Chemical Microscopy Applied to Biological Systems

Marian Navratil,² Gary A. Mabbott,²,³ and Edgar A. Arriaga*,²

Department of Chemistry, University of Minnesota, Minneapolis, Minnesota, 55455, and Department of Chemistry, University of Saint Thomas, Saint Paul, Minnesota 55105

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The ideal bioanalytical method is one that would allow the researcher to carry out sensitive, selective, reproducible, fast, and in situ chemical measurements of the composition and function of biological systems. Such measurements always become more challenging whenever one is investigating micrometer-scale, complex cellular systems. While impressive developments are being made in microanalysis (e.g., microseparations), complementary techniques that would reveal the chemistries within the cell, preferentially in real time, are needed. Chemical microscopy, defined as the spatial representation of chemical information, is an analytical development that promises to meet these needs.

Based on the above definition of chemical microscopy, the authors have decided to review the new developments in, and applications of, fluorescence microscopy techniques including spectral imaging, along with near-field scanning optical microscopy, Raman and IR microscopies, X-ray microscopy, mass spectrometry imaging, and scanning electrochemical microscopy. Since such a large number of publications, mainly on the applications of these techniques, continue to appear in the literature, we have decided to highlight only those reports published in the last three years that present promising methods or instrumentation related to chemical microscopy techniques.

Each section is dedicated to one of the techniques selected and begins with a general overview of that technique, then continues with highlights on technique and instrumentation, and finally provides examples of reports of the use of this technique to carry out chemical measurements.

CONVENTIONAL FLUORESCENCE MICROSCOPY

Conventional fluorescence microscopy is primarily used to determine the spatial distribution of fluorescent probes, which usually describe a given molecular property, subcellular compartment, or function of a biological sample. An overview of the principles, instrumentation, and factors that affect the quality of fluorescence microscopy images was published by Lichtman and Conchello (1). In fluorescence microscopy, chemical fluorescent probes, which now number in the several thousands, make it possible to label more than one cellular component at a time. Fluorescent proteins (i.e., GFP, CFP, YFP, or DsRed) are also widely used as probes thanks to their brightness, resistance to photobleaching, and potential to be fused with virtually any gene product (2). Another aspect of fluorescence microscopy, image enhancement in a software-based manner using deconvolution algorithms, has also been recently discussed (3).

Technique and Instrumentation. Increased interest in fluorescence microscopy has spurred the development of more sophisticated microscope configurations, providing improved resolution and sensitivity. In particular, advances in multiphoton fluorescence microscopy continue to appear. Multiphoton excitation occurs during the simultaneous absorption of two or more photons by a fluorophore, when the total energy of the photons matches the energy required for absorption. Because multiphoton excitation utilizes longer wavelengths of light than does single-photon excitation, it is characterized by less light scattering and...
deeper penetration of the light into the sample and usually entails less damage from photobleaching \(^4\). However, this technique stands out the most for its definition of excitation volume, which occurs only at regions where the photon density is high; this makes the technique less susceptible to out-of-focus emission, resulting in both higher resolution and signal-to-noise ratio \(^5\).

Sánchez and Gratton used two-photon fluorescence microscopy in conjunction with fluorescence correlation spectroscopy (FCS) to measure lipid–protein interactions \(^6\). FCS allows the researcher to quantitatively measure the fluorophore concentration in, and diffusion through, a very small area of illumination. These authors also reviewed the work of other groups in this field and showed that by combining these two techniques one can quantitate the number of fluorescently labeled molecules in a given excitation volume and unequivocally confirm whether a given protein is associated with a lipid membrane both in vitro and in vivo. Multiphoton FCS has also been used to quantitatively analyze preparations from arterial walls with quantum dot nanocrystals \(^7\) and to study drug delivery and pharmacokinetics \(^8\).

Attempts to increase the resolution of conventional fluorescence microscopy have resulted in the introduction of 4Pi microscopes \(^9\) that utilize two opposing objective lenses to illuminate the sample with coherent light focused onto the same spot. While the interference of the counterpropagating waves sharpens the focus, providing a 4–7-fold improvement in resolution, it also produces secondary interference side lobes half a wavelength above and below the focal plane. These lobes can be digitally removed by deconvoluting the 4Pi image \(^10\). The improved resolution of 4Pi microscopes has made it possible to reconstruct high-resolution 3D images \(^11\). The combination of this technique with stimulated emission depletion microscopy allows for further improvements in resolution down to 30–50 nm. These authors foresee this method as becoming widely applicable in the biological sciences within the next few years.

**Selected Applications.** The most common applications include the measurement of intracellular and organellar pH, the detection of reactive oxygen species, and the probing of ion concentration (such as calcium) in living cells. The first two applications have been reviewed by Yeung et al. \(^12\). In the first application, the pH measurements usually are carried out with fluorescent weak bases such as acidine orange, LysoTracker dyes \(^12\) SNARF/SNAFL, and carboxyfluorescein and its derivatives \(^13\), all of which are membrane permeant when deprotonated. Upon protonation, the ability of these bases to exit targeted cellular compartments is greatly reduced, and their local concentrations are proportional to the transmembrane pH gradient. In the second application, Alvarez-Leffmans et al. modified the use of a ratio-metric probe BCECF in order to simultaneously measure changes in cell water volume and intracellular pH \(^14\). This method reportedly provides a time resolution of \(<1\) s, which allows for time-lapse measurements, is sensitive to osmotic changes of \(\sim1\)% and is independent of changes in the concentration of intracellular ions. In the third application, Yip and Kurtz carried out a time course measurement of intracellular pH by extracting the pH-related information from image pairs of BCECF-labeled cells that had been collected at a rate of 4 Hz \(^15\). They then used a similar approach to measure intracellular \(\text{Ca}^{2+}\) concentration in smooth muscle and endothelial cells that had been labeled with two calcium indicator dyes, Fluo-3 and Fura-Red. Here, the emission ratio was proportional to the \(\text{Ca}^{2+}\) concentration. The three-dimensional resolution of any of these methods can be improved by using confocal microscopy, which eliminates out-of-focus fluorescence information \(^15\).

Hug et al. describe a simple and rapid method for counting *Microthrix parvicella* bacteria and *Nocardioform actinomycetes* in activated sludge using fluorescence in situ hybridization and epifluorescence microscopy \(^16\). Here, three Cy3-labeled probes were designed to hybridize with DNA of the bacteria and imaged using a fluorescence microscope. A similar approach has been reported by Perez-Feito \(^17\). Tang et al. present another system for the quantitative measurement of DNA content in chicken and eel erythrocytes \(^18\) and DNA ploidy in rat hepatocytes \(^19\).

They then investigated the binding properties of five anticancer drugs that were studied in the diploid cells using acridine orange as a fluorescent probe with a high-resolution Hadamard transform fluorescence imaging microscope.

McCool et al. discuss measuring the expression of SOS genes, which are induced by the cell in response to DNA damage \(^20\). They used a fusion construct of the green fluorescent protein (GFP) reporter gene with an SOS promoter \((sulA)p\) inserted at the att\(\bar{B}\) site of the *Escherichia coli* chromosome to determine the SOS expression levels in individual cells.

Fluorescence microscopy has also been widely used to study protein interactions in living organisms. Andrawiss and co-workers demonstrated the use of this method to quantitate murine leukemia virus particle assembly \(^21\). To track the assembly of the virus and study protein–protein interactions, they used a fusion protein of the GFP and Gag molecules, the principal structural proteins of retroviral particles. The experiments showed that changes in posttranslational modification of the Gag proteins result in an \(\sim50\)% decrease in efficiency compared to the wild-type Gag.

A study by Freites et al. \(^22\) of the interaction of annexin A1 with phospholipid bilayers is another example of the use of fluorescence microscopy to investigate interactions at the molecular level. These authors show that these interactions are strongly dependent on the domain structure of the lipid bilayer, but only weakly dependent on pH, and that annexin A1 forms networks in the presence of a domain structure.

**CONFOCAL FLUORESCENCE MICROSCOPY**

Confocal fluorescence microscopy has the ability to control the depth of focus by spatial filtering and as a result, it allows for collection of a series of optical sections throughout the sample, while reducing background fluorescence originating from sections that are away from the focal plane \(^23\). A review article on imaging of membrane traffic by spinning disk confocal fluorescence microscopy, a relatively new development, was published by Nakano \(^24\). Major factors affecting the quality of confocal imaging were summarized elsewhere \(^25\).

**Technique and Instrumentation.** In addition to 4Pi microscopy and two-photon microscopy described in the previous section, there are other attempts that have been made to improve the spatial resolution of confocal microscopes. One of the methods is mathematical deconvolution of confocal images by 3D centerline extraction of elongated objects, such as blood vessels, described
by Maddan et al. (26). They used this approach to generate 3D images of complex vascular structures in rat brains. A similar concept was reported by the Wouterlood group; they identified synaptic contacts between neurons in deconvoluted and 3D reconstructed laser scanning microscope images and used them for detection of postsynaptic density proteins, such as ProSAP2/Shank3. This technique allowed them to study molecular interactions in cells and was significantly faster than an electron microscopy method that is typically used to investigate the synaptic contacts.

Eggeling et al. measured photobleaching kinetics of rhodamine 6G by one- and two-photon confocal microscopy and designed a theoretical model of the kinetics (27). They discovered that two-photon microscopy causes negligible off-focus photobleaching, which has been reported previously, but results in severe in-focus photobleaching.

Godlewski and co-workers analyzed kinetic patterns of BAX translocation and Smac/DIABLO release from mitochondria during apoptosis using 4D (time-lapse 3D) confocal microscopy (28). During apoptosis, the release of apoptogenic proteins such as Smac/DIABLO from mitochondria into the cytosol is facilitated by apoptosis promoters such as BAX. They discovered that the BAX aggregation and Smac/DIABLO release coincide and have similar kinetics, which suggests that the release of Smac/DIABLO is correlated with BAX translocation to the mitochondria. The authors also included a brief comparison of time-lapse microscopy, and 2D/time and 4D confocal microscopy techniques.

Other extensions of confocal microscopy include polarized light confocal microscopy and phase-amplitude confocal microscopy. While the former provides additional information about the optical anisotropy of the sample (29), the latter uses partially coherent light and allows one to measure the refractive index of viable cells (30).

**Selected Applications.** Many applications of confocal microscopy are a mere extension of fluorescence microscopy techniques described previously. Confocal microscopy provides higher resolution leading to better knowledge of the spatial localization of the analyte (9, 31). These applications include measurement of intracellular pH (13, 32, 33) or Ca²⁺ ions in living cells (13, 34–36). Yahata et al. described a system for measuring histamine concentration in endothelial cells based on changes in calcium concentration stimulated by histamine addition over several orders of magnitude (35). They also studied and quantified effects of antagonists of the H₂ receptor and blocking of histamine binding to the receptor. Expression levels of heat shock protein HSP60 on the membrane surfaces of epithelial cells were probed by the Pfister research group (37).

Numerous applications of confocal microscopy are directed toward counting or size measurements. Kukavica-Ibrulj et al. described an immunofluorescence system for detection and quantitation of hepatitis A virus in sewage treatment effluent (38). Polyclonal antibodies raised against the virus were used in combination with an Alexa-conjugated secondary antibody and allowed detection of the virus in concentrations as low as 2 × 10⁵ plaque-forming units/mL. This rapid method produces results highly correlated with traditional techniques, and its use can be extended to virtually any type of virus, as demonstrated by other groups that used a similar approach for HIV quantitation (39).

Park and co-workers experimented with measuring the loading density of FITC-labeled glucose oxidase immobilized on gold substrate surfaces for biosensor construction (40).

Confocal microscopy allowed Gabriel et al. to quantitate cell hybridoma yields. The authors used fluorescent markers to label the two cell lines and then quantitated the extent of colocalization after cell electrofusion. The percentage of false positives was estimated by measuring the area of individual colocalized pixels in the confocal images.

Fluorescence microscopy was compared with laser scanning confocal microscopy and two-photon confocal microscopy (2P-CM) for quantitative analysis of activated sludge (41). The authors concluded that only 2P-CM revealed the internal structure of activated sludge flocs and allowed for more accurate quantitation of the sludge viability.

Katano et al. reported on the use of confocal microscopy for the interaction analysis between an anticancer drug, cisplatin, and the copper export transporter, ATP7B (42). They constructed a human ovarian carcinoma cell line expressing ATP7B tagged with cyan fluorescent protein (CFP) and used these cells to study colocalization of cisplatin with ATP7B. Fluorescence intensities of the vesicular structures expressing CFP-ATP7B were measured along with the intensities of fluorescein-labeled cisplatin. The ratio of the two intensities revealed that the degree of colocalization is severalfold higher when cells are treated with cisplatin, which indicates that the drug became concentrated in the vesicles overexpressing ATP7B.

Other examples of confocal microscopic analyses used to study molecular interactions include a measurement of interactions and diffusion constants of oligonucleotide complexes and cationic liposomes (43), determination of multiprotein interactions using a pixel-to-pixel assessment algorithm (44), a study of the interaction between human microvascular endothelial and mural cells (45), and a study of the interaction between fluorescent protein-tagged GABA receptor interacting factor-1 and the kinesin-1 family member KIF5C (46).

Corneal confocal microscopy also allows a researcher to produce high-resolution images of corneal layers in vivo and use them for cell size or density measurements. Several reports on investigating human cornea exist, focusing on measurement of corneal cell (47, 48) or keratocyte (49, 50) density.

Particle linear image velocimetry, a novel method for characterization of microfluidic devices, was introduced by the Chao group (51). A series of 1D images representing a trace of fluorescent particles was captured by a laser-scanning confocal microscope and used to measure steady flow with 1-μm spatial resolution and transient flow with 250-ms temporal resolution.

**FRET, FLIM, AND FRAP MICROSCOPY**

A need for increased sensitivity and resolution has been driving the development of new sophisticated fluorescence-based microscopic techniques. Among them are the following: fluorescence/ Förster resonance energy transfer (FRET), fluorescence lifetime imaging microscopy (FLIM), fluorescence recovery after photo-bleaching (FRAP), and fluorescence loss in photobleaching (FLIP) (4). FRET utilizes a pair of fluorophores (donor and acceptor), and takes advantage of long-range dipole–dipole interactions, when the excitation spectrum of the acceptor overlaps with the
emission spectrum of the donor and the energy is nonradiatively transferred from the acceptor to the donor. This phenomenon only occurs when donor and acceptor are in proximity (1–10 nm) (52). A list of the most common FRET pairs has been published repeatedly (53–55). FLIM measures the fluorescence lifetime of a fluorophore, which is independent of the fluorophore’s concentration, and detects changes in its immediate vicinity. FRAP and FLIP are better suited to determine the kinetics of dynamic processes occurring within the cell.

**Technique and Instrumentation.** While FRET is a widely used technique in in vitro models, its use in in vivo studies has remained relatively unexplored due to the thickness of tissue cross sections that are normally examined. Mills et al. tried to overcome this problem by employing two-photon excitation (2p) in FRET microscopy, the performance of which they compared to that of confocal FRET (C-FRET) microscopy (56). They used the interaction of the preapoptotic protein BAD and prosurvival protein Bcl-xL to study post-traumatic activation of apoptotic cascades in 40-μm-thick rat brain cross sections.

Tramier et al. proposed an interesting concept of picosecond hetero-FRET (57) to probe dimerization of herpes simplex virus thymidine kinase labeled with two fluorescent proteins (58). The authors compared two different fluorophore pairs, CFP/YFP and GFP/DsRed. The latter is only rarely used for FRET due to dimerization/tetramerization, as well as slow and incomplete maturation of the DsRed protein. The use of picosecond FRET microscopy allowed the authors to overcome some of the problems with the complex maturation process of DsRed and study the protein–protein interaction as a function of time and space.

Several reports on single-molecule three-color FRET have appeared recently, offering the ability to measure more than one distance at a time (59) or studying multiprotein interactions (60). 3D live FRET microscopy was described to be exceptionally useful for shedding insight on compartmentalized (i.e., organelar) associations within the cell (55).

**Selected Applications.** Because FRET only occurs between two nearby fluorophores, FRET microscopy is typically used to determine binding partners, conformational changes, and proximity or interaction between two molecules (61). Wallrabe and Pariasamy summarized recent examples of quantitative FRET analysis, including assessing the energy-transfer efficiency and measuring the distance between the two fluorophores (54). The authors used this technique for studying the endocytic membrane trafficking of an iron–protein complex Tfn in canine kidney cells. The transport is mediated by the TFR transmembrane protein, and FRET between the Alexa 488 and Cy3 dyes occurs upon formation of the Tfn–TFR complex. (62) Others analyzed the molecular dynamics and interactions of the T cell receptor–CD3 complexes (55).

Traoré et al. described the use of FRET microscopy to investigate the kinetics of endocytic uptake of fluorescently labeled low-density lipoproteins by living cells. The cells were subjected to a laminar flow, and the effects of shear stress on the endocytosis kinetics were studied.

FRET–FLIM technology has been used in several other recent studies to investigate protein–protein interactions (63, 64). For example, it was used to characterize dimer formation for the transcription factor C/EBPα in the nucleus of pituitary cells. Dimerization resulted in FRET, which consequently reduced the donor lifetime from 2.52 ns down to 1.87 ns (54, 65, 66).

Schuttpelz and co-workers developed a method for identification and quantitation of proteins on protein microarrays using UV–fluorescence FLIM, allowing detection limits in the picomolar range. The method was label-free and used differences in the fluorescence lifetimes of proteins containing amino acids tyrosine and tryptophan as low as 200 ps to detect an analyte. The authors demonstrated the potential of the method for quantitative analysis of monoclonal anti-p53 antibodies.

Photobleaching and photoactivation approaches, such as FRAP and FLIP, are often used in conjunction with laser scanning confocal microscopy, because it allows control of the illumination area (4). These methods have been used to measure diffusional mobilities and kinetic characteristics of various cellular processes. For example, Klein et al. used FRAP microscopy to determine plasma membrane fluidity by measuring lateral diffusion of the lipid NBD-sphingomyelin in human neuroblastoma cells (67). Carrero et al. used FRAP to quantify protein–protein and protein–DNA interactions in the study of proteins that associate with chromatin (68). Other methods that have been reported to be used for analyzing macromolecular dynamics and transport include fluorescent speckle microscopy (69) and fluorescence localization after photobleaching microscopy (70).

**TIRF Microscopy**

Total internal reflection fluorescence (TIRF) microscopy excites only those fluorophores that lie within a very short distance (typically 200 nm or less) of the interface between the sample and the glass on which it rests. Consequently, there is little emission from objects further away that might, otherwise, obscure the image of interest. Recent reviews discuss theory and instrumentation (71, 72) applications to single-molecule detection (72, 73) and multiphoton excitation (74).

**Technique and Instrumentation.** A variable-angle TIRF instrument based on illumination from below and collection from above the sample stage was demonstrated to have high-precision control of the incident angle (75). This device allowed easy switching between TIRF and conventional epi-illumination experiments so that measurements could be made of rhodamine 123 bound to the membrane or inside mitochondria, respectively (76). Using rapid time-gated image intensifiers, the authors were able to obtain fluorescent lifetime data as well as intensity measurements.

Nonideal conditions (surface roughness, particulate scattering of the incident light, beam divergence) can lead to uncertainties in the penetration depth, d, of the evanescent field and cause some “flaring” or enlargement of the illumination volume (74). Consequently, some workers are exploring the use of two-photon excitation techniques (72, 74). The instrumental configuration is similar to that of conventional TIRF, except that a near-IR laser is used as the light source. As in other applications of nonlinear methods, the excitation volume is confined to a small region where the photon flux is high enough for fluorophores to absorb two photons at the same time.

Although predicting the penetration depth for the evanescent field has uncertainties that are difficult to estimate in advance,
Sarkar and colleagues have recently demonstrated that the evanescent field strength can be precisely calibrated as a function of depth (77). These authors fixed quantum dots to the tip of a cantilever from an atomic force microscope (AFM) head placed on the sample stage of the fluorescence microscope. They recorded the fluorescence intensity as a function of distance between the probe and interface in order to calibrate the relative intensity of the evanescent wave as a function of distance. The authors demonstrated their TIRF distance measurement by attaching opposite ends of a polyubiquitin molecule to the TIRF interface and the cantilever. Pulling on the cantilever with a constant force of 100 pN caused the protein to unwind in stages and the probe slipped back away from the TIRF surface in steps. The intensity changes indicated 20.1-nm displacement steps in excellent agreement with the simultaneously acquired AFM data. The authors anticipate that the use of the calibrated intensity scale will be applicable to measuring displacements and conformational changes of molecules inside a cell where an AFM cannot reach.

**Selected Applications.** The combination of TIRF microscopy and FRET has provided a powerful way to study molecular interactions in cell membranes. The Schneckenburger group used laurdan as a probe for sensing environmental polarity for the interior of lipid membranes (72). Its emission spectrum shifts to longer wavelengths the more polar the environment. By coupling fluorescence lifetime, fluorescence polarization, and spectral measurements gathered in TIRF experiments, these workers correlated membrane stiffness and fluidity with temperature and changes in cholesterol content (78). This technique provides a possible tool for studying the influence of various disease states on membrane fluidity and subsequent influences on cellular release of metabolites and uptake of pharmaceuticals.

TIRF microscopy has been a powerful tool in recent studies of impairment to the mechanism of insulin release associated with diabetes. The dynamics of insulin docking with receptors on the surface of pancreatic cells was studied using a GFP-labeled insulin granule (79). In this work, researchers demonstrated that the insulin granules appear in three different states near the membrane of pancreatic cells. The researchers counted the number of granules that docked with the membrane using TIRF. In investigations of the role of autoimmunity in the development of type 1 diabetes, this group showed that exposure of normal pancreatic β-cells to interleukin (but not interferon) decreased the rate of docking of granules rather than the total insulin inside the cells (80).

**SPECTRAL IMAGING**

Although many scientists think of spectral imaging merely as another means for providing image contrast, the ability to obtain a spectrum at each pixel can provide information that enables chemical analysis inside cells. Reviews in the last three years have covered applications of spectral imaging in microscopy for plant sciences (81), live cell studies (82), and membrane dynamics (78).

Because of the high sensitivity of fluorescence methods, spectral imaging for bioanalytical purposes has most commonly employed emission spectra. Spectral imaging presents a three-dimensional data acquisition problem; therefore, efficient data collection is important. Some commercial instruments use a series of filters to restrict the light reaching the detector to a narrow band-pass (83). Images are then recorded with each filter so that the data are accumulated as separate maps of the specimen (in x/y space) at separate wavelengths. The complete data set is sometimes referred to as a spectral cube. In addition to instruments with a set of discrete glass filters, there are also tunable liquid crystal filters and acoustical optical filters in use.

A second approach is to use an interferometer in place of filters. A Sagnac interferometer, a compact device that uses one rotating beam splitter and two fixed mirrors, can be adapted to most fluorescence microscopes (84). In this case, the third dimension in the spectral cube has to be converted into the spectral domain via a Fourier transformation. A third strategy focuses the image from the specimen onto the front of a monochromator. Only a thin section of the image passes through the entrance slit, is dispersed, and is refocused onto a CCD camera. The camera records spectra in parallel for one column of pixels in the microscope’s field of view. Then the frame of light at the front of the monochromator is shifted to send the adjacent slice of the image into the entrance slit. The process is repeated until the entire physical image is recorded.

Most instruments use a sensitive electron multiplier CCD camera or image intensifiers coupled to a CCD camera for capturing images. A photomultiplier tube (PMT) can also serve as a detector in instruments where the excitation source is a tightly focused beam from a laser that is scanned over the surface (84). In that case, the x/y coordinates in the image are derived from the laser control circuitry.

**Technique and Instrumentation.** Recently several groups in this field have combined FRET and FLIM with spectral analysis (83, 85—88). The spectral resolution allows for better distinction between overlapping emission spectra of the donor and the acceptor. Since the lifetime of the donor excited state decreases when FRET occurs, and it is not complicated by spectral overlap, lifetime measurements can be used to signal the contact between proteins. However, FLIs are also influenced by other environmental factors such as pH and polarity. Spectral data can provide complementary information for distinguishing FRET from background interactions (88). The combined FRET and FLIM technique has also been applied to studies of membrane potential (89).

The photophysical properties of streptavidin-conjugated quantum dots were characterized (90). They are attractive reagents for bioanalytical applications of spectral imaging because of their selectivity, high sensitivity, and photostability. The titration of nanomolar levels of the quantum dots was demonstrated by monitoring spectral changes as well as fluorescence lifetime measurements.

Ecker et al. demonstrated that spectral imaging permitted the simultaneous use of as many as eight different fluorescently labeled analytes and enabled the correction for background due to autofluorescence (87). Attempts have been made to quantify phthalocyanine concentrations in vivo via fluorescence spectral analysis (91). Phthalocyanine is used as a photosensitizer in anticancer therapies.

New multianode PMTs have replaced cameras in some of these new instruments because of their rapid response time. These instruments usually are laser scanning systems where the position of the beam spot defines the x/y coordinates of the image. Although the PMT provides only 16 or 32 channels at a time for monitoring...
wavelengths in the focal plane of a monochromator, the high data acquisition rate makes it practical to measure fluorescence rate constants based on modulation and phase shift methods (86, 87).

An increasing number of groups are employing near-infrared lasers for two-photon excitation in combination with spectral imaging (83, 84, 89, 90). In addition to lower photo damage, less background fluorescence is observed since the photon flux is usually too low for nonlinear excitation outside the tiny volume where the laser is focused. Furthermore, the entire visible range is free from Rayleigh scattering from the laser.

**Selected Applications.** Intensities at two emission wavelengths were used to discriminate against contributions from autofluorescence in the monitoring of single molecules of labeled RNA (92). In this work, individual molecules were counted as they were injected from a special pipet into the cytoplasm or into the nucleus of living mouse fibroblast cells.

The fluorescent stain, SYBR Green II, was used to detect soil microorganisms in marine sediment population studies. By recording the emission spectrum, unbonded stain and other background features could be excluded and the number of individual bacteria could be counted by machine (93).

Two nonfluorescence applications deserve mention here. Katsilakis et al. demonstrated that absorption spectra could be extracted from photomicrographs of reflected light (94). They built a simple monochromator with a 6–15-nm band-pass based on a linear variable filter. The authors applied spectral analysis in order to distinguish normal lymphocytes from lymphoblasts of acute lymphoblastic leukemia. In another study, spectral imaging was used to quantify levels of a cytochrome P450 protein (CYP1B1). The spectral method appeared to be a more sensitive and objective way of diagnosing certain tumors (95).

Vejux and colleagues studied the interaction of 7-ketocholesterol (7KC), a potent inducer of apoptosis, with cell membranes (83). 7KC is an important oxidation product found in lipid membranes associated with atherosclerosis. This study showed that cells treated with 7KC accumulate cholesterol (as verified by GC/MS) and have an increased content of polar lipids in the cell membrane based on microscopic measurements made using FRET and spectral analysis.

**NEAR-FIELD SCANNING OPTICAL MICROSCOPY**

The deflection of light by submicrometer objects limits resolution of normal optical microscopes to dimensions of ~250 nm. Near-field scanning optical microscopy (NSOM or SNOM) is a probe technique that promises to provide lateral optical resolution down to 10 nm. In NSOM, the probe acts as a narrow gate through which light is delivered, collected, or scattered. Light crossing between the probe and sample is influenced by interactions with the sample mainly within the immediate neighborhood of the probe. In fact, theory predicts that the principle mechanism for light crossing between the probe and sample is through an evanescent wave of the source light overlapping with the other material (96). However, these methods require that a probe be scanned over the surface of the specimen usually within a distance of several nanometers (i.e., less than the diameter of the aperture, on the order of 10 nm). Until recently, maintaining such tight position control in an aqueous system has not been practical for soft, biological specimens that are easily damaged by probe "overshoot" (97). Some new techniques have overcome this barrier and some biological applications have been reported, but they have been mainly limited to imaging membrane surfaces (rather than chemical analysis). A review by some pioneers in the field summarizes progress up to 2003 (98). Most of the other references in this field cited here were chosen for the potential of their contributions for making future quantitative work possible.

Instead of using the probe to illuminate the sample, one can employ the conventional optics of a fluorescence microscope for illumination and use the probe to collect fluorescence emission. Theoretically, the information obtained by this approach is equivalent to the illumination mode (96). A second alternative is sometimes called apertureless NSOM. In this mode, a metal probe is used to scatter the light from the near-field of the sample. In apertureless NSOM, the sample is illuminated with standard ("far-field") optics. Some of the fluorescent light emitted by molecules in the sample surface does not propagate out into the medium but remains in the sample as a totally internally reflected wave. The associated evanescent field can interact with surface electronic waves (plasmons) in the metal on the probe tip scattering it into a propagating wave that can be collected by an objective (96). Gold or silver nanoparticles are particularly effective for this scattering process and have been suggested as material for capping apertureless probe tips. This mode is free from the practical difficulties associated with making circular apertures on the nanometer scale. Furthermore, it should be capable of lateral optical resolution as fine as 10 nm (99).

**Technique and Instrumentation.** Only recently have advances in position control of the probe made NSOM imaging of live cells possible. Previously, successful control of probe distance was done (only in air) by a tuning fork driven at its resonant frequency, which is dampened by molecules on the surface of the sample. This phase shift between the driving voltage and the oscillation of the tuning fork (as monitored by a separate laser beam) increases as the distance between the probe and specimen decreases (97). Koopman et al. (97, 100) have demonstrated that, in aqueous environments, sensitivity to the surface topography can be regained by keeping the tuning fork dry in a "diving bell" enclosure just above the probe. Hoppener and colleagues introduced a "tapping mode" force sensor in which a tuning fork driven by a piezoelectric crystal is positioned so that its tines were parallel to the sample surface (101). The NSOM fiber probe was attached to the end of the tine closer to the sample with the tip perpendicular to the fork. The probe protruded ~2 mm below the fork. In this manner, the probe was immersed in the sample solution while the tuning fork was kept dry above the liquid. The sensitivity of the probe to shear forces near the sample remained high. Several other groups have done something similar (102).

An alternative method for position control based on ion conductance was reported. Early NSOM work was performed with hollow probes similar to the micropipets used by membrane physiologists. In addition to being easy to make, they can be filled with buffer and used to monitor the ion conductivity between the probe and the sample solution. As the probe nears the sample ion, conductance is partially blocked and the change in conductivity can be used as a measure of the probe to sample distance. This mechanism has been coupled to NSOM for obtaining images of living cells (103). More recently, Rothery et al. exploited the pipet
type of probe not only for position control by ion conductance but also as a unique light source (104). In addition to transferring ions, their “nanopipet” delivered fluor-3, a reagent for calcium that formed a strongly fluorescent complex at the sample surface near the probe opening. Light from an argon ion laser excited the complex that was formed near the end of the pipet. The light from the probe was collected by the objective of an inverted microscope. They showed that the intensity increased 2-fold as the probe came within 70 nm of the surface of the cells that they were imaging. These images were based on optical contrast rather than emission from tagged substances on the surface. They achieved an optical resolution of ~190 nm, and the topographical resolution provided by the scanning ion conductance microscopy was 220 nm. Despite the fact that the resolution in this study was not as good as with other NSOM methods, the pipet may become competitive as workers strive to make smaller probes.

Silica fibers are becoming more common as waveguides, but the preparation of high-performance fiber probes is challenging. After the outside is coated with metal (in order to prevent light leakage), an aperture must be etched in the tip. Reproducibility in making smooth, circular apertures has been a problem for some of the simpler methods. Other methods such as focused ion beam milling can involve expensive equipment. Haumann et al. have described a relatively simple method for electrochemically etching an aperture at the tip of a silver-coated silica fiber that consistently yielded probes with a spatial resolution of 90 nm (105).

One of the challenges in the use of apertureless NSOM is the high level of background light reaching the detector. A method of discriminating the signal from the background was demonstrated by Diziain (106). A vertically vibrating probe can be made to move in and out of the evanescent field creating a modulated signal. Because of the modulation, this enhancement of the propagating wave intensity can be selectively detected using a lock-in amplifier tuned to the oscillating frequency of the probe. Other nonlinear energy-transfer mechanisms are also candidates for this type of modulation enhancement of the NSOM imaging. For example, Muller et al. (99) demonstrated NSOM using the Forster energy transfer from a probe tip supporting a polystyrene bead coated with CdTe nanocrystals. Images could be constructed from both the decrease in fluorescence from the nanocrystal donor and from the increase in fluorescence of an acceptor molecule residing in the sample.

Selected Applications. NSOM is well suited for studying receptor molecules and heterogeneities in cell membranes with little interference from intracellular fluorescence. Ianou and colleagues have attempted to quantify the number of β-adrenergic receptor protein molecules that combine to form channel clusters or “signosomes” on the surface of cardiac myocytes (102). Based on the fluorescence intensity of tagged antibodies targeted to the receptors, they estimated the dimension of the clusters, their density on the cell surface, and the lower limit for the number of receptors (30) that were present in the average complex in rat H9C2 cells.

Koopman et al. were able to detect individual transmembrane DC-SIGN proteins in immature dendritic cells (100). The authors used circularly polarized light to excite the proteins tagged with fluorescent antibodies. A beam splitter and polarized filters in the collection optics allowed them to monitor the polarization of the emitted light with two separate photon-counting avalanche photodiodes (APDs). If signal from a specific site appeared in only one APD, the probability was high that they were observing only one molecule at that moment and location. Simultaneous signals in both detectors implied multiple emitters in different dipole orientation or one emitter with an optical dipole at ~45°. They were able to observe individual proteins in 90-nm domains. Consequently, it was possible to estimate the surface density and spatial distribution of these proteins, whereas confocal images were not able to resolve individual molecules. Furthermore, the background was 10-fold darker in the NSOM fluorescence image because the excitation volume for the NSOM was much smaller.

RAMAN MICROSCOPY

Raman microscopy is a nondestructive technique that provides information on molecular vibrational modes with high spatial resolution. The basic concept consists of illuminating the sampled submicrometer volume with a focused laser (e.g., 1064-nm Nd: YAG laser), which then is inelastically scattered with loss (or gain) of energy associated with the molecular vibrational modes. The spectral dispersion of the scattered light is used to construct a Raman spectrum from femtoliter size volumes. Both resonant (laser excitation at a wavelength suitable for an electronic transition) and nonresonant Raman microscopy have been reported. Selected reports describing the foundations and limitations of Raman microscopes included works by Vyroýkkä et al., Sourisseau and Maraval, and Everall (107–110). The technical review by Everall stresses the issues that are relevant to confocal Raman measurements. Vyroýkkä et al. describes experimentally and theoretically the benefits of matching the index of refraction of the sample with its exterior by using immersion oils and deconvolution that reduce the blurring effect. These details allow for better depth resolution (i.e., 2.1 μm) and better accuracy in determining sample thicknesses. The treatise by Sourisseau and Maraval describes the effects of refraction, diffraction, and spherical aberration under different pinhole apertures, which are required for a true confocal measurement. Using their model, depth profiles of 150–200 μm and depth resolutions of 1.0 μm are reported for thin films.

Coherent anti-stokes Raman scattering (CARS) continues to be an important development for Raman microscopy and imaging (111). This technique, in addition to sensitivity (larger scatter cross section) and three-dimensional spatial resolution, offers high speed, which facilitates monitoring dynamic processes. To observe CARS, it is necessary to use two coherent propagating or counterpropagating pulsed laser beams that provide a pump field and an anti-Stokes field in a small excitation volume (i.e., ~1 μm²). The vibrational constant arises from the signal enhancement when the difference in frequencies of the two laser beams is tuned to a Raman-active vibrational band. Rastering the laser beams over the sample then produces an image. Similar to conventional Raman experiments, resonance may enhance the CARS mode when absorption of one or two photons at the frequency of the laser pump leads to an electronic transition. For a review on the theory and applications, see ref 112.

Technique and Instrumentation. Three exciting new directions are multiplex CARS, CARS correlation spectroscopy (112), and CARS-based optical coherence tomography (113). The first
technique uses a narrow-band and a broadband dye laser for the pump and the Stokes beams, respectively, which then can be used to emulate broad Raman spectra. A related report on nonlinear interferometric vibrational imaging, which shares several features with multiplex CARS, also appeared (114), and Kee and Cicerone have reported an increase of bandwidth up to 2500 cm\(^{-1}\) (115). The second technique is useful to measure fluctuations of the CARS response in the excitation volume. An example of the third technique is a prototype that measures CARS interferometrically over at least 600-\(\mu\)m depth of field and was demonstrated in measuring stacked microlayers of glass–acetone–glass–air and beef fatty tissue (113). This approach has further been used for distinction and identification of the background (nonresonant Raman) from the resonant Raman signal via polarization and phase control in an approach called double quadrature spectral interferometry of polymeric samples (116). The authors acknowledge that technical challenges such as axial chromatic aberration of the microscope objective and limited bandwidth still remain.

A promising technological combination is tip-enhanced Raman microscopy (117, 118). This approach uses a metal-coated cantilever (i.e., from an atomic force microscope) to create an enhanced field at the tip apex, which then makes SERS possible when the tip contacts the surface being explored. In these microscope designs, the excitation source usually uses an epi-illumination/collection scheme, which also allows for measurement of conventional Raman and fluorescence images. There are still technical difficulties that need to be overcome (e.g., attachment of impurities to the tip, difficulties in controlling the morphology of the tip, and the aging of the tip). These advances appear necessary before it can be concluded that this technology provides the enhancement expected from SERS.

**Selected Applications.** The distinction between basal cell carcinoma and normal skin tissues (119), based on the amide I mode and the PO\(_4\)^– vibrational mode, is discussed. This study demonstrates the potential of the technique for a dermatological diagnostic tool.

Beattie used Raman microscopy to investigate the chemical environment of different retinal regions (120). While using 785 nm for excitation, they mainly observed vibrational modes associated with proteins and lipids. When using the 633-nm excitation, they found additionally the presence of cytochrome. By using 514-nm excitation, the features above were even more enhanced. Principle component analysis revealed that each excitation wavelength preferentially defines a given retina subregion. This report clearly shows a strong correlation between the histological map and the Raman counterpart.

While Choi et al. and Beattie et al. observed a section that was microtomed perpendicular to the epidermis, Xiao et al. (121) used confocal Raman microscopy for depth profiling (30–100 \(\mu\)m deep) of the epidermis and for tracking the permeation of deuterated lipids that were delivered in the form of vesicles. The results show the superior spatial resolution of the technique (2–3 \(\mu\)m\(^3\)/pixel) when it is compared with IR imaging (10–12 \(\mu\)m on a plane).

Jarvis and Goodacre reported the use of ultraviolet excitation (i.e., 244 nm) to produce resonance-enhanced Raman signals of bacteria found in common urinary tract infections (122). The short-wavelength excitation has two advantages: (i) the Raman spectra are not subject to fluorescent background and (ii) the spectra show enhanced vibrational features of nucleic acids because this type of compound absorbs strongly at this wavelength. The results illustrate that this approach is a useful tool for bacterial discrimination.

Raman microscopes were also used to investigate single particles and cells (123–129). Although the particle experiments are not in a practical sense true image experiments, they are mentioned because of the use of the instrumentation and because they represent a promising approach to reduce interferences from other neighboring regions. Both particles attached to a substrate (123, 125) and levitated via optical tweezers (124, 126, 127) were reported. The Raman spectra of individual pollens attached to the surface provided compositional data that showed predominantly the vibrational modes of carotenoids (123).

Single microbial cells attached to a surface were also investigated by Raman microscopy (125). This is a promising avenue to investigate metabolic transformations of isotopically labeled compounds (i.e., \(^{13}\)C\(_6\) glucose) in single microbes. It also shows that it is possible to distinguish different species in their stationary and growth phases after using principle component analysis to analyze and interpret the Raman spectra. The Harris group used this approach to obtain Raman spectra from individual liposomes (0.6, 2.0, and 5.0 \(\mu\)m) (124, 126, 127). They demonstrate that each isolated lipid has a spectrum that is characteristic of its phospholipid composition and that it is feasible to investigate leakage through the phospholipid membrane (127). In follow-up reports by the same group, they present an elegant theoretical description of depth profiling and optical trapping (126) and use the technique to investigate lipidosome deformation that is caused by the optical trapping forces of the laser, which is also used for Raman excitation (126).

Using confocal resonance Raman microscopy, van Manen described the localization of heme-containing enzymes (e.g., NADPH oxidase) and studied the associations between phagosomes and lipid bodies in single leukocytes (128, 129). The cytochromes found in these enzymes strongly absorb the 413.1-nm excitation, which then results in Raman enhancement due to resonance. Besides being a valuable contribution to knowledge in cellular biology, this report is clearly an example of the state-of-the-art subcellular imaging based on vibrational spectroscopy.

An interesting combination of biochip technology and surface-enhanced Raman scattering (SERS) was also reported (130). It relies on immobilizing on a chip (e.g., via an antibody bound to the surface) pathogens or toxins. Due to the presence of a SERS-active metal on the surface, an enhanced Raman spectrum would be expected. From the presented results, it is clear that Raman spectral measurements are feasible although it is less clear if indeed SERS is playing a role in the observed response. Another report compared the SERS spectra of six Gram-negative bacteria. Imaging was feasible and characteristic spectra, less complex than normal Raman spectra (likely resulting from only those molecular features close to the nanocluster and with the correct orientation), were observed for each species when they were deposited on gold nanoclusters (131). This study points to the feasibility of using SERS for the characterization of bacteria despite the complexity of these systems. Last, but not least, protein structure changes (e.g., integrins) were successfully investigated after immobilization.
onto rough electrochemically deposited gold (132).

Salient biological applications of the specialized forms of CARS microscopy have been reported in the last three years. There is one impressive report on conventional CARS microscopy for the imaging of lipid droplets in live fibroblast cells. Using one frequency of aliphatic C–H vibrations (i.e., 2845 cm⁻¹), images (512 x 512 pixels) were acquired at a rate of 1 Hz. In addition to the nondestructive nature of the experiment, this example shows that the CARS fast imaging rate is suitable for investigating dynamic processes in vivo. Good descriptions of the theory and predictions of lipid orientations based on CARS were published by Wurpel et al. (133) and Wang et al. (134). The lipid surveying by CARS was later used to investigate lipid buildup as a result of inhibition of a peroxisome proliferator-activated receptor (135), to investigate lipid order in axonal myelin in live spinal tissue (134, 136). In addition the concentration dependence and adhesion to glass effects on the conformation of the extracellular matrix protein (i.e., collagen) were investigated and compared with Fourier transformed IR (FT-IR) and circular dichroism (137). This report shows feasibility but was not used for imaging. The ultimate application of CARS had been the imaging of tissue (i.e., mouse ears) in vivo at video rates (30 frames/s) and maximal depth of 125 μm (138).

IR MICROSCOPY

Recent advances in IR microscopy, another nondestructive technique that provides information on the molecular vibrational modes of the sample, have enhanced the capability to study biological samples on a chemical and microscopic scale (139). The primary reason is that a majority of biological molecules, such as proteins, lipids, carbohydrates, or nucleic acids, have specific and well-defined vibrational fingerprints in the IR region, making it unnecessary to label or tag the sample. This method is complementary to Raman microscopy; however, the intensities of Raman bands are generally several orders of magnitude smaller than those of IR bands. Synchrotron IR microscopy has allowed extension to the lower-energy spectral range (far-infrared) with negligible effects of the synchrotron IR radiation on the sample.

Technique and Instrumentation. The newest advances in FT-IR microscopy, including instrumentation development, data processing algorithms, and the introduction of the method to new fields, were summarized in a comprehensive review by Levin and Bhargava (140). Developments in the area of infrared microscopes, detectors used, sampling and collection techniques, and data acquisition and processing methods are critically reviewed and discussed. Spatial variations of several quantities of hydroxypatite and total protein in bone tissue are one example of numerous biological applications of FT-IR microscopy discussed in the paper. The data acquired by FT-IR microscopy have been proved clinically useful, and the authors foresee further development of the method at an accelerated pace. Additional examples of the use of IR microscopy for biomedical diagnostics were outlined by Bindig et al. (141).

Selected Applications. IR spectroscopy can be limited by the strong and wide infrared absorption bands of water, which tend to mask bands of other molecules. While this is often considered a disadvantage, Bründemann and colleagues have used this feature to measure intracellular water content in living cells (142). They employed a near-infrared (NIR) microscope with a tunable diode laser in the range of 1530–1570 nm, which is close to the peak of overtone absorption of water, to examine the water content in rat hepatocytes. Hypoosmotic treatment of the cells showed that changes in the water concentration can be detected and easily extracted using \( \alpha = -\ln(I/I_0)/L \), if the thickness of the cell is known (L), where \( I \) is the measured intensity and \( I_0 \) is a reference intensity of buffer. Confocal microscopy can provide further improvement by measuring the water content in different sections of a cell.

Baeten et al. explored the possibility of detecting the presence of meat and bone meal (MBM) in compound feeds, an analysis of high relevance to assess the risk of bovine spongiform encephalopathy (aka mad cow disease), by collecting NIR spectra in the 1112–2500-nm range (143). It was shown that the MBM concentrations as low as 0.05% (w/w) can be detected upon deconvolution of the spectral information and construction of a discriminant model by the partial-least-squares algorithm.

Clarke also used NIR microscopy for analysis of pharmaceutical samples (144). A chemical NIR image in the 1100–1700-nm range was used to determine the spatial distribution and cluster size of ingredients in tablets and pretabletting blends. The author believes that an increase in speed of analysis would be useful, but the data processing is still the main hurdle in the development of NIR microscopy.

An intriguing demonstration of the potential of synchrotron IR microscopy was published by Holman and co-workers (145), who used it to discriminate between cells in different growth cycle stages. Another example was detection of the expression levels of the cytochrome P450A1 gene (CYP1A1) upon exposure of human hepatocellular carcinoma cells to a polychlorinated aromatic compound 2,3,7,8-tetrachlorodibenzo-p-dioxin. Similar work presented by Raab and Vogel used synchrotron IR microscopy to predict mutations in the \( \text{pm} \text{d}4 \), \( \text{pm} \text{d}5 \), and \( \text{pm} \text{d}6 \) resistance genes in live leaf, stem, and root tissues of Arabidopsis taliana (146). Dumas and Miller also presented several biological applications of synchrotron IR microscopy (147). IR microscopic analysis of 5μm cross sections of human hair revealed differences in secondary structure composition of proteins in different sections of the hair and that lipids were highly localized in the centermost portion of the hair.

The use of synchrotron IR microscopy to distinguish between cancerous and normal tissue was reported by several groups (148, 149). They found mid-infrared spectra collected from different cancerous tissue samples to be sufficiently distinct from healthy tissue. Mark et al. showed that principal component analysis of the collected data allows one to categorize single cells into one of three groups (149). Yano et al. used glycogen levels measured by this technique to discriminate between cancerous and healthy tissue in patients with lung and colorectal cancers. Two bands at 1045 and 1545 cm⁻¹ were chosen, and the ratio of the intensities at the two wavelengths correlated with the glycogen concentration determined chemically.

A fascinating example of the use of synchrotron IR microscopy was put forth by Cotte et al. (150). They applied this technique to study skin sections obtained from an Egyptian mummy. Characteristic vibrational signatures of calcium oxalate, proteins, and fatty acids identified these compounds in different layers of

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the skin, but provided little quantitative information. Due to the nondestructive nature of IR microscopy, this technique is an essential tool when little amounts of material are available or for samples of great cultural value.

**X-RAY MICROSCOPY**

For the purpose of this review, X-ray microscopy can be divided into two categories: (i) radioautography, which visualizes the localization and concentration of radioisotopically labeled compounds incorporated into living organisms (predominantly cells), and (ii) X-ray microanalysis, which analyzes the total amounts of elements present in the sample. The history, methods, and applications of radioautography have been meticulously summarized by Nagata, one of the pioneering leaders in the field, in his 167-page review (151). Two years later in 2004, he published a just-as-thorough review article on X-ray microanalysis (152).

Typical applications described in his reviews include the use of X-ray microscopy for quantitation of (i) radiolabeled compounds, such as ^3^H-uridine and ^3^H-thymidine, (ii) gold or silver particles upon colloidal immunostaining, and (iii) endogenous elements in cells and tissues, such as sodium, phosphorus, sulfur, chlorine, potassium, calcium, and zinc.

**Technique and Instrumentation.** Recent advances in X-ray microscopy have improved the quality of lenses and focusing optics used, thereby bringing the resolution of X-ray imaging down to 15 nm (153). Miao et al. detailed a new method of X-ray diffraction microscopy applied to cells and cellular structures, providing 3D images of cryoprotected whole cells by the use of a femtosecond flash X-ray source (154). The authors envision this technique to be applicable to imaging of single macromolecules, even when the sample is noncrystalline, but a sufficient supply of exact copies of the structure of interest must be present in the sample. This approach could open a new horizon for imaging macromolecules that are hard to crystallize and it could provide significant structural, but little quantitative information on biologically significant molecules. Further structural information on large biological assemblies can be obtained by combining X-ray crystallography and electron microscopy (155).

Schneider discussed the use of X-ray microscopy for detection of proteins in cells (156). In comparison to transmitted light or fluorescence microscopy, X-ray microscopy has the potential to improve the accuracy of locating proteins within cells, resulting in more accurate information about the distribution of proteins and their mutual interaction. The authors demonstrate this potential on an example by elucidating the spatial relation of the X chromosome and the nuclear protein MSL-1. To complete this effort, however, an increased resolution by collecting more projections and subsequent automated data processing will be necessary.

**Selected Applications.** Current analytical efforts in X-ray microscopy are oriented toward characterization and quantitation of the structural organization in living organisms. Hernandez Cruz et al. proposed and tested quantitative mapping of the orientation of fibron ^β^-sheets in a cocoon silk fiber by scanning transmission X-ray microscopy (157).

Another group used scanning transmission X-ray microscopy to map the quantity of metal species in microbial biofilms cultivated from river water (158). Imaging was achieved using a near-edge X-ray absorption spectrum of the sample, which does not need to be processed in any special way and allows examination of specimens in hydrated environments. The authors demonstrated its practical use and robustness by composing quantitative maps of the iron, nickel, and manganese species in the microbial biofilm samples with a spatial resolution of 50 nm.

**MASS SPECTROMETRY IMAGING**

Mass spectrometry imaging is an extension of other conventional mass spectrometric techniques in which ions are produced by processes such as matrix-assisted laser desorption/ionization (MALDI) or secondary ion mass spectrometry (SIMS). However, in mass spectrometry imaging, the mass spectrum must correspond to ions from specific micrometer-size regions that are produced by virtue of controlling the position of the laser beam (MALDI) or the primary ion source (SIMS). Using these destructive techniques, ions are produced; these are directed to a mass analyzer, which selectively or temporally transmits ions to a detector that then records the spectrum associated with each position. By rastering the laser (214-nm nitrogen laser) or the primary ion probe beam (e.g., gallium source) over the biological surface, the entire surface is sampled and a mass spectrometry image is generated. While MALDI-based imaging can provide spectral details of biomolecules at large m/z values (e.g., >1000 m/z), SIMS is mainly used for smaller molecular fragments or elemental ions that have small m/z values (e.g., <200 m/z). SIMS-based imaging has better spatial resolution (e.g., 0.5–1 μm) than MALDI-based imaging (e.g., 25–50 μm). Sample preparation is highly specialized for each technique. While MALDI requires controlled and reproducible deposition of a matrix (e.g., sinapinic acid), SIMS usually requires cryogenic preparation of the sample (i.e., freezing, cleaving, drying). Reviews on the key features on each of these techniques can be found elsewhere (159, 160).

**Technique and Instrumentation.** Recent reports on technical improvements of MALDI-based imaging are mainly on sample preparation. One report describes the use of histological stains (e.g., Toluidine Blue) on the same tissue cross section that is used for MALDI-TOF-MS imaging. Identification of the tissue features is accomplished by bright-field microscopy and then the sample is subjected to MS imaging. A major technical improvement in this report was the use of indium–tin oxide-coated slides as part of the MALDI plate holder. The transparency of the material makes it suitable for bright-field imaging; its electrical conductivity makes it suitable as a sample deposition target (160). Another salient report conducts a subcellular spatial profiling of single neurons after identifying suitable conditions (e.g., deionized water/air-drying, 50% glycerol/artificial seawater) that prevent distortion of the initial localization of neuroepitides (161). The authors also comment that cation adducts cannot be eliminated with these procedures.

A promising instrument design that resembles the geometry of a true optical microscope was reported (162). In this report, the entire sample surface is illuminated with a pulsed laser to simultaneously cause MALDI in the entire surface. Then, the formed ions are guided by ion optics in parallel trajectories toward a dual microchannel plate. The flight time of ions to reach the microchannel plate encodes the m/z ratios as is done in time-of-flight mass spectrometers. The speed of image acquisition
obtained for patterned peptides (e.g., insulin) in this report suggests that mature versions of this instrumentation can lead to true MALDI-MS imaging.

Recent technical advances to improve the sensitivity in SIMS imaging of biological systems include the use of buckminsterfullerene (C_{60}^-) as a primary ion source and the use of a matrix to enhance the ion yield (i.e., matrix-enhanced MALDI) (163, 164). The use of C_{60}^- as a primary ion source for the analysis of lipid films results in a 1000-fold improvement over Ga^+ and In^+ sources (163). A report on matrix-enhanced SIMS used 2,5-dihydroxybenzoic acid as the matrix and an indium liquid metal ion gun to develop methods to profile lipids in ganglia and neurosecretory organs (164). The matrix was delivered to the tissue sections by electrospray deposition, which causes microdroplet formation and decreases the risk of image distortion by redistribution of the analytes in the tissue. This report demonstrates improvements in sensitivity over conventional SIMS imaging and in spatial resolution over MALDI imaging. One drawback was that imaging of a given m/z value was undermined by the topography of the sample.

In efforts to address the difficulty in interpretation of TOF-SIMS images from biological samples, both model membrane Langmuir–Blodgett films and enhanced data analysis algorithms were reported (165, 166). The model membrane studies revealed that proton-donating molecules (e.g., cholesterol) have the ability to mask the signal associated with certain phospholipids (e.g., dipalmitylophosphatidylethanolamine) (165). Keenan and Kotula demonstrated that common data processing steps (e.g., normalization of the pixel intensities, and autoscaling or variance scaling of the mass spectra) lead to less satisfactory results. They show that use of an optical scaling approach allows for estimation of the number of pure components, for segregation of the chemical information, and for estimating small features from noise (166).

An interesting report for SIMS sample preparation based on pattern imprinting was presented (167). In this report, the authors transferred the freeze-dried cells to a silver foil and subjected the pattern to SIMS imaging with a gallium source. The authors state resolutions approaching 100 nm, which are almost sufficient to investigate the functional rafts of differential lipid composition.

**Selected Applications.** Selected applications of SIMS-based imaging include the following: The direct visualization of intracellular distribution of mineralization elements in rat osteoblasts (168, 169), ^{3}B-containing drugs in malignant melanoma MeWo cells (170), mutagens in breast cancer MCF-7 cell lines, and fatty acids in the rat retina (171). SIMS imaging was also utilized to investigate the quality and homogeneity of surfaces used in biosensors (172–178).

**SCANNING ELECTROCHEMICAL MICROSCOPY**

The most common method of obtaining a scanning electrochemical microscopy (SECM) image involves physically rastering a probe above the specimen and measuring the faradaic current associated with the oxidation or reduction of some electroactive compound or ion freely diffusing in the surrounding buffer. Platinum, gold, and carbon electrodes (usually a disk with a typical diameter of 1–10 μm and surrounded by an insulating sleeve) are commonly used as probes. This technique offers the possibility of mapping the spatial and temporal variations in the concentration of compounds and ions that are released from, or that interact with, a cell. For example, recent publications have included studies of catecholamine release from model neurons (PC12 cells) (179). In this case, the current for the oxidation of catecholamine was measured while holding the electrode at a constant voltage (amperometry). Stimulating the cell produced bursts of ejected catecholamine. The imaging mode (based on oxygen reduction current) was used to position the probe before stimulation and then the catecholamine oxidation current was monitored at the stationary electrode. Molecular oxygen (180–184), serotonin (185), and NO (186) are examples of other electroactive analytes that have been recently studied directly by amperometric SECM.

In addition to observing the direct electrolysis of an analyte, amperometric methods have been developed for measuring enzyme activity (187), membrane permeability (188), and intracellular redox activity (189). Potentiometric probes have also been used in SECM. An ultramicro pH sensor based on antimony was reported for monitoring enzyme activity on membrane surfaces (190).

**Technique and Instrumentation.** An independent measure of the probe-to-specimen distance is often critical to the interpretation of the current signal. To address this issue, Kurulagama et al. chose to use an impedance measurement as an independent means of monitoring the probe-to-surface distance (179).

Another group has maintained a fixed probe distance by a feedback mechanism based on the resonance vibrational frequency of a carbon fiber probe. The stagnant aqueous layer adjacent to the cell surface imposes a greater shear force on the probe and changes the resonant frequency (191). A feedback mechanism was employed to maintain a chosen probe frequency.

If respiration rates are steady, then the current for oxygen reduction correlates well with its distance above a cell. Isik and co-workers used a dual amperometric probe in which they measured the oxygen current at a 10-μm Pt electrode to determine the probe distance. They monitored the nitric oxide released from human endothelial cells at a 50-μm electrode modified with a nickel porphyrin complex where it was selectively oxidized (186).

Holt and Bard miniaturized two popular voltammetric techniques in investigating dosage levels of silver nitrate as a bactericide (192). They employed an ultramicro Clark electrode (a polymer-covered oxygen sensor in combination with a reference electrode) as an SECM probe to measure cell respiration. Using a bare Pt disk electrode on a separate probe, the workers monitored the uptake of micromolar levels of silver by anodic stripping voltammetry.

A novel SECM sensor for ATP was introduced by Keung and co-workers (193). Although direct oxidation of ATP at an electrode surface is possible, high background signals, electrode fouling, and high pH requirements present challenges to measuring ATP by amperometry at an SECM probe. To circumvent those problems, these workers immobilized glucose oxidase and an ATP-dependent hexosekinase in a polymer film over a Pt electrode on the probe to carry out an indirect measurement.

**Selected Applications.** SECM has recently been applied to the study of detoxification processes in hepatocytes (194). Cellular efflux of menadione (2-methyl naphthaquinone) and its glutathioneylated metabolite (thiodione) was detected by different oxidation potentials by cyclic voltammetry. Assuming the enzymatic conver-
sion of menadione to be very fast, these workers were able to obtain an estimate of the rate of efflux of thiodione per cell.

Mirkin’s research group has studied the use of membrane permeable mediators as a means of characterizing the redox activity of cells (189). In their work a redox mediator, such as \( N,N,N,N\)-tetramethyl-1,4-\( \alpha \)-phenylenediamine (TMPD), is added to the buffer surrounding the specimen. The oxidation of TMPD at the SECM probe produces a species, \( O \), that can diffuse into the cell nearby. Intracellular redox centers can regenerate the starting material that also diffuses out of the cell.

\[
R^{\text{red}} + O \rightarrow R^{\text{ox}} + O^{\text{red}}
\]

The cellular regeneration of the reduced mediator creates a higher steady-state concentration of that form, \( R \), near the SECM probe. Consequently, the oxidation current increases (compared to the background signal). Using a simplified model, a value for the heterogeneous rate constant, \( k \), for the overall regeneration process can be calculated from the oxidation current. These workers have shown that metastatic breast cancer cells could be distinguished from normal breast cells by their redox activity. The work described in this reference was aimed at detecting a metastatic cell in a layer of otherwise normal tissue (189).

**Table 1. Qualitative Comparison of Chemical Imaging Techniques**

<table>
<thead>
<tr>
<th>Imaging Technique</th>
<th>( x-y ) Resolution (( \mu \text{m} ))</th>
<th>( z ) Resolution (( \mu \text{m} ))</th>
<th>Depth (( \mu \text{m} ))</th>
<th>Chemical Information</th>
<th>Speed</th>
<th>Destructive Power</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional Fluorescence</td>
<td>0.5</td>
<td>1</td>
<td>100</td>
<td>Low</td>
<td>Fast</td>
<td>Low</td>
</tr>
<tr>
<td>Confocal Fluorescence</td>
<td>0.5</td>
<td>0.25</td>
<td>100</td>
<td>Low</td>
<td>Medium</td>
<td>Low</td>
</tr>
<tr>
<td>FRET</td>
<td>0.5</td>
<td>0.25</td>
<td>100</td>
<td>Low</td>
<td>Fast</td>
<td>Low</td>
</tr>
<tr>
<td>TIRF</td>
<td>0.5</td>
<td>0.2</td>
<td>0.2</td>
<td>Low</td>
<td>Fast</td>
<td>Low</td>
</tr>
<tr>
<td>NSOM</td>
<td>0.1</td>
<td></td>
<td>Surface</td>
<td>Low</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Raman</td>
<td>1.0</td>
<td>1.0</td>
<td>200</td>
<td>Medium</td>
<td>Slow</td>
<td>Low</td>
</tr>
<tr>
<td>Confocal Raman</td>
<td>1.0</td>
<td>0.3</td>
<td>200</td>
<td>Medium</td>
<td>Slow</td>
<td>Low</td>
</tr>
<tr>
<td>CAR</td>
<td>1.0</td>
<td>0.3</td>
<td>200</td>
<td>Medium</td>
<td>Fast</td>
<td>Low</td>
</tr>
<tr>
<td>IR</td>
<td>1.0</td>
<td>10</td>
<td>200</td>
<td>Medium</td>
<td>Slow</td>
<td>Low</td>
</tr>
<tr>
<td>X-ray</td>
<td>0.015</td>
<td></td>
<td></td>
<td>Medium</td>
<td>Slow</td>
<td>Low</td>
</tr>
<tr>
<td>MALDI-MS</td>
<td>50</td>
<td></td>
<td>Surface</td>
<td>High</td>
<td>Slow</td>
<td>High</td>
</tr>
<tr>
<td>SIMS</td>
<td>0.05</td>
<td>Surface</td>
<td>High</td>
<td>Slow</td>
<td>High</td>
<td></td>
</tr>
<tr>
<td>SECM</td>
<td>0.5</td>
<td>Surface</td>
<td>Medium</td>
<td>Slow</td>
<td>Medium</td>
<td></td>
</tr>
</tbody>
</table>

*The resolution values are approximate and depend on the wavelength and optics used.*

A potential user needs to consider the plane (\( x-y \)) resolution, depth (\( z \)) resolution, the penetration or depth of the measurement into the sample, the complexity of the chemical information, the speed of the imaging process, and how well the sample will tolerate the destructive nature of the techniques included in this review. Table 1 qualitatively compares these criteria for the current techniques.

Obviously, for in vivo measurements, only nondestructive techniques (e.g., fluorescence and Raman microscopies) are adequate. On the other hand, the user may wish to compromise \( x-y \) resolution and select a technique that provides rich chemical information (e.g., MALDI-MS imaging) when the dynamic aspect of biological systems does not need to be investigated. Last, under some circumstances one may want to consider imaging only surface properties (for instance, by using NSOM) instead of obtaining deep profiles (by using confocal fluorescence microscopy).

Indeed, improvements in all these techniques are still being sought. It is particularly important to develop techniques that can provide superior images with high chemical complexity while monitoring in vivo processes. CARS is improving greatly in terms of speed and may become the preferred optical technique in the long run. However, mathematical algorithms capable of disentangling the spectral complexities of this technique must also be made available.

When direct microscopic analysis cannot resolve the multiple components of a given biological sample (e.g., colocalized fluorescent metabolites with similar spectral characteristics) or when the sought for information cannot be directly provided by the imaging technique (e.g., sequence of unknown DNA), other hybrid approaches may still be necessary. Some of these emerging approaches include microscopy guided mass spectrometry (160), laser capture microdissection followed by PCR techniques (195), or microsampling prior to a separation technique (196).

Improved sample preparation protocols are also likely to continue appearing in the literature for applications in which environment significantly affects instrumental response. For example, probes with analytical responses that are insensitive to subcellular variations are greatly needed. Even in techniques where the detected species result from high-energy processes (e.g., SIMS), there are significant effects due to the sample environment (i.e., membrane lipid composition).

Last, in addition to the clever designs that are appearing for some traditionally sluggish imaging techniques (e.g., MALDI imaging) (162), faster analyses and high-throughput approaches are also emerging. These further developments will slowly make sensitive, selective, reproducible, fast, and in situ chemical measurements associated with the composition and functioning of biological systems a reality.
ACKNOWLEDGMENT
The authors thank the National Institutes of Health, the University of Minnesota, and University of St. Thomas for support. E.A.A. is supported by NIH Career Award K02-AG21453. M.N. is supported by NIH AG20866.

Marian Navratil is a postdoctoral fellow in the Department of Chemistry, University of Minnesota. He received his M.S. (1998) and Ph.D. (2001) in biotechnology at the Slovak University of Technology, Slovakia. He worked as a postdoctoral fellow in the Department of Physics, Chemistry, and Biology at the Linkoping University, Sweden. His current research interests focus on the analysis of mitochondria and mitochondrial proteins with respect to aging.

Gary A. Mabbot is a Visiting Professor in the Department of Chemistry, University of Minnesota, and he has been Professor of Chemistry at the St. Thomas University, Minnesota, since 1989. He earned his B.S. degree at the College of Wooster, Ohio, and his Ph.D. at the University of Wisconsin. His scientific research interests include spectral imaging techniques for bioanalytical and forensic applications.

Edgar A. Arriaga is a professor in the Department of Chemistry and in the Department of Biomedical Engineering, University of Minnesota. He received a Licenciatura in chemistry at the Universidad del Valle de Guatemala (1985) and his Ph.D. in the same field from Dalhousie University, Canada (1990). He worked as a postdoctoral fellow at the Department of Physiology, University of Kansas Medical Center, Kansas City, and at the Department of Chemistry, University of Alberta, Canada. His current interests include the development and application of bioanalytical techniques for colocalization applications. These techniques are based on both microspectroscopy and biological imaging.

LITERATURE CITED
