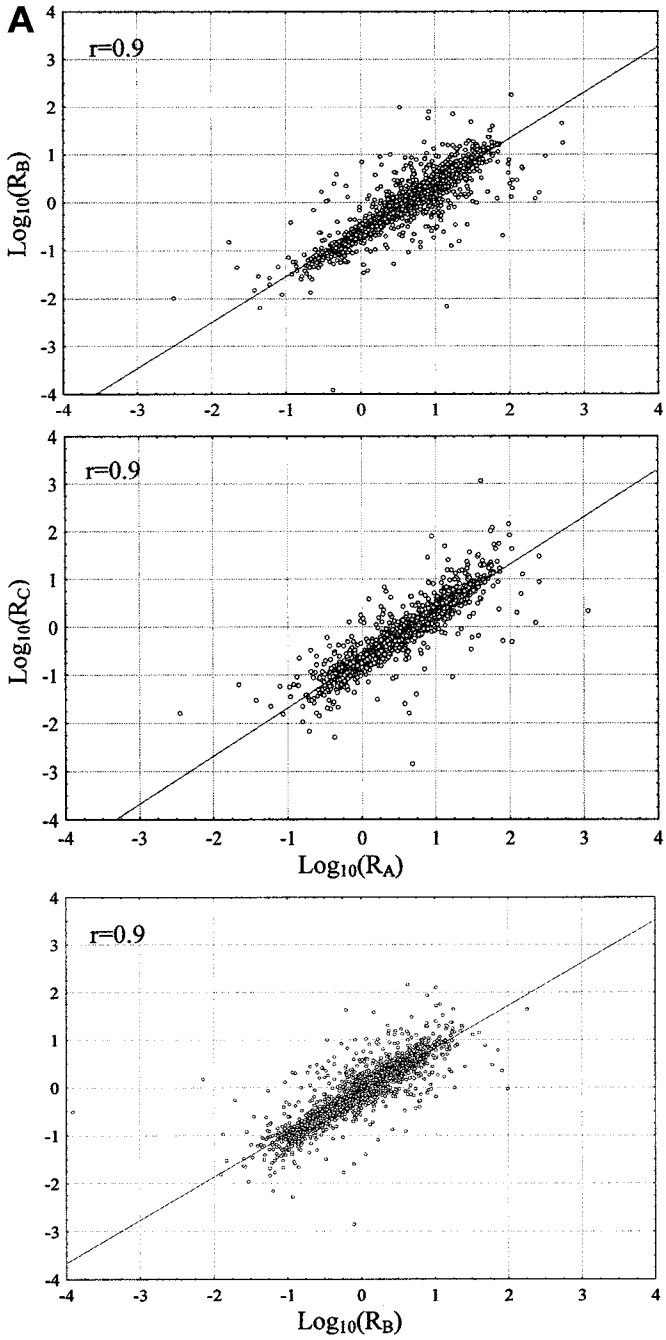

Fig. 2. Correlation analysis among three scanners at **(A)** 20 µm and **(B)** 10 µm resolution. The correlation coefficients *(r)* are labeled in each of the comparisons.

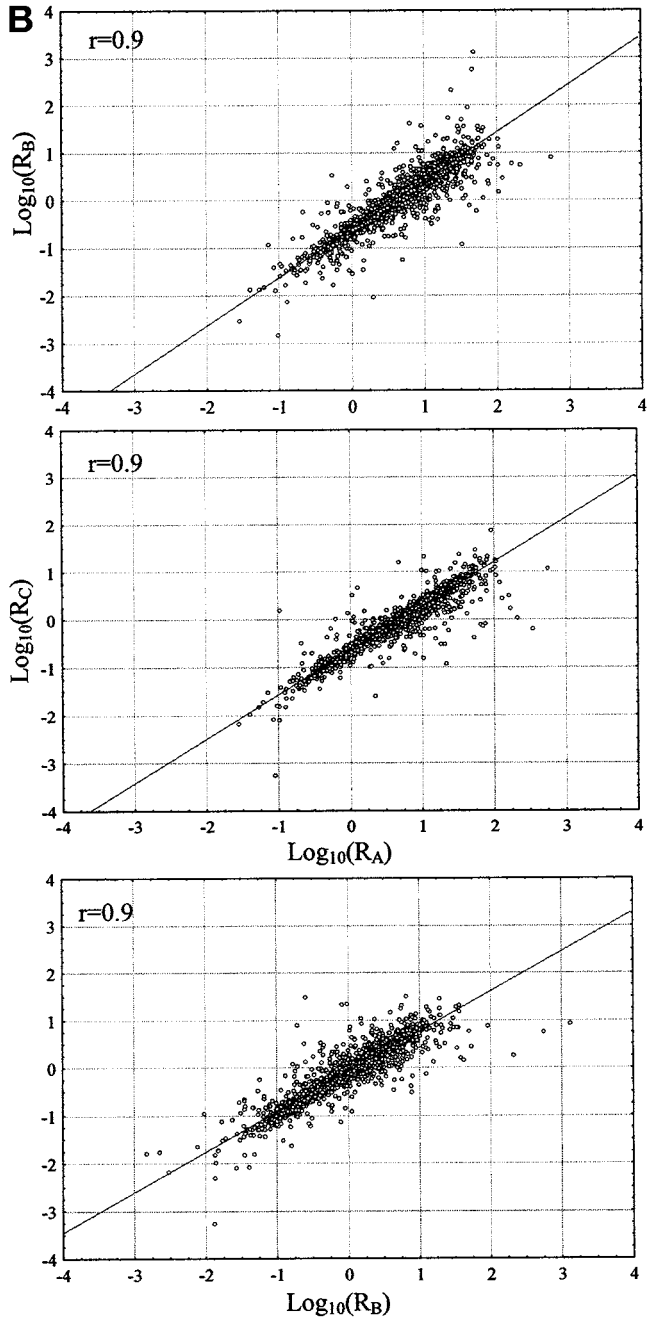

Fig. 2. *(Continued)* R_A, R_B, and R_C represent data obtained from scanners A, B, and C, respectively. $Log_{10}(R)$ is the logarithm-transformed intensity ratio of the two channels. A linear regression line is shown in each plot.

To evaluate the performance of different scanners and compare the results, we scanned a single microarray slide on three different scanners. Our analysis involved the GeneTAC LSIV scanner (A), which combines a laser-beam source, dark-field imaging, sequential scanning, and PMT detection, the GenePix 4000B scanner (B), which uses a laser source, simultaneous scanning, and PMT detection, and the ScanArray scanner (C), a laser scanner with confocal-imaging technology and PMT detection *(7)*. We scanned the slide on the three devices using comparable resolution, retrieved the tagged image file format (TIFF) images, and quantitated the thousands of spots on the array using the spot-finding software, ArrayVision (Imaging Research Inc., Ontario, Canada). The fluorescence intensities from each spot on the array were measured as sVol, which was the background-corrected volume, where volume is the density of each spot multiplied by its area and density is the average of all pixel intensities in the spot. The ratios of sVols from the two channels (Cy3 and Cy5) were used as measures of the relative expression levels, and the data were transformed into logarithmic values. The log-transformed intensity ratios from the three different instruments were then compared. When all three scanners were used, the correlation coefficient was between 0.90 and 0.96 (*see* **Fig. 2**); when images and data were obtained using the same scanner at different times, the correlation coefficient was approx 0.93. Thus, the instruments used did not cause any variability in results. We also compared the genes that were differentially expressed between the two cohybridized samples acquired from the three scanners. Among the 160 most differentially expressed genes, 95% of the genes were identified from all three analyses. Thus, all three instruments identified nearly all of the same differentially expressed genes.

3.5. Setting Scanning Parameters

This subsection tests the steps that need to be considered when using a sequential scanner, which combines dark-field imaging and laser-beam scanning with PMT detection, to obtain raw signal intensity data.

1. The computer and the scanner are turned on in that order to prewarm for scanning.
2. The scanning program controls the action on the laser scanner. Thus, using the program, the machine is set ready to scan. The microarray slides are loaded on the slide holder and, when ready, the shutter is closed to latch (*see* **Note 1**).
3. *Scan dimensions:* Scan dimensions, which define the area on the slide to be scanned, are set and the image captured. For a standard slide (25 mm × 76 mm), the dimensions depend on the area on the slide that the actual array occupies (approx 20 mm × 70 mm) and the values 1248 × 2348 are set on the program for 20 μm resolution.

4. *Scan resolution:* The resolution of the image is set from 5 to 50 µm, which also determines the scan dimension. For a preview of the image, a low resolution is set, but for actual measurement, the spot size and the number of pixels in the spot determine the scan resolution. To obtain valid data, the pixel resolutions should be such that the spot size is greater than 5 times the pixel size and preferably 10 times. Thus, for a spot size of 100 µm, the pixel size should be 10 µm.
5. *Pan and zoom:* Pan and zoom is another option that is available to increase the resolution of a small area on the array.
6. *Gains:* With the sequential scanner discussed here, there are no adjustments to the power of the laser. However, an adjustment to the gains can be made, which dictate the amplification of the signal as it passes through the dynodes and thus influence the signal (*see* **Note 2**).
7. *Palettes:* Another option on this arrayer is the viewing palette, which can assist in determining the optimum gain to be set for scanning. Generally, setting a red or green palette for Cy5 and Cy3, respectively, will produce individual images with red and green spots and a composite image that is mostly yellow if most of the expression is the same in both the samples. However, detecting the saturation of the spots is not possible with this color setup because the human eye cannot differentiate shades of green and red very well. For this reason, there is a feature that allows one to set a color to indicate saturation as white (*see* **Note 3**).
8. *Offset:* This is another option to modify the background to better visualize the spots. By changing the zero value to a finite offset value, the contrast between the spots and the background can be improved. However, this causes a filtering effect, and in order to obtain all the raw data from the array, it is best that this number remain at zero during scanning.

Several other scanners provide the option of varying the PMT voltage and the laser power to improve the signal-to-noise ratio of the spots. For example, in the GenePix 4000 scanner, the lasers operate at full power; hence, the voltage of the PMT controls the gains of the PMT.

4. Notes

1. The glass arrays need to be handled very carefully. While scanning, the slides are always handled with gloves. Care has to be taken not to touch the inner surface of the slide, as this can destroy the poly-L-lysine coat on the surface.
2. Caution must to be taken in increasing the gain, because this affects both signal and noise without discrimination. Also there is serious loss of information from the spots on the microarray as the spots become saturated in intensity. For example, we scanned a slide at a particular gain and several five-increment intervals of gain and analyzed the data. As the gain increases, more and more spots fall in the saturated range and expression variation for all the spots goes to a minimum (*see* **Fig. 3**; Color Plate 14, following p. 274). For best results, the signal intensities from the two channels should fall in the linear range of detection *(8)*.

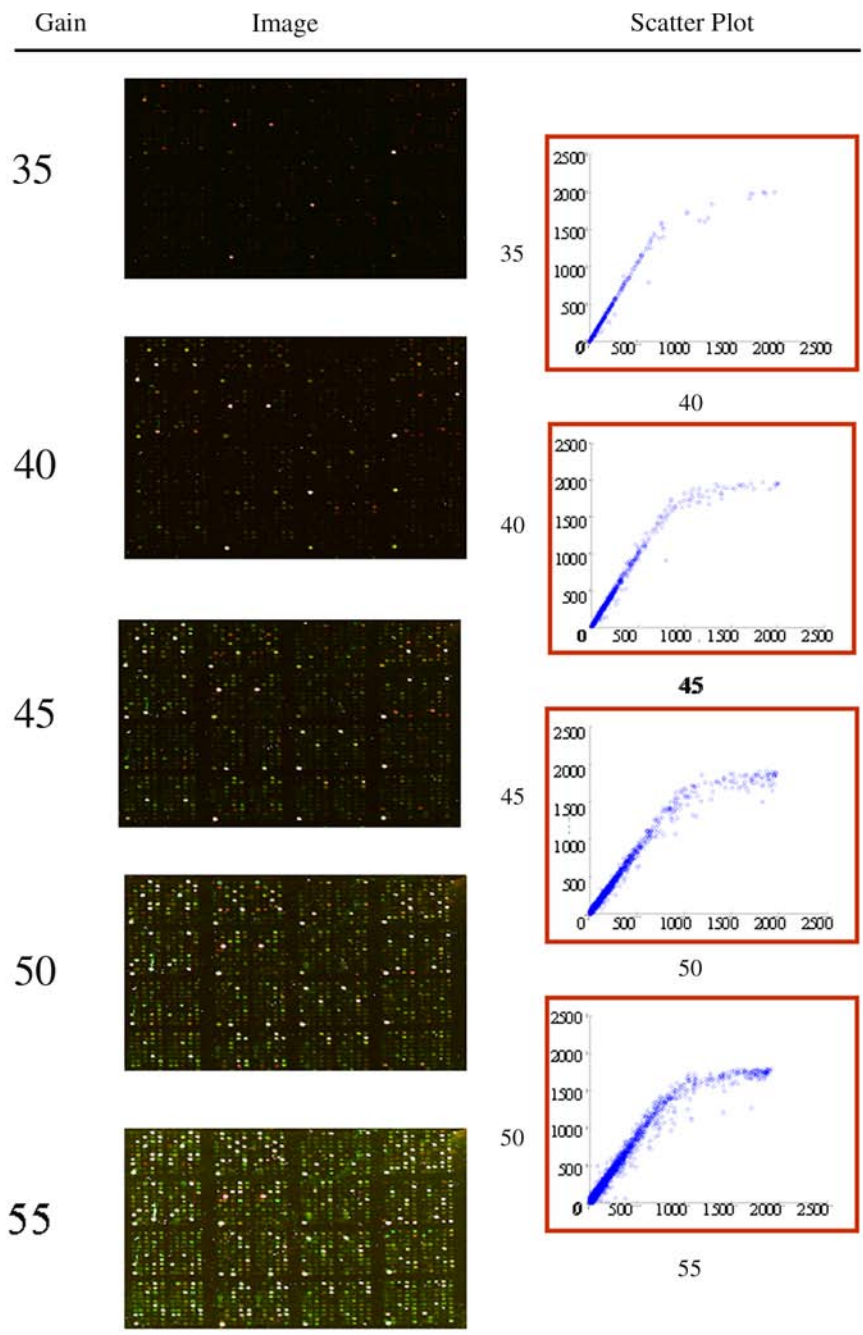

Fig. 3. The images for different gains used for scanning and the corresponding scatterplots are shown. The saturated spots in the images are shown as white spots. The scatterplot is the comparison of the signal intensities between two gains, and as the gain increases, the number of spots that are saturated in intensity increases. (*See* Color Plate 14, following p. 274.)

3. The palette option of red or green to white, where white indicates saturated spots, is a good choice while scanning. Reasonably high gains need to be used while scanning in order to see the spots of low fluorescence intensities. The best choice is the spectral palette with spectral colors that cover the whole range in the 16-bit image.

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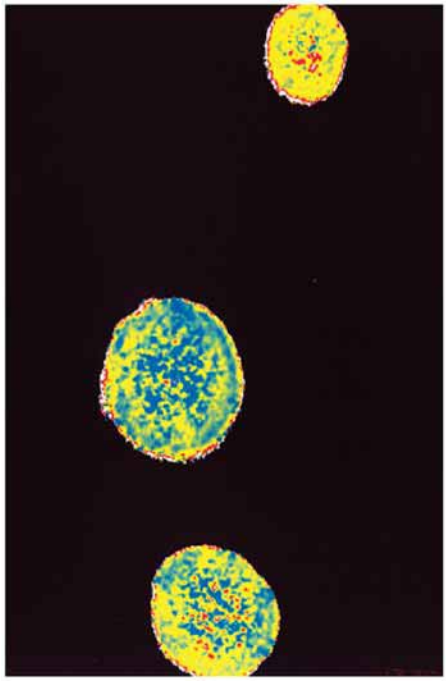
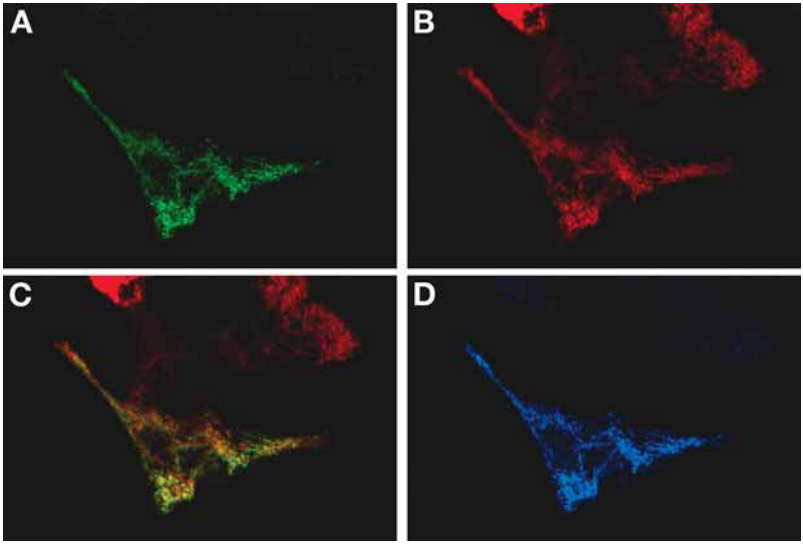
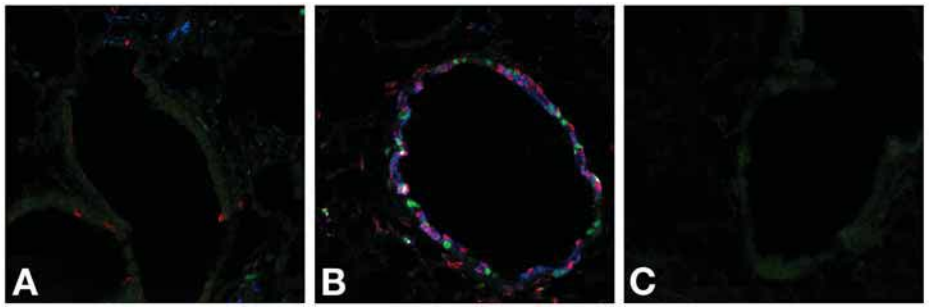


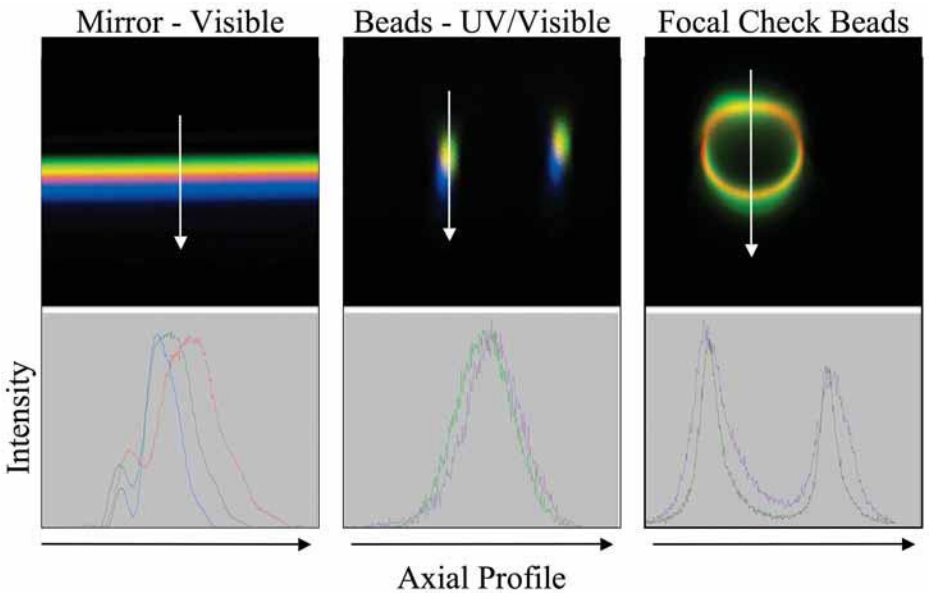
Color Plate 1, Fig. 1. Pseudocolored image of mouse islets loaded with Fura-2. (*See* full caption in Ch. 3 on p. 52 and discussion on p. 51.)



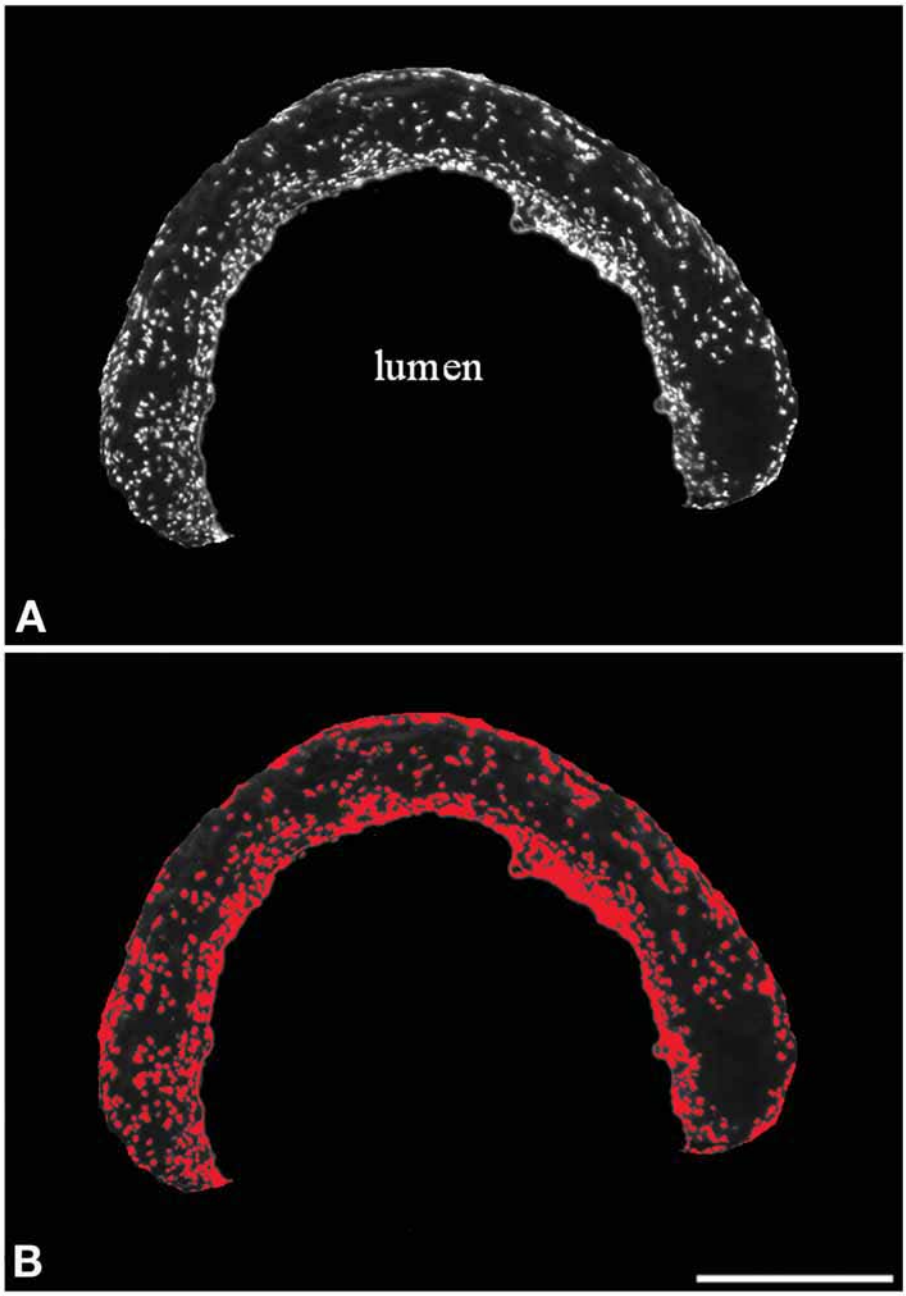
Color Plate 2, Fig. 3. Expression of mitochondrially-targeted ratiometric pericam (RPC-mt) in neuroendocrine cells: laser scanning confocal images of AtT20 cells. (*See* full caption and discussion in Ch. 3 on p. 58.)



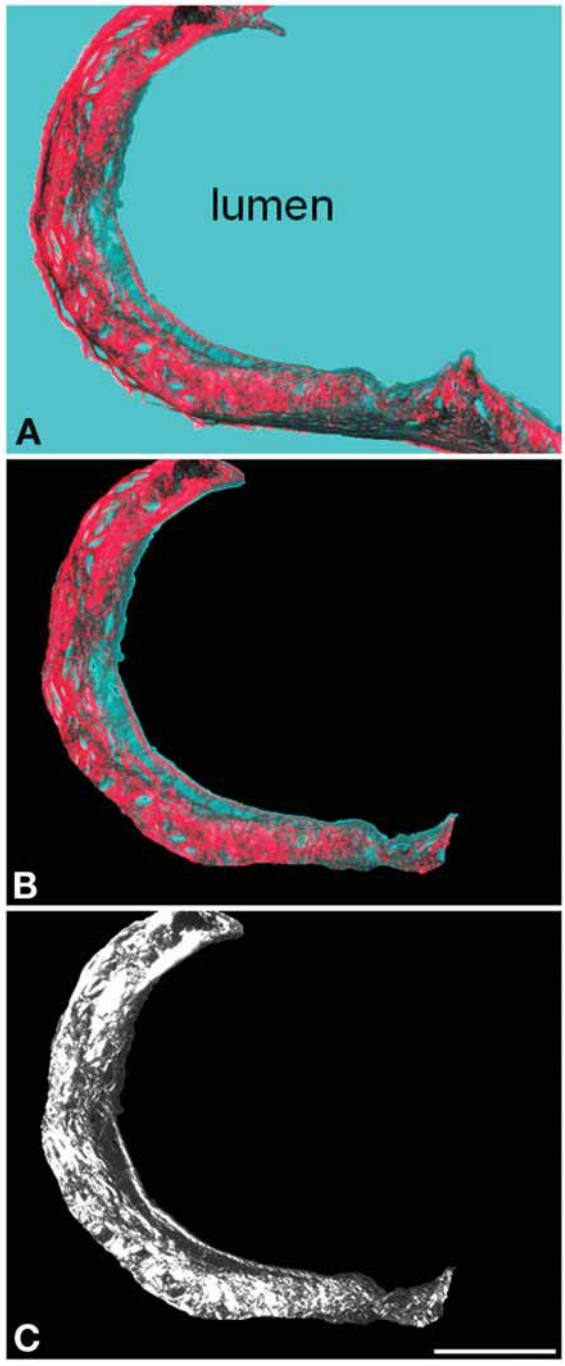
Color Plate 3, Fig. 1. Triple-labeling using mouse monoclonal PKC (blue), rabbit polyclonal p-ERK (red), and rat monoclonal Ki-67 (green) anti-bodies on sham animals **(A)** and animals exposed to crocidolite asbestos for 4 days **(B)**. A negative control omitting the primary antibody is shown in **(C)**. (*See* full caption in Ch. 4 on p. 74 and discussion on p. 73.)



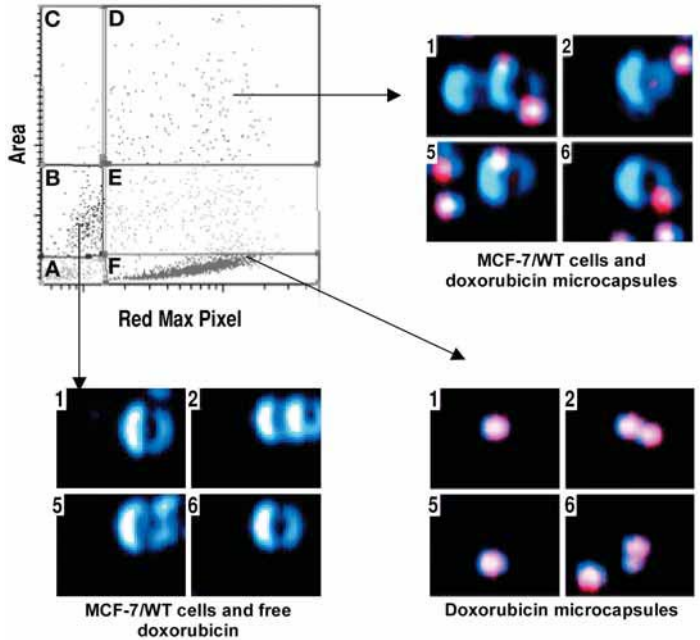
Color Plate 4, Fig. 6. Methods used to check spectral registration of different laser lines. (*See* full caption in Ch. 5 on p. 93 and discussion on p. 92.)



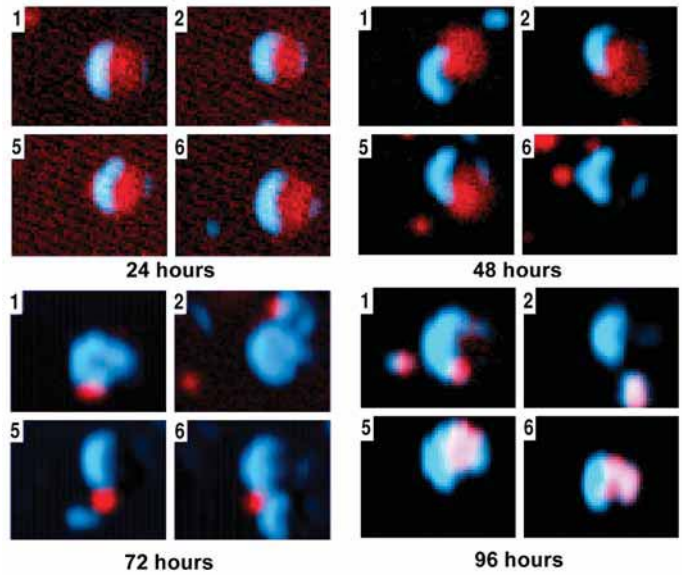
Color Plate 5, Fig. 2. Gray-scale fluorescent capture of DAPI-stained nuclei in mouse atherosclerotic lesion **(A)**, and with red pseudocolor overlay **(B)**. (*See* full caption in Ch. 6 on p. 144 and discussion on p. 142.)



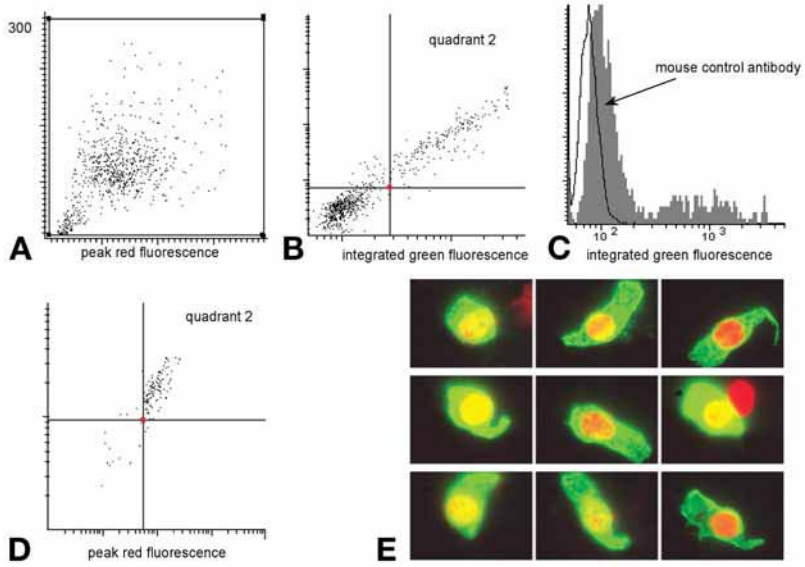
Color Plate 6, Fig. 3. Polarized light microscopy assists in more accurately discriminating atherosclerotic lesion borders. (*See* full caption in Ch. 6 on p. 149 and discussion on p. 146.)



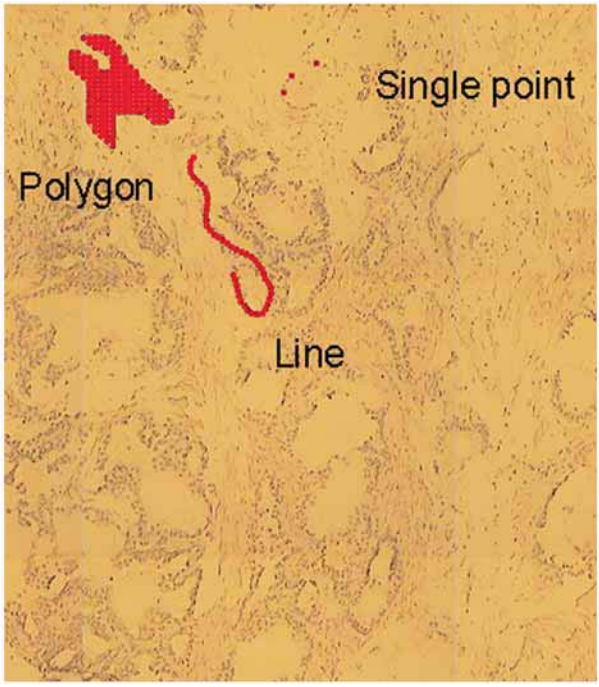
Color Plate 7, Fig. 3. Assay of cells incubated with either free drug or drug-encapsulated microspheres by laser scanning cytometry. (*See* full caption in Ch. 9 on p. 201 and discussion on p. 200.)



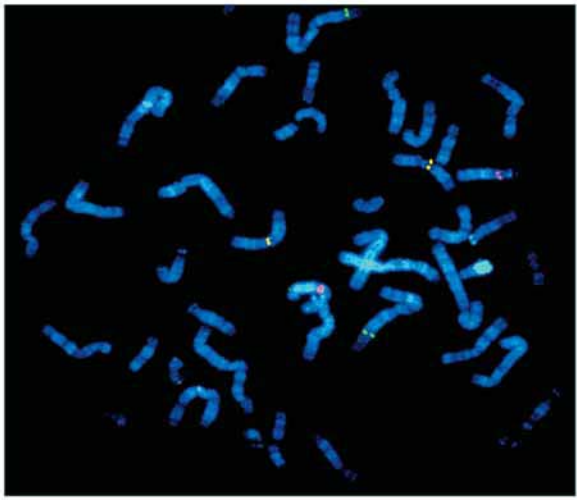
Color Plate 8, Fig. 4. Time-course experiments of doxorubicin encapsulated microspheres. (*See* full caption in Ch. 9 on p. 203 and discussion on p. 202.)



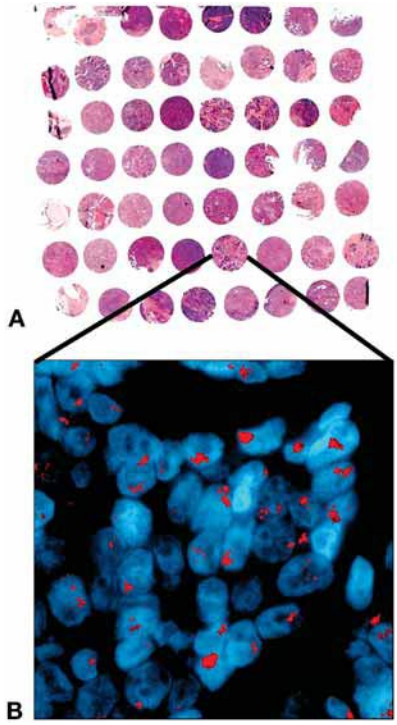
Color Plate 9, Fig. 9. Gate settings for the analysis of sputum bronchial epithelial cells by laser scanning cytometry. (*See* full caption in Ch. 9 on p. 210 and discussion on p. 207.)



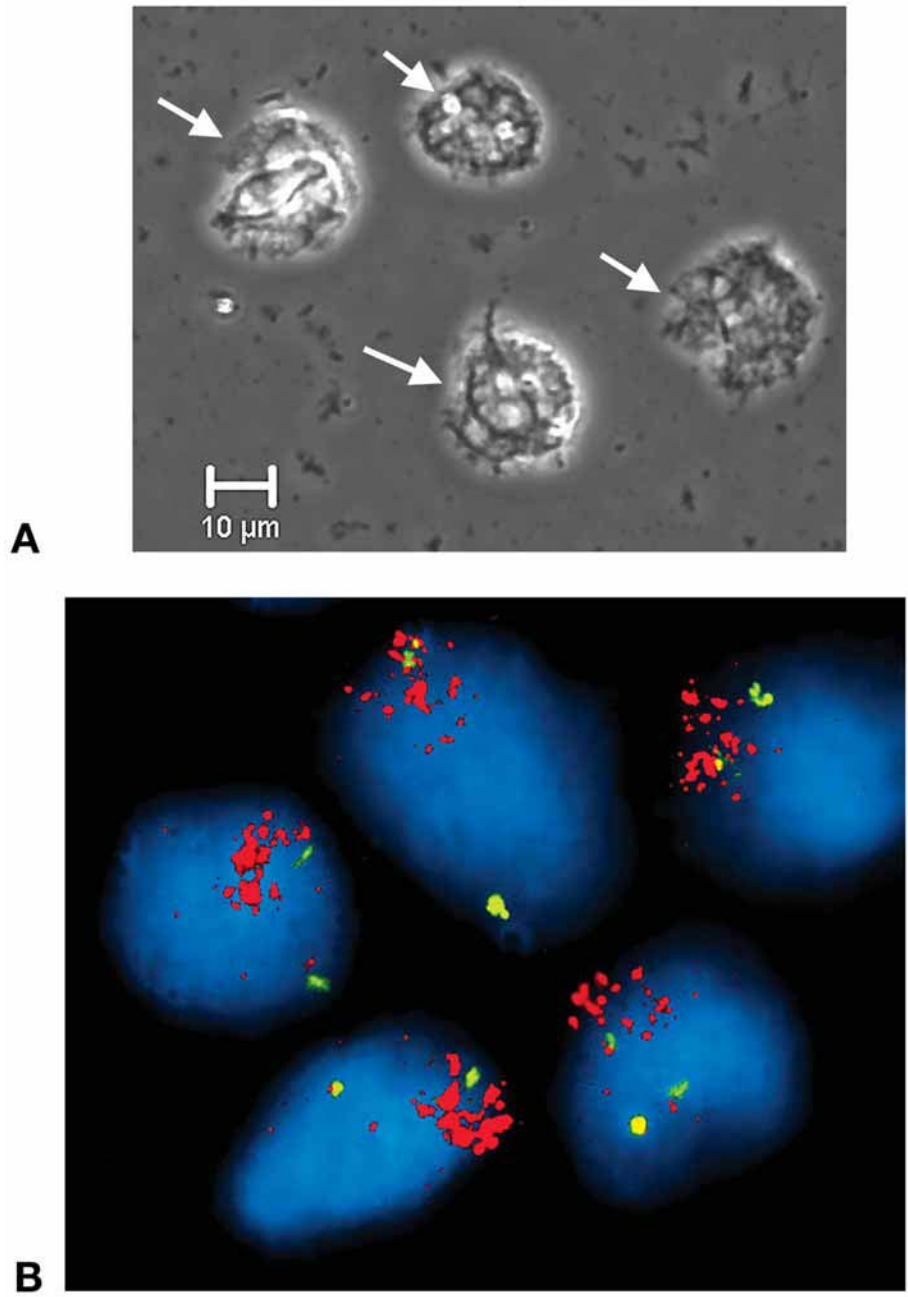
Color Plate 10, Fig. 6. Annotation of stitched images on AutoPix system. (*See* full caption in Ch. 10 on p. 224 and discussion on p. 223.)



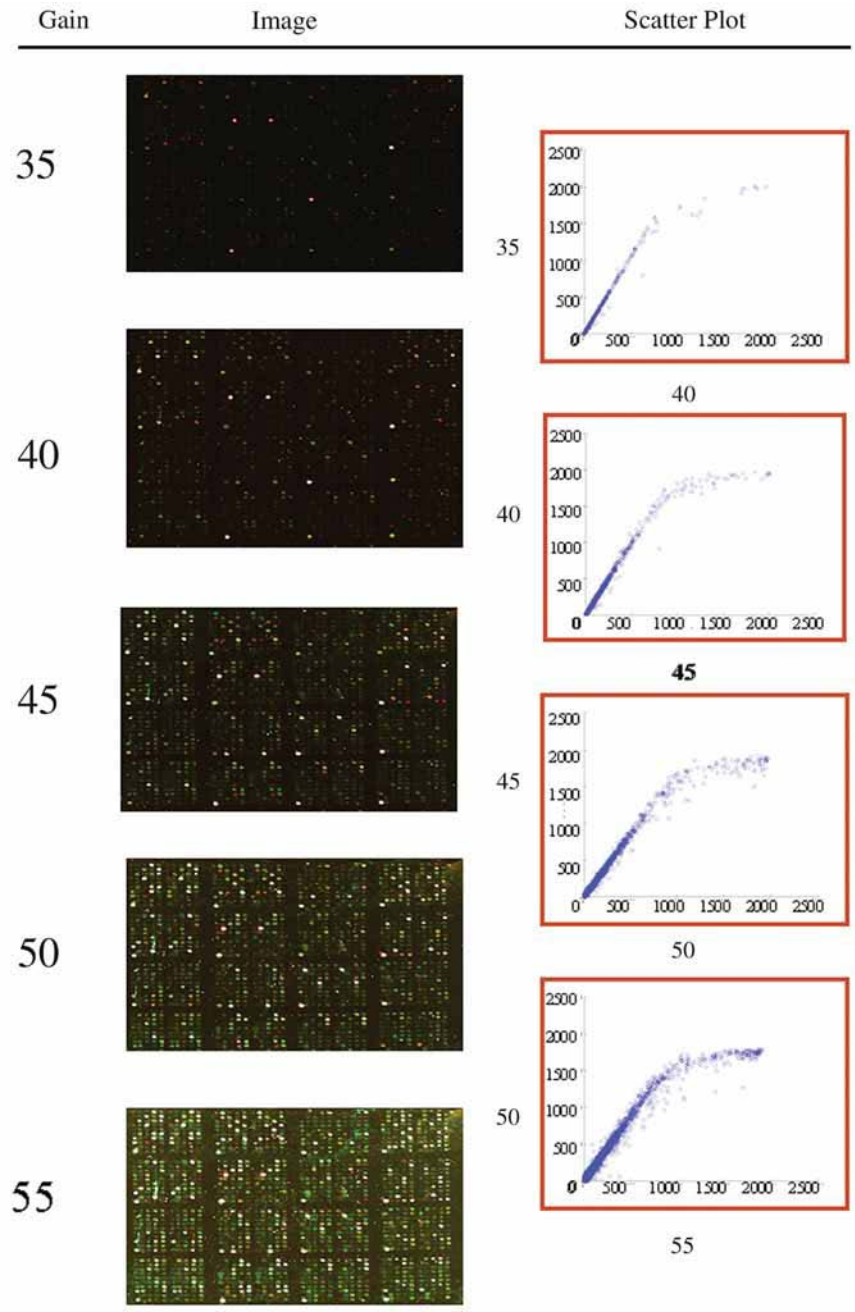
Color Plate 11, Fig. 1. Metaphase analysis with multiple single-gene probes. Multicolor FISH showing simultaneous localization of three human genomic BAC probes. (*See* full caption in Ch. 12 on p. 238 and discussion on p. 237.)



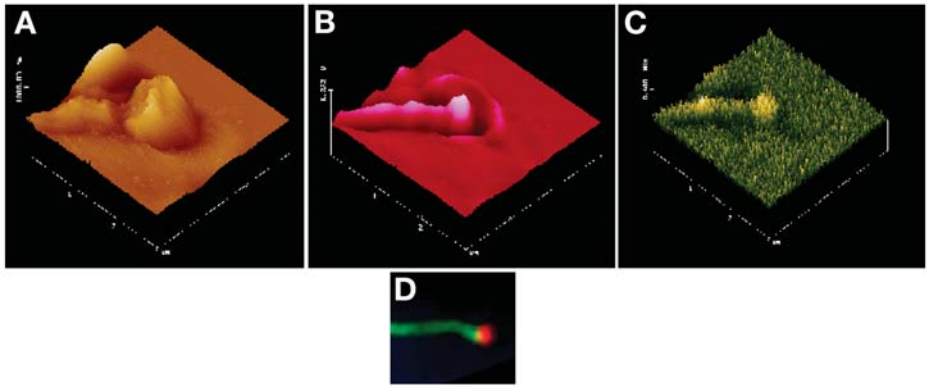
Color Plate 12, Fig. 2. FISH of tissue microarray breast carcinoma specimens. (*See* full caption in Ch. 12 on p. 243 and discussion on p. 242.)



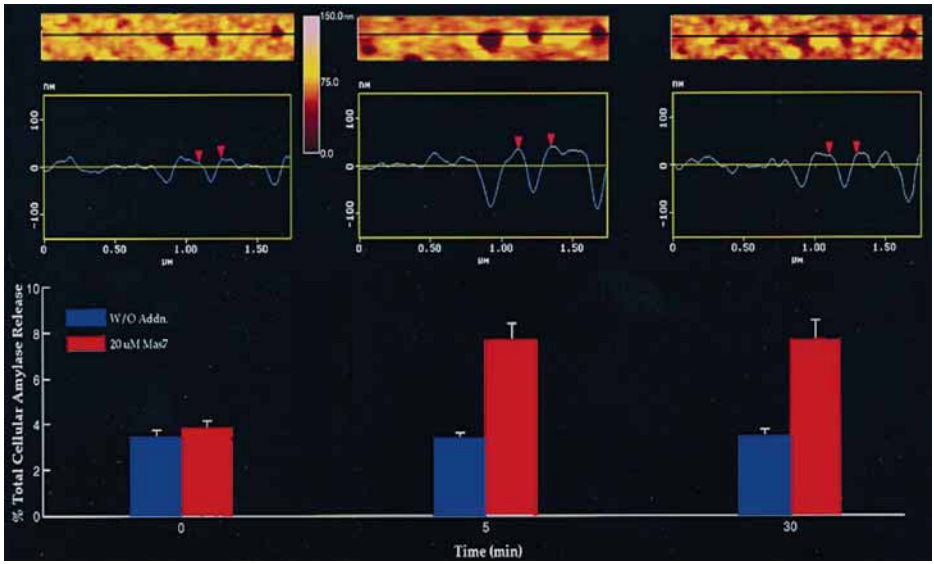
Color Plate 13, Fig. 3. FISH of LCM-prepared nuclei. Naked nuclei as photographed by phase contrast microscopy are shown in **(A)**. (*See* full caption in Ch. 12 on p. 244 and discussion on p. 242.)



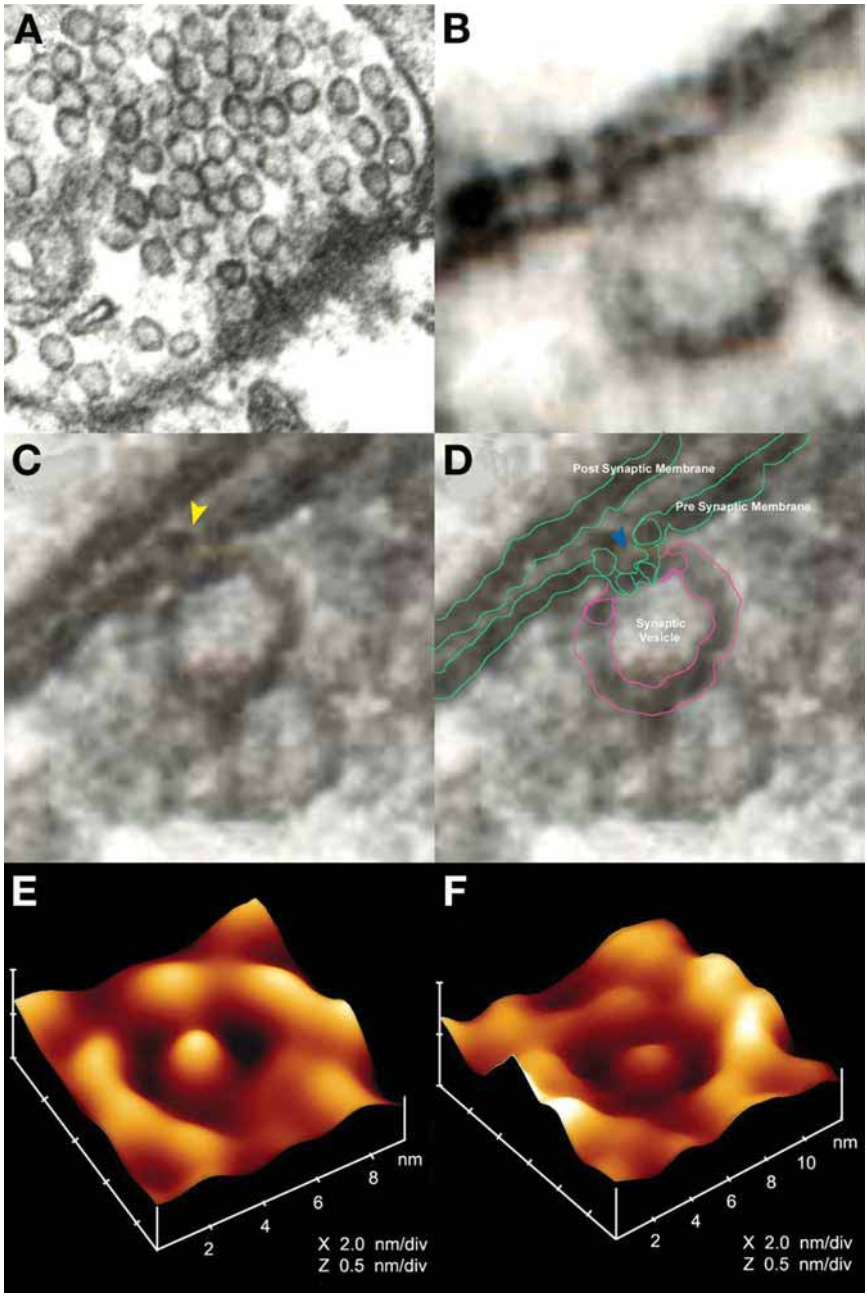
Color Plate 14, Fig. 3. Setting parameters for microarray image scanning: images for different gains and corresponding scatterplots. (*See* full caption in Ch. 13 on p. 272 and discussion on p. 271.)



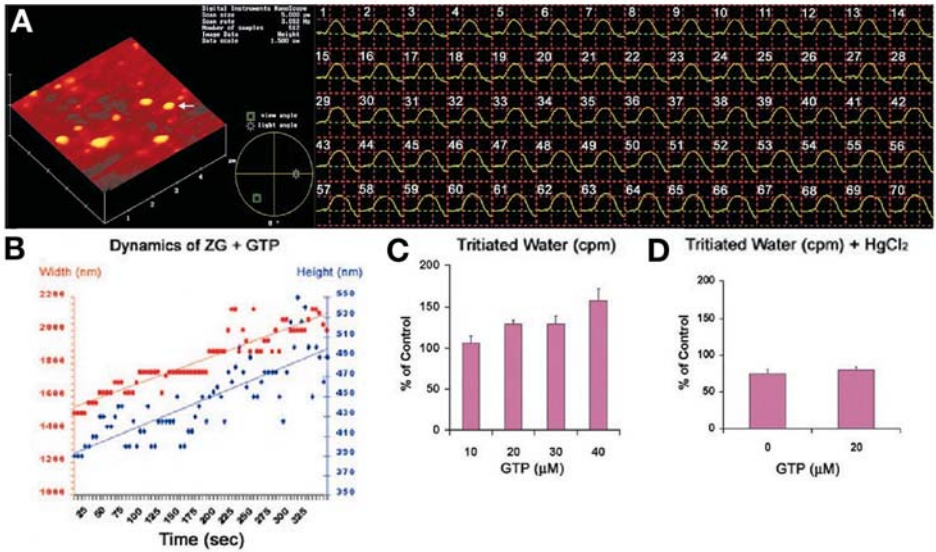
Color Plate 15, Fig. 6. NSOM images of the telomeric region of a human meiotic chromosome core after immunostaining of TRF2 by Cy3-labeled antibodies. (*See* full caption in Ch. 14 on p. 288 and discussion on p. 287.)



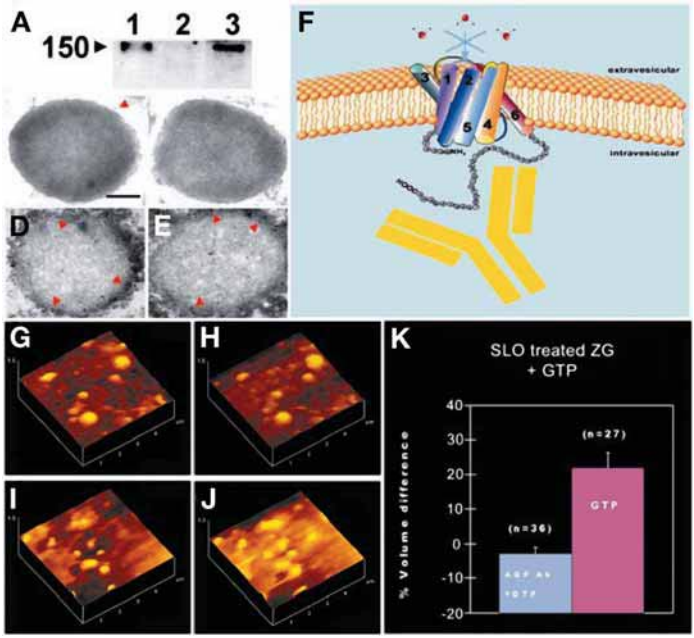
Color Plate 16, Fig. 3. Porosomes: dynamics of depressions following stimulation of secretion. (*See* full caption in Ch. 15 on p. 304 and discussion on p. 302.)



Color Plate 17, Fig. 5. Electron micrograph of porosomes in neurons. **(A–D)** AFM images of fusion pore in nerve terminal **(E)** and isolated neuronal porosome reconstituted into lipid membrane **(F)**. (*See* full caption in Ch. 15 on p. 306 and discussion on p. 304.)



Color Plate 18, Fig. 6. Monitoring height and width of zymogen granule (arrow) after exposure to GTP. (*See* full caption and discussion in Ch. 16 on p. 325.)



Color Plate 19, Fig. 7. Interaction of AQP1 antibody with zymogen granules. (*See* full caption in Ch. 16 on p. 327 and discussion on p. 326.)