Molecular Fluorescence, Phosphorescence, and Chemiluminescence Spectrometry

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This review covers the approximately two-year period since our last review (*A1*), roughly from January 2004 through December 2005. A computer search of *Chemical Abstracts* provided most of the references for this review. Other citations were found through individual searches by the various authors who wrote a particular section of this review. In an effort to more effectively accomplish this goal, we have included authors who are experts in the various subtopics of this review. Coverage is limited to articles that describe new developments in the theory and practice of molecular luminescence for chemical analysis in the ultraviolet, visible, and near-infrared region.

Citations may be duplicated between sections due to articles with contents that span several topics. However, in an effort to reduce the length of this review, we have attempted to limit this kind of duplication. In general, citations are limited to journal articles and usually do not include patents, proceedings, reports, and dissertations.

We have tried to focus on important advances of general interest and relevance to the field of analytical chemistry, rather than extensions of previous advances. This was done in an effort to continue our recent attempts to significantly reduce the length of this biennial review. In addition, we have also expanded our description of individual citations for better clarification of content where necessary.

Although we are not able to provide extensive coverage of developments of relevance to broad areas such as chromatography and biological sciences, we have tried to include major review articles and chapters relevant to these topics. If you feel that we omitted an important article published during the above referenced time period, please forward the reference to one of us and we will be certain to consider it for the next review.

BOOKS, REVIEWS, AND CHAPTERS OF GENERAL INTEREST

There were numerous review articles and book chapters on the use and application of luminescence techniques in the last two years. Fluorescence correlation spectroscopy (FCS) can be applied in a number of disciplines to determine very low analyte concentration or investigate molecular binding or structural, physical, and functional properties of macromolecules. The historical development of FCS from inception in the 1970s to date was reviewed by Elson (B1). Brock reviewed the general application of FCS in isolated as well as intact cells with emphasis on the use of time-correlated FCS (B2). Reviews related to the optical and photophysical factors that may have significant effects on the FCS measurement and the measured analyte diffusion coefficient (B3), and general problems associated with FCS measurements, particularly in heterogeneous samples (B4), are available in the literature. Vukojecvic et al. reviewed the current applications of FCS for the study of biochemical processes in living cells (B5). Application of FCS for the investigation of physical and chemical characteristics of protein and protein analysis (B6), in vitro and in vivo characterization of singlemolecule dynamics (B7), and drug discovery (B8) were also reviewed.

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The use and application of two-photon microscopy and FCS to study lipid-protein interaction (*B9*) has been reviewed, and a book chapter was devoted to the application of two-photon FCS to study hapten-antibody binding (*B10*). A general application of fluorescence recovery after photobleaching, FCS, and fluorescence cross-correlation spectroscopy (FCCS) for the monitoring of intracellular material was reviewed by Weiss and Nilsson (*B11*).

The recent developments, methodological advancement, instrumentation, comparative analysis of theoretical and experimental data, challenges, and potential future utility of single-molecule spectroscopy (SMS) were reviewed (B12-B17). There were specific reviews on the use of SMS to study protein folding (B18), conjugated polymers (B19), DNA sequencing (B20), gene expression (B21), and detection of molecules in microfluidic channels (B22). In a review by Higgins and Collinson, the application of SMS to the investigation of heterogeneity within nanoscale environments of organically modified silicate films was discussed (B23).

Schneckenburger reviewed the application of wide-field microscopy, with particular emphasis on the application of fluorescence lifetime imaging, energy transfer, and total internal reflection fluorescence microscopy in cell biology and photobiology (*B24*). A general review of the photophysical properties of nonlinear optical single-molecule emitters for SMS in cellular as well as polymer systems was also published (*B25*).

Significant advances in the design of new optical sensors have been made possible with developments of new light sources, detectors, and methods of data processing. A few examples of reviews in this area describe progress in optical probe development (*B26, B27*). A general discussion of evanescent wave fluorescent biosensors, describing their function and application, has been reported (*B28*). Cao and Heagy review synthetic carbohydrate chemosensors reported since 1992 (*B29*), and the Tan research group explains fundamental principles and biological applications of molecular beacons as DNA probes (*B30*).

The use of NIR dyes as fiber-optic sensors has been reviewed by the Patonay group (B31), and recent advances in tumor imaging applications with near-IR fluorescent tags have been considered by Ballou et al. (B32). NIR fluorophores are finding applications in other fields including separation science. Methods of labeling biomolecules with NIR dyes, through covalent and noncovalent means, reduces matrix interferences and has been reviewed (B33).

Possible energy-transfer or energy-hopping processes through intramolecular interactions in multibranched, multichromophoric dendritic systems were reviewed (*B34*). A second review emphasized the effect of such energy-transfer processes on the resulting nonlinear optical effects (B35).

Several biological and medicinal applications of fluorescence detection at surfaces are reviewed (*B36*). And, femtosecond time-resolved spectroscopy to resolve ultrafast solution-phase dynamics is reviewed by Tahara (*B37*).

GENERAL INSTRUMENTATION

Many prototype instruments and improvements to existing instruments have been reported in the last two years. A novel fluorescence spectrometer with no moving parts is reported for the simultaneous and instantaneous detection of multiple-fluorophore species (*C1*), and a single-beam mini-rapid-scan spectrophotometer that collects absorption, reflection, and luminescence measurements over a spectral range extending from the UV to the infrared region was developed and patented (*C2*). A prototype detector for an analytical ultracentrifuge was reported (*C3*), and a universal ellipsoidal mirror device for fluorescence-detected circular dichroism was developed to eliminate polarization artifacts (*C4*).

A compact system combining two nontraditional low-cost solutions for excitation light sources and emission filters in fluorescence measurements was reported. High-power blue and green light-emitting diodes (LEDs), used to excite cell monolayers and thin emission filters with uniform properties, no autofluorescence, high durability, and good flexibility, were fabricated (*C5*). High-power blue and UV LEDs can now be used as light sources for sensitive fluorescence detection techniques, including chipbased flow cytometry, capillary electrophoresis, and single-molecule imaging. These sources are available at a fraction of the cost of an Ar ion laser and can be used in applications that demand portability, low cost, and convenience (*C6*).

Another report described a vertical cavity semiconductor device for fluorescence spectroscopy in biochips and microfluidic platforms that integrates, into a single unit, the three functions a microfluorometer must perform: optical pumping, optical detection, and optical filtering of weak light sources. The device is designed to emit light when driven in forward bias mode and to detect light of longer wavelength when driven in reverse bias mode (*C7*). Single-photon detectors with high-quantum detection efficiency, low noise, and high time resolution are required in single-molecule spectroscopy. A compact and versatile single-photon avalanche diodes, working with a monolithic integrated active quenching and active reset circuit and cooled by a Peltier element (*C8*) was reported.

In the field of microscopy, an instrument that breaks Rayleigh's diffraction limit has been reported (*C9*). Another instrument, applicable to biological systems, that combines simultaneous wide-field imaging and spectroscopy of localized single fluorophores (*C10*) has also been reported. High-throughput single-cell fluorescence imaging and spectroscopy of bacterial cells moving through a capillary tube was also reported. The cells were imaged by a $20 \times$ objective, and the fluorescence was dispersed by a diffraction grating. An intensified CCD camera simultaneously recorded the zero- and first-order images, yielding both the single-cell image and spectra (*C11*).

The combination of annular illumination microscopy, timecorrelated single-photon counting, and multichannel detection allowed Prummer et al. to deduce 14 independent parameters from measurements of photon arrival time, wavelength, and polarization for a single fluorophore. The intensity, polarization, and spectral dynamics were analyzed on a nanosecond time scale and the mean values monitored with submillisecond time resolution (*C12*).

Fluorescence lifetime imaging is a powerful technique to investigate spatially inhomogeneous samples; yet, there is a need to increase the speed of data acquisition. Along these lines, a time-resolved multifocal multiphoton microscope with adequate data acquisition rate made possible the measurement of both intensity and lifetime images (C13), and a high-speed wide-field time-gated

endoscopic fluorescence-lifetime imaging instrument capable of fluorescence-lifetime imaging at rates of as many as 29 frames/s was reported (*C14*). Also, a frequency-domain fluorescence anisotropy optical microscope based on intensity-modulated linearly polarized laser light fed into the epifluorescence port was described (*C15*).

Two new intensity modulatable LED light sources were reported. Frequency-domain fluorescence spectroscopy of proteins and protein-related fluorophores using 280- and 300-nm LEDs was performed to evaluate the use of LEDs as an excitation source for frequency-domain time-resolved measurements (*C16*). The use of ultrabright LEDs in time-resolved fluorescence of crude oil contaminated samples was demonstrated (*C17*).

While both time- and frequency-domain fluorescence spectroscopy instruments and microscopes have the potential to provide very useful and accurate information on dynamic processes, experimental artifacts may have disastrous consequences. An overview of potential difficulties encountered in the use of timeand frequency-domain instrumentation as well as practical remedies were reported (*C18*).

Several time-resolved systems based on fast-time-gated detection principles have been reported. One article describes the technical setup of a time-resolving instrument with either a fixed time-gated detection principle or a direct fluorescence lifetime detection principle for lifetime-based fluorescent assays (*C19*). Other reports detail gated systems taking advantage of CCD detectors for time-resolved fluorescence spectroscopy (*C20–C23*).

Several advances in research grade FCS instrumentation have also been reported in the literature. In one such advancement, a single-molecule tracking confocal microscope was combined with force spectroscopy for gene expression analysis. The instrument allows for simultaneous measurements of fluorescence anisotropy, energy transfer, and correlation (C24). With the ultimate goal of tracking individual molecules diffusing in solution, a multifocus confocal laser scanning microscope was reported (C25), and a multipoint FCS system was developed to measure correlation functions at multiple points inside a cell simultaneously (C26). Another instrumental advancement has come in the form of scanning FCS. An instrument was designed to perform multiple FCS measurements simultaneously by rapidly directing the excitation laser beam in a repetitive uniform circular scan across the sample (C27).

A sample volume-controlled FCS instrument taking advantage of a motorized variable beam expander to adjust sample volume was reported (*C28*). The use of a solid immersion lens was shown to provide higher light field confinement as well as better collection efficiency compared to a conventional confocal setup (*C29*).

Advances in detection systems for FCS and single-molecule instruments have also been reported. Thus far, singlemolecule detection has been limited to dual-color detection; however, triple-color coincidence analysis was demonstrated on a single-molecule level using two-photon excitation (*C30*). The same group reported a filterless multicolor detection unit for FCS that allows up to 15 detection channels covering the entire visible spectral range (*C31*). Other advancements in FCS detection report parallel single-molecule detection and FCS experiments using fully integrated complementary metal oxide semiconductor single-photon 2×2 detector arrays (*C32*). To reduce cross-talk in fluorescence cross-correlation spectroscopy, a new method using pulsed excitation with detection on shorter time scales than the residence time in the focal volume was developed (*C33*). A paper discussing the difference between dual-color FCS and dual-color photon-counting histograms (PCHs) reported PCHs also reduce cross-talk, allowing a binary dye mixture to be resolved (*C34*).

Improvements in excitation have also been reported. A prism setup for multiple-color confocal fluorescence experiments was designed and constructed such that the colors emerging from the prism are optimally overlapped in the focus of a high-numericalaperture objective, a geometry commonly used in single-molecule detection (C35). A new total internal reflection FCS system based on an epi-illumination configuration was reported to achieve photon count rates as high as commonly achieved with confocal FCS instruments (C36). Also, single-fluorophore detection at higher concentrations with increased signal-to-noise ratios was attained using focal volume confinement in submicrometer-sized fluidic channels (C37). The observation volumes created by these channels are ~ 100 times smaller than observation volumes obtained with conventional confocal optics, and molecules are restricted to diffuse through the central regions of the excitation volume. Applications for high-throughput, singlemolecule detection in drug screening and genomic analysis are discussed.

Several instruments designed for clinical imaging have been reported. The accuracy of fluorescence molecular tomography was investigated in vivo by imaging translucent plastic tubes containing a turbid solution with a known concentration of Cy5.5 implanted subcutaneously in nude mice to simulate the presence of a tumor (*C38*). To investigate normal and atherosclerotic portions of the aorta wall, a dual-modality instrument for simultaneous optical coherence tomography imaging and laser-induced fluorescence (LIF) was reported (*C39*). In addition, a compact optical fiber-based time-domain LIF spectroscopy apparatus for in vivo studies of atherosclerotic plaques and brain tumors was reported (*C40*).

A new fluorescence imaging probe with a resolution of 5 μ m over a scanning area of several square millimeters was proposed for in vivo applications (*C*41), and a prototype confocal MACRO-scope system, with a 22 mm × 70 mm field of view, was designed for imaging large tissue sections in a single frame (*C*42). A compact spectroscopic instrument designed to excite and measure fluorescence in the oral cavity (*C*43) and a prototype instrument capable of attachment to the eyepiece of an endoscope was designed for fluorescence imaging applications in vivo and in vitro (*C*44, *C*45). Another method applicable to the development of submillimeter diameter endoscopes used spectral and frequency encoding to obtaining fluorescence images with a high number of resolvable points (*C*46).

Other developments to instrumentation for clinical analysis have also been reported. The cross-talk problem on multichannel microfluidic chips used in microanalyical systems such as pointof-care diagnostic systems (C47) was investigated. Also, a new ratiometric fluorophotometer for fluorescence-based viscosity measurements of biofluids using molecular rotors (C48) was reported during this time frame.

LASER-BASED TECHNIQUES

Laser-based techniques such as multiphoton excitation, FCS, and confocal microscopy/spectroscopy have become important tools in many fields. The utility of these tools has, in many ways, become routine as evidenced by the number of publications employing them to investigate important problems. At the same time, continued development of individual techniques or the combination of two or more techniques, to investigate more complex problems, also continues to be a major area of research. Unfortunately, only a small percentage of recent developments can be described here. Several advances in instrumentation related to these techniques may also be found in other sections of this review.

A novel Fourier transform method for measuring two-photon excitation spectra and its applications to microscopy has been reported (*D1*). Two raster scanning algorithms suited for fast imaging and for spectroscopy in a two-photon microscope were developed (*D2*). Imaging could be performed at a rate of 1-100 Hz/s, or a slow scanning algorithm was used for PCH and lifetime analysis. An enhanced two-photon fluorescence excitation technique using resonant grating waveguide structures, eliminating the need for highly focused laser excitation light, was also reported (*D3*).

There are many references that combine the use of two-photon excitation with FCS. A rapid new method of determining uracil DNA glycosylase activity in human cell extracts using FCCS with two-photon excitation was reported (*D4*). The technique may be useful for automated rapid screening of large samples. Two-photon FCS was also used to investigate the mobility as well as the molecular interaction between calmodulin, an important transducer of intracellular Ca²⁺ signaling, and Ca²⁺/calmodulin-dependent kinase II both in solution and in living cells (*D5*).

The Berland group discusses excitation saturation in one- and two-photon imaging and FCS applications. It was shown that excitation saturation can dramatically alter the effective imaging point spread function (PSF) in two-photon fluorescence microscopy with drastic implications for resolution in fluorescence imaging even at relatively modest excitation levels (D6). Saturation leads to both an increased fluorescence observation volume and an altered spatial profile for the PSF, decreasing the resolution. A computational approach to accurately quantify these effects was reported. In an extension of this work, a computational approach to characterize the effect of excitation saturation on observation volume is presented and used to accurately interpret FCS measurements (D7). In additon, the authors developed a quantitative model to characterize how excitation saturation, and its effect on the fluorescence observation volumes, affect one-photon confocal microscopy and FCS (D8) and discuss the broader importance of excitation saturation in fluorescence fluctuation spectroscopy (D9). In related work, the Zare group has proposed an improved model, correcting the observation volume profile for out-of-focus emission, that was shown to adequately fit experimental PCH data when a three-dimensional Gaussian observation volume could not (D10).

The development and application of FCS for various studies continues to be of great interest and many innovations were reported. In what may signal a coming of age of the technique, the design and construction of two simple and relatively inexpen-

sive single-molecule confocal fluorescence and FCS instruments useful for both research and advanced laboratory courses were reported (D11, D12). At the same time, new variations of FCS, such as polarized FCS (D13), are continually being developed. An improvement to a commercially available instrument was also reported, where the Carl Zeiss CofoCor 1 FCS microscope was upgraded to take advantage of time-correlated single-photon counting (TCSPC). Individual photon events were recorded without the on-the-fly data reduction performed by hardware correlators, thus preserving the full information content of the measurement (D14). Another new instrument takes advantage of TCSPC to deliver traditional FCS or FCCS and fluorescence lifetime data simultaneously while preserving all the original data inherent to single-photon counting techniques (D15). In related work, a new method was developed to eliminate afterpulsing effects in FCS measurements by using TCSPC (D16).

Developments of other fluctuation-based alternatives to FCS have been made. For example, alternating-laser excitation allows the monitoring of fast dynamics or simultaneous monitoring of a large number of individual molecules (D17). Two-photon excitation fluorescence fluctuation spectroscopy with PCH analysis was reported as a new tool to study the binding of globular proteins to colloidal particles in situ (D18). Traditionally, fluorescence fluctuations are evaluated by FCS analysis, but PCH analysis is advantageous when particle concentrations of a multicomponent system are of interest and the particles can be distinguished through differences in particle brightness. Other advancements in the analysis of FCS data have been reported. A new version of the autocorrelation function of the temporal fluorescence intensity fluctuation was derived and compared to the previously formulated statistical physics-based autocorrelation function method (D19).

A position-sensitive FCS instrument that calculates correlation functions as a function of lag time was used to detect the direction and speed of a flowing dye solution and the position of an immobilized particle (*D20*). Simultaneous two-beam FCCS and single-beam autocorrelation spectroscopy was used to investigate the dynamic equilibrium between the folded and unfolded conformations of single-stranded DNA hairpin molecules flowing sequentially between two spatially offset microscopic detection volumes, eliminating the need of a control sample (*D21*). The first use of confocal microscopy in combination with FCS to study the fluid velocity profile within poly(dimethylsiloxane)–glass microchannels was reported (*D22*, *D23*). This technique was found to be capable of two-dimensional mapping of velocity in a microfluidic system.

It is reported that data from a laser scanning microscope contain hidden time structure that can be exploited to measure fast dynamics of molecules in solution and in cells. The analysis used to process laser scanning images to obtain diffusion constants in the microsecond to second time scale was reported (D24). In similar work, another report combined imaging and scanning FCS to characterize microsecond fluctuations in the fluorescence emission of a sample (D25). A laser scanning confocal microscope was operated in the customary way while recording the time each photon was detected using a low-cost counting board.

A new line-scanning FCS technique was described that provides an easy and reliable measure of the spatial resolution and sensitivity of a confocal microscope without the need to modify the instrument (D26). An FCS microscope, providing an automatic real-time readout of the location in the confocal image, was also developed (D27).

SENSORS

Many novel fluorescence sensors for detection of various chemicals of biomedical, industrial, and environmental importance have been reported since the last review. Nakata et al. developed a two-site-selective sugar-binding lectin protein, modified with fluorescein and coumarin fluorophores, for the ratiometric sensing and imaging of mannose in breast cancer cells as well as determining glucose concentration inside HepG2 cells (E1). Schaferling et al. developed a simple and reversible glucose sensor, involving the coadsorption of glucose oxidase and europium(III) tetracycline that were incorporated into a hydrophilic polymer, applicable at neutral pH (E2). The glucose detection is based on the lifetime-resolved fluorescent imaging of hydrogen peroxide generated by the glucose-assisted oxidation of glucose. In addition, several nanoparticle-based glucose sensors were reported. Rossi et al. immobilized glucose oxidase in amino acid functionalized magnetite nanoparticles and the rate of oxygen consumption, sensed by a ruthenium phenanthroline fluorescent complex, was used to monitor the enzymatic oxidation of glucose (E3). Cappuccio et al. synthesized various glucose-sensing pyranine and sulfonamide derivatives containing boronic acid functional viologens for use in in vivo, real-time glucose monitoring (E4). A hydrolase- and oxidase-encapsulated optical array biosensor immobilized in a sol-gel was constructed for the accurate and simultaneous screening of urea, creatinine, and glucose in fetal calf serum (E5). The array-to-array biosensor reproducibility was found to be highly stable, with no cross-interference of analytes over a period of three months.

Efforts were also made in the development of novel fluorescence sensors for the detection of biological warfare agents (BWAs) and chemicals of homeland security interest. Jonsson et al. employed a UV laser pulse irradiation and photomultiplier tube array detector for sensing the presence of BWA simulant particles (*E6*). The use of a range-gated laser-induced fluorescence spectrometer for the detection of *Bacillius subtilis* var *globiggi* and *Erwinia herbicola* bioaerosol simulants was reported by Buteau et al. (*E7*). A novel molecularly imprinted polymeric material containing europium was fabricated to detect the presence of organophosphorus Sarin and Soman species in water (*E8*).

Two groups have developed new sensors for the determination of nitrogen oxide during the time period. Zhang and Zhang synthesized a novel 1,3,5,7-tetramethyl-8-(4'-aminophenyl-N-(2"amino)phenzyl)difluoroboradiaza-s-indacene probe for direct in vivo sensing of NO in S-nitrosocysteine (E9). Ye et al. developed a new method of determining NO in animal tissue by placing 4,5diaminofluorescein and a sample solution containing NO, each frozen in dry ice, in contact with each other (E10). In this way, common interferences in NO determination, such as dehydroascorbic acid and ascorbic acid, were eliminated due to their low diffusion across adjacent frozen blocks.

Continued efforts were also made to develop new oxygen sensors. Erler et al. reported the use of a novel fluorescently labeled hemocyanin sensor for single-molecule oxygen detection in respiratory proteins (E11). Dissolved oxygen in rat C6 glioma

cells was monitored in vitro by a ratiometric PEBBLE (probes encapsulated by biologically localized embedding) nanosensor embedded within an organically modified silicate matrix (*E12*). Jorge et al. reported an optical fiber sensor based on the fluorescence quenching of a ruthenium complex immobilized in a sol-gel solid matrix for simultaneous determination of temperature and oxygen concentration in a gaseous environment (*E13*). And, an oxygen sensor was developed by Im et al. that took advantage of dual lumophores embedded within monodisperse polystyrene beads (*E14*).

Various sensors were developed for the detection of protein, protein activity, and nucleic acids. Cy5-labeled protein, bovine serum albumin, fibrinogen, and lysozyme adsorbed to glass microscope slides previously coated with silanes and detected using total internal spectroscopy was reported (E15). Pinto and Schanze described two different sensors to monitor peptidase and thrombin activity: a "turn on" sensor based on the fluorescence enhancement of a peptide substrate labeled with *p*-nitroanilide, and a "turn off" sensor based on the fluorescence quenching of a rhodamine substrate derivatized with a peptide (E16). Strongin and co-workers reported a novel fluorescence bioassay technique involving the interaction of saccharide-substituted oligopyrrolic macrocycles with lectins to sense concanavalin activity (E17). A protein sensor based on the sensitization of incorporated Eu³⁺ into polymerized liposomes by aminosalicylic acid was developed by Santos et al. (E18), while a fluorescent sensor based on phototinduced energy transfer between a 4-aminonaphthalimide reporting group and a triplex-select intercalator recognizing group was used for triplex DNA detection (E19). A modular aptameric sensor, using a malachite green signaling domain, for the recognition of ATP, FMN, and theophylline was reported by Stojanovic and Kolpashchikov (E20).

Berns et al. have developed a novel sensor for the determination of polarity within live cells based on a photochromic fulgimide (*E21*). A rapid and sensitive laser-induced fluorescence optical fiber sensor, designed to enhance spectral differences between normal and malignant tissue, has been reported by Kim et al. (*E22*).

Fluorescence sensors were developed for the detection of various metals. The use of bis-*N*-carbazolyldistyrylbenzene for the detection of various metals was developed by Vaganova et al. (*E23*). A reversible fluorescence metal sensor based on inner filter effects was developed where a spiropyran absorber and porphyrin emitter were immobilized in a polymer matrix. Copper(II) concentrations as low as 0.15 μ M could be determined (*E24*). Fluorescence sensors for the determination of Co²⁺, Ca²⁺, and Hg²⁺ in water, whereby fluorescent self-assemble monolayers were deposited onto a glass substrate, were reported (*E25*). Wang et al. employed organic fluorescence nanoparticles for direct determination of Cr⁶⁺ in wastewater (*E26*).

A new fluorescent probe, 1,3,5,7-tetramethyl-8-(4'-aminophenyl)-4,4-difluoro-4-bora-3a,4a-diaza-s-indacence (TMABODIPY), has been developed for the determination of trace nitrite in drinking water and vegetables without the need of prior extraction (*E27*). Intermittent fluorescence from oligo(phenylenevinylene) was correlated with environmental rigidity, where a more rigid environment hinders twisting about the vinyl linkages (*E28*). By controlling the rigidity of the sample matrix, the authors were able to tune the blinking frequency. Using a two-dye system to monitor oxygen and temperature, a pressure-sensitive coating was developed (E29). The fluorescence emission of a ruthenium dye changes with temperature while the emission of a porphyrin dye is affected by temperature and pressure. The authors also discuss a three-dye system that includes an intensity reference dye.

SAMPLE PREPARATION, QUENCHING, AND RELATED PHENOMENA

Because of its sensitivity, fluorescence quenching techniques are known to be effective methods by which to investigate substrate conversion or ligand binding. As such, the majority of significant developments in this area have an emphasis in biochemistry or medicinal applications.

Bocedi et al. investigated the binding properties of HIV protease and reverse transcriptase to human serum albumin (HSA) (*F1*). Based on a series of studies monitoring the quenching of intrinsic tryptophan214 fluorescence, the authors determined that, in the presence of proteins found in the plasma, the concentration of anti-HIV drug required for 90% reverse transcriptase and protease inhibition is a minimum of 1 order of magnitude greater than when no plasma proteins are present. Jezewska and co-workers studied the binding of nucleotide analogues with RepA hexameric helicase from plasmid RSF1010 (*F2*) and determined that there was negative cooperativity among the six binding sites.

Using tryptophan phosphorescence lifetime measurements combined with acrylamide quenching experiments, sucrose was found to minimize fluctuations in protein structure primarily when the protein is thermally expanded and internally hydrated (F3). The structure and dynamics of glycolipid transfer protein is studied using fluorescence techniques for the first time (F4).

Using the ratiometric response of two fluorophores, a reporter fluorophore whose fluorescence is quenched and a remote fluorophore whose fluorescence is unperturbed, Li et al. studied intramolecular dynamics of large biolomecules at the single-molecule level (F5). The rate of molecular motions determined using the quenching method was comparable to that determined using FCS. Investigations of biopolymer conformation dynamics, operating on the nanosecond time scale and subnanometer length, were performed by Doose and co-workers by utilizing resonance energy transfer between organic dyes and tryptophan (F6).

The combination of FCS and peptide quenching probes allowed Scheffler et al. to simultaneously monitor peptide diffusion and peptide—antibody binding (F7). Monitoring miniprotein dynamics on a nanometer scale was demonstrated by selectively quenching tryptophan fluorescence by an extrinsic label (F8). Using FCS, the authors were able to resolve a hierarchical folding transition of Trp-cage not previously observed.

A study of the fluorescence quenching of a series of mutant tropomyosins by acrylamide provide the first direct evidence to support the hypothesis that tropomyosin, a protein involved in muscle contraction, has a coiled-coil structure (F9). By altering the oligophenylene bridge between perylenebisimide chromophores, Holman et al. systematically studied photoinduced intermolecular electron transfer (IET) through the bridge (F10). The authors noted a pronounced switching of IET with an increase

in solvent dielectric constant and were able to establish parameters to control the observed IET.

A direct method to measure base excision repair activities in cell-free extracts, purified proteins, and cultured cells using molecular beacons has been reported by Maksimenko et al. (*F11*). To investigate GTPase functions, BODIPY was coupled to three GTP analogues and was shown to behave like a low molecular weight guanine nucleotide exchange factor in the presence of Mg²⁺ ions (*F12*). The real-time observation of nucleotide exchange occurred as a result of the increase in BODIPY fluorescence intensity. The fluorescence behavior of five pterins was investigated over a wide pH range in a variety of buffers. Results indicate the fluorescence of the acid form was effectively quenched by select anions while quenching of the basic form was not observed or negligible in comparison (*F13*).

Liao et al. developed a real-time fluorescence assay to monitor the uptake of nonesterified long-chain fatty acids (LCFAs) into cells using a labeled fatty acid and nontoxic quencher unable to enter the cell (F14). Plate readers or conventional fluorescence microscopy can be used to obtain unprecedented temporal resolution of LCFA uptake kinetics. A simple and sensitive fluorescence self-quenching assay of disulfide reduction activity of protein disulfide isomerase has been developed (F15). The authors note this method is capable of detecting one turnover of enzyme when no reducing agents are present and observed an insignificant blank rate. A new protein assay has been developed by Cui et al. (F16). By monitoring the static fluorescence quenching of bovine serum albumin or HSA by 1-benzoyl-4-p-chlorphenyl thiosemicarbazide, the determination of the proteins in BSA or HSA yielded similar results observed by Coomassie Brilliant Blue G-250 colorimetry.

DATA REDUCTION

The development and application of data reduction and analysis methods continues to track the increasing complexity and order (dimensionality) of fluorescence measurements. There is a long history of appropriation and modification of methods that originate in other fields such as chemometrics, signal processing, machine learning, and image analysis for the analysis of fluorescence measurements. Consequently, several papers that describe the use of linear regression methods such as principal components regression and partial least squares to map fluorescence measurements of sample composition were reported during the review period. These applications appear over a wide range of fields including food analysis (*G1*), pharmaceutical analysis (*G2*), environmental analysis (*G3*), clinical analysis (*G4*), and quality assurance (*G5*).

There also were a number of reports describing applications of linear classification methods such as principal components analysis followed by clustering and discriminant analysis to fluorescence measurements. These reports tend to appear in applications that require qualitative rather than quantitative analysis, such as clinical analysis. A report by Brereton and Devonshire, describing the classification of single-nucleotide polymorphs using fluorescently labeled DNA probes, illustrates the performance of linear classification methods (G6).

In most cases, the linear regression and classification methods discussed above are applied to collections of steady-state spectra

(emission, excitation, or synchronous) acquired under varying measurement conditions to disperse the sample component responses. However, a few of the reports outlined above (G2, G4) apply these methods to excitation-emission matrixes (EEMs). There also is a long history of applying linear curve resolution methods to EEMs. The report by Antunes and da Silva describing the resolution of the spectra of humic substances extracted from soil and recycled waste (G7) illustrates this methodology. However, these analyses can be more difficult to implement because there can be an infinite number of solutions to the curve resolution problem for single EEMs due to rotational ambiguity. Consequently, many more investigators use parallel factor analysis (PARAFAC) models to resolve and quantify spectra from threeway data sets. The application of PARAFAC models to three-way (and higher order) data is a comparatively new field; consequently, data analysts continue to invest research effort to improving these methods and understanding the factors that impact their performance. Tomasi and Bro (G8) described and compared two methods for dealing with missing values in PARAFAC models, and Olivieri (G9) described a procedure for calculating the standard errors of the concentrations predicted by PARAFAC models as well as the limits of using net analyte signal theory to compute sensitivity and selectivity measures. Several applications of PARAFAC models also were reported during the review period, but only a few are listed here. Damiani et al. (G10) compared the performance of PARAFAC (as implemented by Bro) to bilinear least squares in determining ciprofloxacin in human urine. Hall et al. used PARAFAC and N-way partial least-squares/discriminant analysis to classify the water in the ballast tanks of ships entering U. S. waters (G11). They report that EEM fingerprinting can distinguish sampling sites that are fewer than 10 miles apart.

Several reports described the development and application of methods for analyzing three-way (and higher order) data sets in which one of the measurement axes is time. The analysis of kinetic measurements can present special challenges, often due to the collinearity of the exponential basis set. Vega-Montoto and Wentzell demonstrated how three-way analysis methods may be modified to accommodate the measurement error of such matrixes (*G12*). Applications of higher order kinetic measurements also appear in the evolution of EEMs in a variety of fields including food analysis (*G13*), clinical analysis (*G14*), and biological analysis (*G15*).

Another time-dependent measurement requiring specialized data analysis methods is FCS and fluctuation analysis. Some papers describe expanded methodologies that provide information similar to conventional FCS but require modified data acquisition and analysis. Notable among these is the development of raster image correlation spectroscopy, which achieves the temporal resolution of single-point FCS while preserving long-range spatial correlations, by Gratton's group (G16). The development of photon arrival-time interval distribution analysis of time-resolved fluctuation data by Laurence et al. (G17) also expands the capacity of fluctuation measurements to distinguish subpopulations of complex systems. Two papers describe alternative brightness measures based on photon moments (G18, G19). A number of papers report data analysis schemes for specialized fluctuation measurements, including those arising from two-photon excitation (G20). Enderlein et al. (G21), describe a method for estimating the precision of diffusion coefficients calculated from autocorrelation functions. Other papers described powerful statistical analyses for fluctuation measurements including those using Bayesian inference (G22, G23) and the maximum entropy method (G24). Two groups (G25, G26) describe comprehensive data analysis software approaches for fluctuation spectroscopy measurements.

The rapid development of many types of fluorescence imaging systems has also spawned the development of specialized data analysis methods. Even a sparse summary of the developments in this area during the review period would require more space than is available here. We only point out a few trends likely to be significant soon. Several papers illustrate the recent progress in optical tomography using fluorescent contrast agents (G27). In fact, 3D tomography using near-infrared fluorescent contrast agents (G28) was reported during the review period. Several papers describe the application of multivariate analysis methods to hyperspectral images (G29). In particular, the paper by Kong et al. (G30) describes the application of a wavelet transform to compress the image (spatial) dimension and PCA to compress the spectral domain prior to using a neural network to classify healthy skin and tumors of poultry. Now that adapters that convert conventional microscopes to hyperspectral are available commercially, applications of this methodology are likely to increase. Several papers that describe decay analysis methods that provide more efficient or accurate analysis of lifetime (G31-G33) and resonance energy-transfer images (G34) also appeared during the review period. Finally, many of the reports describing image processing (G35), classification (G36), and quantification (G37) were based on Bayesian inference. It is likely that this trend will continue because Bayesian methods allow the analyst to incorporate as much information as is available into the calculation.

ORGANIZED MEDIA

Organized media is a broad term that refers to macrocyles or molecular assemblies that can compartmentalize the solvent into regions of varying properties and sequester solutes in environments that are distinct from the bulk. The list of media includes cyclodextrins, surfactant self-assemblies, lipid aggregates, dendrimers, polymeric surfactants, polymer films, sol-gels, and selfassembling peptides. Investigators typically use luminescence measurements in one of three ways: (1) to characterize fluorescent solute-medium interactions, (2) to characterize the medium using fluorescent probes, or (3) to detect analytes via changes induced in a fluorescent solute (which may not be the analyte) or medium by analyte association. Only a few examples of the numerous reports published between 2004 and 2005 are included in this summary.

Guo et al. (*H1*) used fluorescence to measure the inclusion constants of meso-tetrakis(4-hydroxylphenyl)porphyrin (THPP), a potential photodynamic therapy agent, in a series of β -cyclodextrins. Interestingly, they observed that the 1:1 complex between THPP and hydroxypropyl- β -cyclodextrin (HP- β -CD) exhibited a 300-fold fluorescence enhancement, indicating the potential utility of HP- β -CDx in THPP analysis. Al-Soufi et al. (*H2*) used FCS to analyze the thermodynamics of the interaction between β -cyclodextrin and two structurally similar pyronines. They found that the association rates of the dyes were similar and the difference in the inclusion complex stability was due to very different dissociation rates.

Chiral recognition via cyclodextrin complexation continued to be a recurring theme during the review period. For example, Fakayode et al. have reported a method to accurately determine enantiomeric composition of phenylalanine samples using chemometric fluorescence spectral data analysis of cyclodextrin– guest host complexes (*H3*). The method is potentially useful in the pharmaceutical industry for high-throughput determination of enatiomeric purity of drug molecules and for rapid online screening of hundreds of potential drug candidates. D'Anna et al. (*H4*) demonstrated chiral recognition of a series of amino acids using complexes of fluorophores, e.g. xanthone, and β -cyclodextrins.

Maggio et al. used the fluorescence of the fungicide carbendazim to compare its association with β -cyclodextrins and several surfactant micelles in order to determine which medium and conditions produce the largest fluorescence enhancement (H5). Raghuraman and Chattopadhyay used micelles, red-edge excitation, and rotational dynamics measurements to investigate the impact of membrane surface charge on the conformation of the amphiphilic hemolytic peptide melittin (H6). Gramlich et al. also used surfactant micelles, as well as POPC liposomes, as membrane models (H7). In their report, a probe that mimics reactive membrane radicals was used to study the antioxidant properties of a-tocopherol. Other micelle reports describe investigations that use fluorescence to study the relationship between surfactant and micelle structure. Wang et al. monitored the impact of varying the properties of the spacer in gemini surfactants on the properties of their micelles (H8). They found that more flexible, hydrophilic spacers promote micelle formation, decreasing the critical micelle concentration and increasing the aggregation number. Griffiths et al. used time-resolved fluorescence and other spectroscopic techniques to study the consequences of changing the hydrophobicity and hydration of surfactant headgroups (H9).

Luminescence studies of lipid aggregates during the review period have been dominated by the intense discussion about the existence and nature of lipid rafts, protein-lipid aggregates enriched in sphingolipids and cholesterol. Kenworthy et al. did not find evidence of raft protein clustering in fluorescence recovery after photobleaching measurements of labeled proteins on the surface of kidney cells (*H10*). On the other hand, Rao and Mayor observed evidence of protein clustering in small (<5 nm) rafts (H11). It is interesting that most studies of rafts are carried out using labeled proteins or other raft markers, such as cholera toxin B subunit bound to ganglioside (H12), rather than labeled lipids. One reason for this stems from concerns that fluorescence labels change the structure and membrane distribution of lipids. The recent report by the Thiele group indicates that, while these concerns may be well founded (at least in the case of NBD- and BODIPY-labeled lipids), a new class of fluorescent probes, polyenelipids containing five conjugated double bonds, exhibit few differences from their unlabeled counterparts (H13).

The Wirth group has investigated the ability of a transmembrane protein to bind to a synthetic ligand within a lipid bilayer by single-molecule spectroscopy. Polyacrylamide brush layers of specific thickness were grown on fused silica, and using vesicle fusion, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine lipid bilayers were formed on bare silica as well as each polyacrylamide brush layer. Using additonal fluorescence techniques, the authors determined optimum polymer thickness to maximize dye diffusion and electrophoretic mobility within the lipid bilayer (H14).

The development of heterocyclic membrane probes that do not mimic lipid structure continues. For example, Jin et al. have reported a membrane staining dye able to distinguish liquidordered phases from liquid-disordered phases coexisting in model membranes (H15). The Demchenko group has found that derivatives of 3-hydroxyflavone are responsive to probe hydration as well as polarity (H16, H17). They attribute the bimodal distribution observed for one probe in a variety of lipids under many conditions to distinct probe forms: a hydrogen-bonded form located at the membrane interface and a free form closer to the bilayer cascade region. They posit that hydrogen bonding and bimodal probe distributions are features of many probes, such as PRODAN or Nile Red, that carry hydrogen-bonding groups.

Polymeric surfactants (molecular micelles), dendrimers, and polymer films add the stability of covalent bonds to the architectural elements of noncovalently associated media. The use of molecular micelles by the Warner group for use in analytical separations illustrates this feature. During this review period, this group reports that increasing micelle polarity by nonionic surfactant incorporation (H18) enhanced the resolution of test analytes, and Akbay and Shamsi describe the effect of changing alkyl chain length on polymer characteristics (H19). Using a variety of solvatochromic probes, the solvation environment within a novel self-assembling vesicle is reported by Pandey (H20). Lee et al. (H21) describe mixtures of hydrophobically modified poly(acrylic acid) and photosensitive surfactant that can be controlled reversibly by UV and visible light irradiation. Tinnefield et al. (H22), Masuo et al. (H23), and Thompson et al. (H24) use this feature in dendrimers to investigate the details of the interactions between chromophores in a variety of systems. Three generations of dyelabeled dendrimers with terminal carboxyl groups were used by the Harris group to study diffusion in sol-gel thin films (H25, H26). The Tucker research group has used a pyrene derivative, pyrenebutyric acid (PBA), to study solution- and solid-state interactions within nanometer-scale molecular hosts (H27). The authors conclude that C-hexylpyrogallol[4]arene encapsulates two PBA molecules where specific interactions with capsule walls maintain separation between the two guests.

Many of the reports of luminescence measurements of solgels that appeared during the review period describe the correlation between changes in the emission of probes with changes in the microenvironment associated with the phase transition. Mabuchi et al. (H28) ascribe the sensitivity of benzoquinolines to sol-gel transitions to spectrally distinct excited states, i.e., neutral, ion-pair, and protonated species, that can be isolated numerically and monitored throughout the transition. In a similar application, Gupta et al. used Hoechst 33258, a bisbenzimidazole derivative, to monitor the aging of a tetraethyl orthosilicate-based sol-gel (H29). Other reports during the review period described the development of sol-gel-based fluorescent sensors. For example, Hussain et al. demonstrated that encapsulation of yeast hexokinase sequestered the protein from serum quenchers that obscured the dependence of the protein's intrinsic fluorescence on the glucose concentration (H30).

Reports describing the application of fluorescence technology to supramolecular systems based on peptides constitute the smallest class of papers that appeared during the review period. Stupp's group reported the synthesis of peptide amphiphiles, peptides that have been conjugated to lipid or surfactant groups, which form cylindrical nanofibers. They used pyrene emission, excimer formation in particular, as an indicator of nanofiber formation (H31). They also used pyrene in combination with the intrinsic tryptophan to probe the interior of the aggregate.

LOW-TEMPERATURE LUMINESCENCE

Considerable activity continues in the use of low-temperature and related high-resolution luminescence techniques. However, only a handful of those recently published reports have direct analytical implications. A general review of line-narrowing techniques was produced by Purchase et al. (11) with particular reference to photosynthetic chromoproteins and autofluorescent proteins. Grubor and colleagues provided two interesting reports of the application of low temperature with fluorescence immunoassay biosensors. They described a monoclonal antibody-gold biosensor chip for detection of DNA-carcinogen adducts at low temperature with detection limits in the low-femtomole range (I2). They successfully demonstrated the temporal resolution of two structurally similar benzo[a]pyrene-derived DNA adducts using low-temperature, time-resolved fluorescence. The same group also used low-temperature fluorescence to explore the cross-reactivity and multispecific functionality of the monoclonal antibodies (13).

Campiglia's group continues to be a leading contributor in the application of Shpol'skii line-narrowing techniques to environmental samples. Goichoecha et al. have described the use of parallel factor analysis with phosphorescence spectra of tetracholorodibenzodioxin in water samples collected using Shpol'skii matrixes (*I4*). Yu and Campiglia have reported the use of time-resolved Shpol'skii fluorescence for the direct and unambiguous detection of dibenzopyrene derivatives with both solid—liquid extraction (*I5*) and HPLC (*I6*).

TOTAL LUMINESCENCE AND SYNCHRONOUS EXCITATION SPECTROSCOPIES AND RELATED TECHNIQUES

Total luminescence and synchronous fluorescence spectroscopy continue to be routinely employed for the characterization and differentiation of natural organic matter. For example, Sierra et al. determined that an evaluation of single excitation or emission spectra combined with synchronous spectra can indicate the source of organic matter as well as relative age of the humic material (*J1*), and Cilenti et al. observed a significant reduction in fluorescence intensity of synchronous spectra with increasing soil salinity (*J2*). In a different study, Swietlik and Sikorska found that the oxidation of natural organic matter with ClO_2 or O_3 resulted in the formation of smaller, less aromatic fragments (*J3*) as evidenced by changes in excitation—emission spectra.

The analysis of polycyclic aromatic hydrocarbons (PAHs) in environmental matrixes is accomplished using total luminescence or synchronous scan techniques. Second-derivative synchronous spectroscopy allowed Lage-Yusty et al. to simultaneously identify and quantify 13 of 16 PAHs in hexane (*J4*). With the aim of improving selectivity without a concomitant loss of sensitivity, Lin et al. developed a derivative nonlinear variable-angle synchronous fluorescence spectroscopic technique for the simultaneous determination of PAHs in complex aqueous mixtures (*J5*).

Several clinically important applications of total luminescence and related techniques have been described in the literature. A new CdS nanoparticle, capable of detecting human and bovine serum albumin as well as human γ -IgG, has been reported by Wang et al. (*J6*). The authors reported significant increases to the synchronous fluorescence intensity of the composite CdS nanoparticle in the presence of protein at a pH of 6.90. A sensitive and inexpensive method to detect DNA has been reported by Li et al. (*J7*), where the intensity of synchronous fluorescence spectra of cysteine-functionalized ZnS nanoparticles is enhanced in the presence of DNA.

SOLID SURFACE LUMINESCENCE

Hurtubise et al. published a recent review of solid-phase, roomtemperature phosphorescence as applied to both organic and inorganic compounds (K1). Also, Enderlein reviewed singlemolecule fluorescence measurements on solids as well as in solution (K2).

A number of reports appeared during this review period describing the application of solid surface luminescence in the analysis of agricultural and food products. Granger et al. report the direct quantification of protein absorbed at the oil–water interface in food product emulsions (K3). Front-face fluorescence was reported for the analysis of process cheese (K4), the authentication of the botanical origin of honey (K5), and the characterization of edible vegetable oils (K6, K7).

Llorent-Martinez et al. describe a flow-through fluorescence sensor coupled to an on-line solid-phase separation for the simultaneous detection of pesticide mixtures (*K8*). In this article, they demonstrate the simultaneous determination of the common pesticides carbendazim, carbofuran, and benomyl with detection limits in the parts-per-billion range. Jin and co-workers used solid surface fluorescence to study the adsorption characteristics of pyrene adsorbed to titanium dioxide from water—alcohol mixtures (*K9*).

The Hurtubise group reported the use of both solid-matrix fluorescence quenching (*K10*) and solid-matrix phosphorescence (*K11*) for the sensitive and selective detection of polycyclic aromatic hydrocarbon–DNA adducts and metabolites. The solid-matrix fluorescence quenching results suggest the potential to characterize PAH–DNA adducts based on the number of aromatic rings. Tawa et al. used surface plasmon fluorescence to directly study the hybridization of two single-stranded DNA oligonucleotides at a solid–solution interface and demonstrated the ability to simultaneously analyze a two-component target–oligo mixture hybridization with probe–DNA at the sensor surface (*K12*).

Bosch et al. report the use of polyurethane–acrylate-based adhesive polymer films doped with fluorescent probes as potential humidity sensors (*K13*). Yamashita and co-workers investigated the solvation dynamics of a coumarin dye immobilized on a water– mica surface (*K14*). Data suggest the presence of a rigid layer on the surface due to hydrogen bonding. Yao and Li report the use of total internal reflection fluorescence with synchronous scanning to study the adsorption behavior porphyrin compounds onto a glass surface from aqueous solution (*K15*).

LUMINESCENCE IN CHROMATOGRAPHY, ELECTROPHORESIS, AND FLOW SYSTEMS

The Zare laboratory has analyzed the chemical contents of a single cell on a picoliter scale using an integrated microfluidic chip. The device was capable of handling cells, metering and delivering chemical reagents, lysis of cells, and chemical derivatization, followed by separation and detection of the compounds with laser-induced fluorescence (L1). Shelby et al. report the ability to control and monitor the rotational motion of single microparticles and living cells optically trapped in a microfluidic device (L2).

The fabrication of plastic microfluidic devices for the capillary electrophoretic separation—multiwavelength fluorescence detection of various proteins is described by Hsiung et al. (L3). This microdevice was able to discriminate multiple protein samples labeled with different fluorescent dyes within a single channel in one run and also measure sample speed. The Soper group separated near-infrared dye-labeled oligonucleotides in poly(methyl methacrylate) microchip electrophoresis devices to investigate the applicability of fluorescence lifetime discrimination of two labeling dyes during microchip separation (L4). They also examined the feasibility of a two-color time-resolved detection scheme. A dual-color optical fiber-based time-resolved near-IR microscope coupled lifetime and color discrimination in order to increase multiplexing capabilities for DNA sequencing applications (L5).

By calibrating the photobleaching rate of a fluorescent dye, Wang was able to calculate fluid velocity within a microfluidic device from measured fluorescence intensities. This method was successfully applied to pressure-driven and electric-driven microfluidic systems (*L6*). A variety of fluorescence techniques were employed to characterize the performance of a microfluidic mixer (*L7*). The fast mixing time, 8 μ s, allowed the authors to study fast protein folding kinetics on a time scale that was previously inaccessible. Continuous-flow microfluidic mixers designed to measure the rate of protein folding based on endogenous tryptophan fluorescence allow the acquisition of information about protein structure in real time (*L8*). Using a combination of continuous-flow and stopped-flow mixing experiments, Welker et al. studied the earliest folding behavior of single-tryptophan mutants of ribonuclease A (*L9*).

Protein concentration, as well as changes in size or aggregation, has been measured with an HPLC method developed by Shihabi et al. (*L10*). In this study, the authors used a molecular exclusion column and native fluorescence to detect tamm-horsfall, a large glycoprotein, in urine. Spectroscopic studies were used to detect G-quartet structure in a DNA aptamer stationary phase, providing evidence of intramolecular G-quartet structure on an aptamer-coated capillary (*L11*). Real-time dynamics of amino acid concentration was monitored in vivo using a microdialysis method. The separation of 17 amino acids, continuously collected via microdialysis and derivatized on-line, was achieved in less than 30 s using micellar electrokinetic chromatography with LIF detection (*L12*).

CE-LIF has been employed for the analysis of individual mitochondria from a single, semimembraneous muscle fiber cross section (*L13*). The direct injection of fluorescently labeled mitochondria was achieved by the application of 11 kPa of negative pressure for 3 s while the capillary end was in contact with the prestained muscle tissue. UV absorbance or LIF whole column imaging detection (WCID) increases detector resolution and

speed. An immunoassay of viruses developed using capillary isoelectric focusing coupled to a LIF-WCID was preferred over UV absorbance-WCID (L14).

The Lin research group has combined on-line concentration techniques with sweeping-micellar electrokinetic chromatography (sweeping-MEKC) –low-temperature fluorescence detection (at 77 K) for the separation and identification of *trans*-resveratrol in red wine (*L15*) and *E,E-*4,4'-bis(2-sulfostyryl)biphenyl found in synthetic detergents (*L16*). Using sweeping-MEKC, the authors noted an almost 1500-fold increase in sensitivity for the detection of *trans*-resveratrol.

A recent trend has been the use of imaging and single-molecule spectroscopy to investigate chromatographic processes. The Yeung group has used fluorescence imaging to determine the electrophoretic mobilities of individual DNA molecules (L17). They also used DNA molecules to probe chromatographic surfaces (L18) and variable-angle evanescent field microscopy to study the motion of DNA molecules at a liquid-solid interface (L19). Tallarek used confocal laser scanning microscopy and fluorescent tracer molecules to study electrokinetic transport in a bed of porous glass beads (L20). The Geng laboratory has used laser scanning confocal microscopy to monitor the structural changes of polyelectrolyte multilayer coatings used in open-tubular capillary electrochromatography (CEC) (L21) and to observe dynamic processes, such as bubble formation, in packed-column CEC (L22). In a continuation of this work, confocal FCS imaging and single-molecule spectroscopy was used to investigate the adsorption sites of C-18 silica under reverse chromatographic conditions (L23).

Several detection systems for use with separation techniques have been developed over the review period. On-the-fly fluorescence lifetime detection was reported for multiplex single-strand conformation polymorphism analysis by CE (L24). A silica capillary coated with Teflon AF was used as the separation channel as well as liquid core waveguide for the detection of isoelectrically focused fluorescent markers and proteins labeled with fluorescein isothiocyanate (L25). This detection system did not require additional focusing optics or filters. Ahmadzadeh et al. modified a commercially available CE instrument for the detection of single particles using on-column LIF detection (L26). The authors noted similar variation in migration times of single particles using data acquisition rates of 4 or 100 Hz. However, the faster acquisition rate resulted in reduced fluctuation in fluorescence intensity at the expense of higher signal-to-noise ratios. Kamei et al. demonstrated the utility of an integrated hydrogenated amorphous silicon fluorescence detector for use with a microfluidic CE device (L27). A micro-CE LIF-intensified charge-coupled device (LIF-ICCD) with multichannel detection was developed by Qin et al. (L28). Using a tunable dye laser pumped by a picosecond pulse nitrogen laser as the excitation source, the authors observed an increase in detection sensitivity. Tung et al. designed a microfluidic detection channel, with simultaneous multicolor excitation capability, for use in microflow cytometry (L29). The observation channel, employing solid-state lasers and silicon-based pin photodetectors, can simultaneously collect two independent fluorescence measurements to increase the signal-to-noise ratio. In addition, multiple cytometric measurements can be collected at a single interrogation point at the same time.

Several important and interesting chromatographic or electrophoretic separations have been developed over the last two years. In the majority of the papers, analytes must be derivatized before fluorescence detection can be employed. Because of the intense interest in this area, specific separations or advances cannot be included in this review. However, the interested reader is directed to reviews discussing different derivatization techniques that have been developed (*L30, L31*).

DYNAMIC LUMINESCENCE MEASUREMENTS

Dynamic luminescence measurements remained an indispensable characterization technique for systems ranging from transition metal–ligand complexes (M1, M2) to biological entities (M3-M10). Researchers used these methods to explore the photophysics of fluorophores (M11-M15), solvent effects (M16-M18), complex formation (M19, M20), and conformational changes (M21-M23).

Some of the fundamental studies included the investigation of the dynamics and energetics of the intramolecular excimer formation of diarylsilane, di-9H-fluoren-9-yldimethylsilane (M24). Fluorescence self-quenching and fluorescence correlation spectroscopy were used to probe the possibility of transient structures forming in an unfolded protein, in contrast to the conventional random coil model (M25). The light-harvesting ability of benzophenone- and norbornadiene-labeled poly(aryl ether) dendrimers was shown and was demonstrated to be generation dependent (M26). The noncovalent interactions and complexation formation of carboxylate-terminated poly(amidoamine) dendrimers with meso-tetrakis(4-N-methylpyridinium)porphine were studied (M27). Using single-molecule fluorescence techniques, fluctuations of one Cy5 molecule at the air-glass interface were examined (M28). And, the nonexponential tryptophan fluorescence decay in proteins was attributed to electron-transfer quenching by a nearby amide group in the peptide bond (M29).

Interesting applications included illustrating that steady-state and dynamic fluorescence measurements could be used to evaluate the efficacy of decontaminating bacterially laden surfaces with disinfecting agents (M30). Using a pH-sensitive dye encapsulated in poly(ethylene glycol) microparticles, it was shown that fluorescence lifetime spectroscopy with multiply scattered light may be an ultrasensitive approach for toxin screening (M31). An inexpensive sensor using a lifetime-assisted ratiometric method was developed for measuring fatty acid levels, which are an indicator of a number of medical disorders (M32). Time-resolved, laser-induced fluorescence spectroscopy was used to determine margins of brain tumors for future intraoperative demarcation (M33). The photochemical and photophysical characteristic of photosensitizing agents used in phototherapy were explored. Time-resolved spectroscopy was proven to be a rapid method for the quantification of surface binding of labeled molecules to gold and silver nanoparticles (M34). The localized photoconversion of EosFP, a novel fluorescent protein from the stony coral Lobophyllia hemprichii, was employed to demonstrate its utility for resolving intracellular processes (M35). A new fluorescence-based approach for quantification of the extent of antibiotic-induced conformational changes in ribosomal RNA was developed (M36). Nanohybrids of functional single-wall carbon nanotubes, negatively charged pyrene derivatives, and watersoluble enzyme model systems in aqueous solution were also reported (M37).

Advances in instrumentation used for dynamic measurements have been made throughout the review period. For example, an advanced time-correlated single-photon counting technique that provides fluorescence lifetimes and fluorescence correlation/crosscorrelation information simultaneously was developed, utilizing new electronics (*M38*).

FLUORESCENCE POLARIZATION, MOLECULAR DYNAMICS, AND RELATED PHENOMENA

Fluorescence polarization continues to be an important and widely used technique, as evidenced by numerous applications of fluorescence anisotropy/polarization that were reported in the literature during the period of this review. For the purposes of this review, we use the terms polarization and anisotropy synonymously.

Single-molecule analysis continues to be a rapidly growing area in the scientific community, and a number of studies reported polarization measurements at the single-molecule level. This type of measurement capability has only been realized in the past halfdecade, and these recent developments have led to several biological applications, including the probing of the nanosecond protein motions of calmodulin, a regulatory protein for calciumdependent cell signaling (N1). A photon time-stamping technique that combined the capability for both subnanosecond time resolution of time-correlated single-photon counting and single-molecule time trajectory recording was demonstrated. In another study, the diffusion behavior of a single fluorescent perylene molecule in a nematic liquid crystal mixture was studied using single-molecule fluorescence correlation spectroscopy (SM-FCS) (N2). Because a large anisotropy in the translational diffusion was observed, the authors suggest the observed results can be potentially applied in the regulation of single-photon light sources and for singlemolecule data storage in the future. Quinlan et al. used singlemolecule total internal reflection fluorescence polarization microscopy to study the orientation and dynamics of myosin by measuring the fluorescence polarization of single molecules and ensembles of myosin decorated actin filaments (N3). Johnson et al. also utilized single-molecule fluorescence spectroscopy to characterize the dynamics of calmodulin and probe its conformation when bound to oxidatively modified plasma membrane Ca²⁺-ATPase (N4). A new approach was presented for measuring the 3D orientation of individual macromolecules using single-molecule fluorescence polarization microscopy (N5). The approach utilizes the unique polarization of evanescent waves generated from total internal reflection to excite the dipole moment of individual fluorophores.

Several papers reported developments in DNA analysis using fluorescence anisotropy and molecular dynamics (MD). With the effective completion of the human genome project, the efficient analysis of oligonucleotides will play a critical role in the translation of new disease-related genetic information into improvements in health care. The Lakowicz group reported a strong two-photon induced fluorescence emission of Cy5–DNA within the tunable range of a Ti:sapphire laser (*N6*). The two-photon cross section for Cy5–DNA was \sim 3.5-fold higher than for rhodamine B. They also observed an enhanced two-photon induced fluorescence of Cy5–DNA deposited on silver island films, with a \sim 100-fold brightness attributed to enhanced local field. Development in the area of DNA analysis was also reported by Barone et al. (*N7*), where they evaluated the DNA 7-hydro-8-oxoguanine (8-oxoG) modification of the structural and dynamical properties of the G6 sequence of the HPRT gene in the mismatch repair of defective cells. Fluorescence polarization anisotropy and MD showed that the 8-oxoG increased the DNA rigidity and constrained movement of the single-stranded region at the single/double stranded DNA junction of the DNA replication template/primer. Another development in this area was reported by Yamana et al. (*N8*). The structural basis for the different fluorescence properties of pyrene-modified DNA/DNA and pyrene-modified RNA/RNA duplexes was presented. The use of DNA aptamer-based bioanalysis of IgE by fluorescence anisotropy was also reported (*N9*).

Sexton et al. developed a fluorescence assay to monitor the protein-protein interaction of B lymphocyte stimulator (BLyS), a tumor necrosis factor family member and a key regulator of B cell responses (*N10*). The assay, composed of BLyS labeled with a ruthenium chelate and TACI-Fc, a soluble form of a BLyS receptor, demonstrated the potential of short peptides to disrupt high-affinity cytokine-receptor interactions. The use of molecular aptamers for real-time protein-protein interaction studies using fluorescence anisotropy and resonance energy transfer (RET) was described (*N11*).

There were several reports of the use of fluorescence anisotropy imaging in monitoring cellular activities. Fluorescence lifetime, anisotropy decay, and associated parameters indicated differential cellular activity (N12). Measurements on individual cells were carried out using a specially designed combination of a frequency-domain microscope and a picoliter well-per-cell array. Squire et al. reported a novel approach for the dynamic measurement of homo-RET in live cells (N13) using steadystate fluorescence anisotropy microscopy to acquire anisotropy images at main-band and red-edge excitation of enhanced green fluorescent protein. Axelrod et al. also reported a new RET method based on polarization that requires only one camera exposure, thereby offering the opportunity for improved time resolution of dynamic associations among subcellular components, unlike the common use of separate camera exposures (N14). A RET detection approach, based on detecting depolarized sensitized emission by fluorescence polarization anisotropy, was reported (N15). In the absence of RET, the emission from a donor fluorescent protein is highly polarized; however, in the presence of energy transfer, the depolarization of fluorescence emission is observed. Contrary to alternative approaches, this method was able to distinguish RET between linked and unlinked Cerulean and Venus fluorescent proteins in living cells with a larger dynamic range.

The application of spectrally resolved fluorescence lifetime imaging microscopy, polarization modulation, high-resolution colocalization in combination with coincidence analysis for the structural and dynamic investigation of function protein assemblies was reported (N16). The intrinsic fluorescence anisotropies of six photosensitizers in homogeneous solution were compared with those in tumor cell monolayers using polarization-sensitive laser-scanning confocal microscopy (N17). The intrinsic anisotropies in solutions ranged from 0.2 to 0.27. When observed in cells, however, an alternating high and low anisotropy was observed.

There were also reports of the use of fluorescence polarization microscopy to determine how the transition dipole of a fluorophore is oriented with respect to the membrane in giant unilamellar vesicles (*N18*). Polarization fluorescence microscopy was also used to characterize the emission and nonlinear absorption dipole of single CdSe/ZnS quantum rods (*N19*).

A number of studies were reported using fluorescence anisotropy in evaluating various aspects of chiral recognition. Xu and McCarroll have reported a new method where steady-state fluorescence anisotropy measurements were carried out to examine the thermodynamics of enantioselective binding (N20). A mathematical model developed from fundamental principles was reported that related differential fluorescence anisotropy measurements to chiral selectivity and allowed evaluation of the thermodynamic parameters of the enantioselectivity. The determination of enantiomeric composition by fluorescence anisotropy was reported by the same group (N21), where β - and α -cyclodextrin were used as the chiral selector. In this work, a theoretical model was presented that predicts a linear dependence on the selectivity of the chiral selector, the concentration of free selector, and the enantiomeric composition. Billiot et al. also reported the use of fluorescence anisotropy to evaluate chiral separation systems in electrokinetic chromatography (N22).

The Bright research group employed time-resolved and steadystate fluorescence anisotropy to study the behavior of pyrene (Py) end-labeled poly (dimethylsiloxane) (Py-PDMS-Py) polymer in low concentrations of 1-butyl-3-methylimidazolium bis(trifluoromethyl)sulfonylimide as a function of temperature and added cosolvent (*N23*). They also investigated the response of a three-armed PDMS-based junction that was site-selectively labeled with a dansyl junction in neat and cosolvent-modified CO₂ (*N24*). There were several reports from the Brennan group that employed timeresolved fluorescence anisotropy to probe interactions of silica surfaces (*N25–N30*). The data obtained in these studies suggest that the technique can be useful in the characterization of new chromatographic stationary phases and nanocomposite materials.

The effect of temperature on the fluorescence anisotropy decay and solvation dynamics of coumarin 153 (C153) in γ -cyclodextrin aggregates was also evaluated (*N31*) The large steady-state and residual anisotropy observed for C153 was attributed to the formation of large linear nanotube aggregates of γ -cyclodextrin linked by the dye. The rotational dynamics of C153 in supercritical fluoroform, through measurements of subpicosecond fluorescence anisotropy decay curves using fluorescence upconversion technique, were found to be consistent with data obtained from steadystate electronic spectral shifts (*N32*).

CHEMILUMINESCENCE

Many innovations of chemiluminescence detection techniques for various analytes were reported during the time period of this review. Novel methods of determining molecules of biomedical importance and drug molecules in human serum or biological fluids, while reported in great numbers, are generally not included in this review due to space limitations.

The use of Ce^{4+} —Tween 20 chemiluminescence detection for salicylic acid and resorcinol phenolic compounds in Dermatitis Clear Tincture and *p*-hydroxybenzoic acid in apple juices following

the HPLC separation was reported (O1). A chemiluminescence method of glucose-6-phosphate dehydrogenase (G-6-PD) activity detection in healthy, heterozygous, and enzyme-deficient people was developed by Gumuslu et al. (O2). Compared to the ultraviolet G-6-PD detection, the new chemiluminescence detection method was found to be more sensitive. The use of a hybrid flow injection analysis, involving the measurement of chemiluminescent signal intensity generated by the reaction of hydrogen peroxide and luminol, for a fast glucose assay was described (O3).

Huang et al. described the use of a chemiluminescence imaging detection method for haptoglobin phenotyping in human blood serum following polyacrylamide gel electrophoresis separation (O4). A simple, rapid, and reproducible novel chemiluminescence technique involving the measurement of light emission produced from the reaction of luminol-hydrogen peroxidehorseradish peroxidase and lauric acid ester substrate was used to assay triacylglycerol lipase activity from *Candida cylindracea* and porcine pancreas (O5). Several studies report new methods to measure reactive oxygen production using chemiluminescence detection. Guzik and Channon review advantages and disadvantages of vascular reactive oxygen detection including the use of newly developed chemiluminescence probes (O6).

New chemiluminescence methods of determining chemical oxygen demand (COD) for effective monitoring of water quality was reported by two groups. Hu and Yang developed a cost-efficient chemiluminescence photodiode detector that measures chemiluminescence emission produced by the luminol–water– Cr^{3+} reaction (*O7*). Using a flow injection ozonation chemiluminescence method involving ozone and UV oxidation of α -naphthol, Jin et al. also determined COD in natural water (*O8*).

Marley et al. constructed a novel instrument for the simultaneous determination of atmospheric nitrogen oxide and peroxyacetyl nitrate (PAN) at part per trillion levels (*O9*). The detection of the analytes is based on the chemiluminescence detection of the NO–PAN–luminol reaction at the gas–liquid interface on a solid support surface.

NEAR-INFRARED FLUORESCENCE

During the last two years, near-infrared (NIR) luminescence techniques reported in the literature were overwhelmingly medical or biologically related analyses. NIR is inherently advantageous in these applications due to diminishing autofluorescence of the sample of interest in this spectral region. Fluorescence in vivo imaging has become a major focus of interest. More and more researchers recognize the advantages of this wavelength region for penetrating tissues of the whole body. Detailed review articles describe several cancer detection applications using NIR fluorescence (*P1*) and the present use of quantum dots (*P2*).

Significant developments were reported in the area of NIR imaging of tumors. This area is especially important in light of the increasing importance of early cancer diagnoses as well as more efficient surgery in minimizing surgical boundaries to preserve as much healthy tissue as possible. Citrin et al. discussed the use of in vivo tumor imaging using near-infrared-labeled endostatin molecules (*P3*). Godavarty et al. used NIR fluorescence tomography utilizing molecularly targeted, lifetime-sensitive,

fluorescent contrast agents to show that it has applications for early-stage cancer diagnostics (P4). A two-stage approximate extended Kalman filter reconstruction algorithm was used to interpret data with a Bayesian tomography algorithm to obtain three-dimensional images. Jose et al. synthesized and studied a novel near-infrared fluorescent estrogen conjugate for noninvasive imaging of breast cancer (P5). The development of a selective urokinase-type plasminogen activator NIR fluorescent imaging probe was described for cancer diagnoses in another paper (P6). Wang et al. described near-infrared optical imaging of integrin avb3 in human tumor xenografts using an integrin avb3targeted peptide labeled with fluorescence dyes (Cy5.5 and IRDye800) (P7). Detection of protein overexpression in tumor cells is another important approach. Hilger et al. used Cv5.5-labeled anti-HER-2 antibodies for this purpose (P8). The probe consisted of multiple peptide motifs terminally capped with Cy5.5 or Cy7 fluorophores.

Chen et al. reported near-infrared fluorescence-labeled folate probes for in vivo imaging of arthritis using a lipopolysaccharide intra-articular injection model (*P9*). This receptor-targeted imaging method can be useful for improved arthritis diagnosis and early assessment of the disease progress by providing an in vivo characterization of active macrophage status in inflammatory joint diseases. Using confocal laser scanning microscopy, the commercially available dye Cy5.5 was used to identify arthritis in murine antigen-induced arthritis (*P10, P11*).

Several advances in protease sensor development were made by the Weissleder group. A peptide-based NIR fluorescence probe, consisting of an NIR fluorescence emitter (Cv5.5), an NIR fluorescence absorber (NIRQ820), and a protease-selective peptide sequence, was designed to sense protease activity (P12). A dual fluororophore probe for imaging proteases was also described (P14). This dual fluororophore enzyme-activatable probe has an NIR fluorophore that is activated by protease activity as well as a second protease-resistant fluorophore that serves as an internal standard. And, a novel method for imaging apoptosis in living animals using a caspase-1 near-infrared fluorescent probe was reported (P15). Duan et al. reported two new NIR fluorophores, phthalocyanine and naphthalocyanine analogues, having a single isothiocyanato group suitable for conjugation to proteins (P13). Cell-permeable near-infrared fluorogenic substrates for imaging β -lactamase activity were reported by Xing et al. (P16).

Indocyanie green (ICG) continues to be used in NIR fluorescence imaging. Shibata et al. described an optical method for imaging cerebrospinal fluid space in mice using near-infrared fluorescence imaging by ICG bound to high-density lipoprotein that was injected into the lumber subarachnoid space of nude mice (*P17*).

Using a two-reagent system composed of anionic tetracarboxy aluminum phthalocyanine and polycationic polylysine, nucleic acid concentration was determined using NIR fluorescence recovery (*P18*). The same authors described a method for DNA determination where the fluorescence of a cationic heptamethylene thiacyanine was enhanced by Triton-X and quenched by the addition of DNA (*P19*).

Aggregation has been a major drawback of carbocyanines in aqueous systems. An interesting new class of NIR labels has been

synthesized by Zhang and Achilefu (P20). These polyhydroxylated near-infrared carbocyanine molecular probes, derived from indocarbocyanine dye and galactose, are useful because the presence of a nonionic polyhydroxyl moiety between hydrophobic groups enhances solubility, possibly minimizing aggregation. Novel bis(heptamethine) cyanines were synthesized by Patonay et al. and characterized as to their analytical utility (P21, P22). These dyes form inter- and intramolecular H-aggregates in polar solvents, even at very low concentrations. The intramolecular dimeric form of the dye can be described as a clam shell complex with two interacting hydrophobic carbocyanine moieties. In the presence of HSA, the H and D spectral bands are decreased and the monomeric band is increased, with concomitant increase in fluorescence intensity, suggesting that clam shell H-aggregates open up in the 1:1 complex with HSA. These dyes can have significant bioanalytical utility. Novel monofunctional carbocyanines suitable for biomolecule labeling were prepared by the Weissleder group using improved synthetic procedures (P23). Perlitz et al. compare two highly water soluble tricarbocyanies for fluorescence optical imaging applications (P24).

The synthesis and characterization of the near-infrared fluorescence oxazine dye AOI987, which readily penetrates the intact blood-brain barrier and binds to amyloid plaques, was described by Hintersteiner et al. (*P25*). Amphiphilic squaraine dyes were developed to characterize the presence and absence of the organized media (*P26*). An NIR fluorophore-labeled peptide ligand derived from the amino terminus of α -2-antiplasmin was used in another study as a fluorescent contrast agent that covalently links to thrombi to help clinical diagnosis of acute thrombi (*P27*).

A near-infrared fluorescence affinity sensor, Alexa647 conjugated to concanavalin A, for glucose monitoring was described by Ballerstadt et al. (*P28*). The authors also published a similar article (*P29*) reporting a fluorescence resonance energy-transferbased near-infrared fluorescence sensor for glucose monitoring. The sensor operates by resonance energy transfer between an NIR chromophore linked to concanavalin A and an NIR fluorophore linked to free dextran.

Platinum-containing NIR chromophores have found use in analytical chemistry as well. A ratiometric fiber-optic sensor for the detection of inter- and intracellular dissolved oxygen was developed by Park et al. (*P30*). Oxygen quenching is utilized in these studies where sensitive platinum(II) octaethylporphine ketone was used with a reference dye, octaethyl porphyrin, or bodipy maleimide.

Although conventional conjugated organic molecules dominate in the majority of NIR analytical references, other types of fluorescers are reported as well. Kim et al. published an interesting application of NIR quantum dots (*P31*). In this study, nearinfrared fluorescent type II quantum dots as exogenous contrast agents were used for sentinel lymph node mapping. In addition to quantum dots, single-walled carbon nanotube (SWNT) optical sensors have been reported. Barone et al. developed in vivo glucose detection using a nanotube-based optical sensor (*P32, P33*). These articles describe the development of such sensors, and the optical properties of commonly used organic and nanoparticle fluorescent probes are compared with respect to quantum yield, human tissue penetration, and photobleaching stability. It is important to point out that photobleaching is practically zero for SWNT optical sensors. Another application of SWNTs was reported by Cherukuri et al. (*P34*), which describes near-infrared fluorescence microscopy of these nanoparticles in phagocytic cells.

A more conventional competitive immunoassay using NIR fluorescence detection to analyze microliter biological samples with an attomole limit of detection was described by Zhao and Shippy (*P35*). The fluorescence of the blotted spots is detected with an NIR-sensitive photon counting system that is optimized to detect \sim 30 000 fluorophore molecules. And finally, a very interesting combination of two detection methods was reported by Zhang et al. (*P36*) utilizing radioactivity and NIR fluorescence. The authors prepared NIR monomolecular multimodal compounds consisting of a heptamethine carbocyanine dye and ¹¹¹In-DOTA chelate that served as antennas for optical and scintigraphic imaging, respectively.

LUMINESCENCE TECHNIQUES IN BIOLOGICAL AND CLINICAL ANALYSIS

Although several examples of tumor diagnosis using fluorescence techniques are reported in the literature, only a few representative studies are included in this review. Brewer et al. used a combination of fluorescence spectroscopy, confocal microscopy, and optical tomography to differentiate between normal and abnormal epithelial ovarian cancer tissue (Q1). The thickening of tumor mucosa and enhanced spectral absorption of tissue hemoglobin allow for the microscopic differentiation of normal and tumor colonic tissue using helium-cadmium laser-induced autofluorescence spectroscopy (Q2). Skala et al. described the use of fluorescence spectroscopy for in vivo precancer diagnosis in squamous epithelial tissues (Q3). Both the normal and the precancer tissues were correctly classified using nonparametric analysis of spectral data. The use of correlation coefficient mapping was also reported to be an effective method for sample classification and cancer detection (Q4).

There were several reports on the clinical diagnosis of various diseases. Christov et al. demonstrated that autofluorescence emission measurements of amyloid β and collagen could be used to detect structural changes in brain tissue resulting from Alzheimer's disease (*Q5*). It is well established that low levels of free fatty acid (FA) may be used as a diagnostic marker for neurode-generative diseases. A relatively inexpensive ratiometric fluorescence FA sensor, capable of detecting oleic acid at $0.02-4.7 \ \mu$ M concentration range, was described (*Q6*). A highly sensitive and specific magnetic bead-based sandwich immunoassay for abnormal prion protein, a biomarker for neurodegenerative transmissible spongiform encephalopathies, has been developed (*Q7*).

Based on the autofluorescence of collagen and macrophage foam cells in rabbit, a novel in vivo technique for the detection of macrophage, a potential marker for antherosclerotic plaques, was demonstrated (Q8). Paschalidou et al. demonstrate that variations of an N-terminal tetradecapeptide sequence of human angiotensinogen can effectively detect picomolar concentrations of renin and may provide an effective means to evaluate renin inhibition for the treatment of hypertension (Q9).

The development of novel detection techniques for enzymatic activity, protein activity, and changes in protein conformation in

biological samples continues to attract significant interest. A novel quantitative fluorescence technique for selective determination of sialidase/neuraminidase activity on indoxyl-derivatized N-acetylneuraminic acid substrates in influenza viral neuraminidase was developed (Q10). Using a green fluorescent labeled protein, a rapid in vitro and in vivo screen of protein expression in Escherichia coli was developed (Q11). Kohl et al. demonstrated the utility of a dual-labeled caspase-3 substrate to elucidate protease reaction in situ (Q12). Le et al. combined a synthetic modular genetic system and an FCS strategy for the real-time determination of RNA profiling in prokaryotes (Q13). Neuweiler et al. have developed an oxazine fluorophore-labeled protein for the direct assay of human p53 antibodies in homogeneous solution using FCS (Q14). The influence of sodium pentachlorophenolate (NaPCP) concentration on DNA, RNA, and proteins as well as cytoplasmic membrane polarization in bacterium Sphingomonas sp was studied using a fluorescence method (Q15). There was no observable negative effect over a long period of time, demonstrating the high resistance of bacterium Sphingomonas sp to NaPCP. Jayasinghe and Langen investigated the interaction of islet amyloid polypeptide (IAPP), implicated as a critical chemical in type II diabetes pathogenesis, with a lipid membrane containing negatively charged phosphatidylserine using circular dichroism and fluorescence spectroscopy (Q16). The authors concluded that conditions that encourage weakly stable α -helical conformations can also facilitate IAPP aggregation. Changes in folding and structural dynamics of tryptophan residues of gramicidin as the degree of hydration changed were studied using red-edge excitation techniques in reverse micelles (Q17). Structural investigations of transthyretin (TTP), a protein that has been associated with various amyloid diseases, are challenging because of aggregation formation as a result of misfolded TTP. Using static and kinetic techniques, Lindgren et al. investigated physicochemical and structural characteristics of TTP aggregates throughout the aggregation process using several fluorescent dyes (Q18). The authors note that 4-(dicyanovinyl)julolidine, a molecular rotor applied for the first time to study amyloidogenic processes, can be effectively used early in the oligomerization process. Using fluorescence techniques as well as near- and far-UVcircular dichroism, the reversible conformational changes of E. coli glutamine-binding protein was investigated by Staiano et al. (Q19).

New techniques of diagnosis of tissue and cells were reported. Using a mobile fluorescence detector, Hansch et al. studied the structural and functional properties of several mice organs (Q20). Pitman et al. investigated the structural properties of various thyroid tissues using surface scanning fluorescence spectroscopy for the first time (Q21). A ratio imaging technique was used for the determination of intracellular viscosity of a single cell of a rat embryonic aorta (Q22).

Resolving the depth of signal within biological tissues is of interest in clinical imaging. A confocal fluorescence spectroscopy system was used to study the depth-resolved autofluorescence in biological tissues. The authors demonstrate that depth-resolved fluorescence may be more accurate for the diagnosis of tissue pathology (*Q23*). The same group also investigated the use of single- and two-photon spectroscopy as well as second harmonic generation to investigate autofluorescence from tissue (Q24, Q25). Other investigators have shown that the use of an angled fiber-optic probe facilitates depth-sensitive fluorescence measurements in turbid media (Q26) and that angleresolved polarized fluorescence provides depth-resolved fluorescence measurements in a turbid medium when excited with linearly polarized light (Q27). A simple time-domain optical method for estimating the depth and concentration of florescent inclusions in a turbid medium (Q28) as well as a frequency-domain fluorescence optical diffusion tomography instrument to reconstruct the fluorescence yield and lifetime in turbid media (Q29) was reported.

REAGENTS AND PROBES

Progress in modern analytical chemistry would be impossible without new selective reagents and probes. The field of molecular recognition and sensing provides tremendous tools for monitoring molecular interactions and detecting analytes with very different molecular structures, and fluorescence techniques afford the required sensitivity and selectivity. As a result several new fluorescent reagents and probes have been developed over the review period.

The Lakowicz group has developed a new group of aqueous ratiometric and colorimetric fluorescence probes able to detect fluoride ions even in the presence of significant background interferences (*R1*). The same group also developed quinoline—boronic acid-based colorimetric and fluorescent probes capable of detecting aqueous cyanide. The probes, while able to detect cyanide concentrations in the range of 10 mM, showed minimal affinity for monosaccharides (*R2*).

Monotoring pH with the use of fluorescent dyes continues to be an area of active research. The Lakowicz group reported the first use of enhanced ratiometric pH sensing using silver metal surfaces (R3). Jobsis et al. developed a two-photon excitation ratiometric probe for pH sensing in vitro and in fibroblast cells (R4). Marcotte and Brouwer report the optical spectroscopic properties of a derivative of 1,4-benzenedicarboxylic acid, a promising fluorescent pH probe, investigated by steady-state and time-resolved spectroscopy (R5).

Intracellular magnesium ions continue to be of interest to pharmacologists and cellular biologists. Komatsu et al. describe three novel Mg²⁺ fluorescent probes that allow the real-time determination of changes in free magnesium concentration (*R6*). Chang et al. have developed a fluorescent probe based on a tautomeric seminaphthofluorescein for intracellular Zn²⁺ imaging (*R7*). Numerous probes and reagents have been developed for the detection of thiols such as gluthatione and cysteine (*R8–R10*). A simple method to detect homocysteine or cysteine at physiologically relevent concentrations without preparative separations was developed by the Strongin group (*R11*). The method employs new probes based on fluorescein aldehyde derivatives, cinnamaldehyde and methyl viologene.

New developments in glucose montoring have been made over the review period. In a review article, the Lakowicz group describes a disposable contact lens for tear glucose sensing. This aqueous boronic acid-based sensor continuously determines tear glucose with an approximate response time of 15 min and shelf life longer than three months (R12). Kawanishi et al. developed a boronic acid-based anthracene glucose sensor and describe several methods to immobilize this probe to a solid support in order to ensure its sensing properties are maintained (*R13*). In another report, the Strongin research group developed xanthene-based sensors for the selective detection of fructose or glucose (*R14*).

Abbotto et al. have developed a novel two-photon absorbance probe with a high affinity for DNA and negligible toxicity that is suitable for microscopy and imaging applications (R15). Direct protein detection using newly developed benzazole isothiocyanate fluorescent dyes was demonstrated (R16). Species-dependent differences in serum albumins from seven species were characterized using a dual color ratiometric fluorescence probe (R17). Use of multiple individual fluorophores as a new strategy for fluorescent DNA labeling was explored by Cuppoletti et al. (R18). The Lakowicz group investigated the fluorescence properties of silverbound, fluorescein-labeled DNA oligomers (R19). In this report, the authors provide the fundamental understanding required in order to implement metal-enhanced fluorescence sensing. A novel fluorescent nucleobase capable of base-discriminating is described by Okamoto and co-workers (R20). Resonance energy transfer from the naphthodeazaadenine donor to the fluorescein acceptor was observed only in the presence of the complementary base cytosine. The restoration of fluorescence of tetramethylrhodamine upon binding to double-stranded DNA allowed the detection of specific DNA sequences in solution (R21). Fluorescence labeling methods for aryl halides with a fluorescent arylboronic acid was developed based on the Suzuki coupling reaction (R22). The highly sensitive and selective method provided for the detection of clofibrate present in human plasma.

Fluorescence techniques have found applications in clinical analysis as well as diagnostic tools. In an interesting report by Quach et al. (*R23*), fluorescein isothiocyanate fibrinogen was able to assess improvement in fibrinogen cross-linking by plasma in liver transplant patients. The temperature-induced conformational change of recombinant prion protein was studied using heme as a spectroscopic probe (*R24*).

OTHER TECHNIQUES AND APPLICATIONS

New fluorescence reagents to develop latent fingerprints (*S1*) or contrasting agents to image them was reported (*S2*). Reddy et al. determined the total and free pool of amino acids using a liquid chromatography-laser-induced fluorescence separation method in order to develop a unique fingerprint for each opium source in India (*S3*). And, three-dimensional fluorescence spectroscopy was shown to be a suitable alternative to monitor fading pigments in artwork (*S4*).

The luminescence of native organic volatile compounds emitted from the surface of fruits can be used to study the ripening and aging of fruits (*S5*). Laser-induced fluorescence spectroscopy was used to assess changes in pigment contents of select fruits and vegetables stored in the cold (at 0 °C for carrots and 4 °C for apples) and at room temperature (*S6*).

Fluorescence continues to be an important technique for a variety of environmentally important studies. Leachates from landfills, rich in dissolved organic matter, were effectively detected using fluorescence techniques. The authors observed a strong correlation between leachate fluorescence intensity and ammonia, total organic carbon, and biochemical oxygen demand, common groundwater quality determinants (*S7*).

Kristin A. Fletcher received her B.S. in mathematics and B.A. in chemistry (1995) and M.S. in analytical chemistry (1997) from the University of North Texas. She worked in the field of forensics before receiving her Ph.D. from New Mexico Institute of Mining and Technology under the supervision of Dr. Siddharth Pandey in 2004. She is currently a postdoctoral researcher in Dr. Isiah Warner's research group at Louisiana State University. Her research interests include applications of molecular spectroscopy to probe physicochemical properties, understanding mechanisms of chiral recognition, and the development of novel stationary phases for chemical separations.

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Mark Lowery received his B.A. in chemistry from Central College in Pella, IA, in 1998. He then pursued graduate studies under the direction of Professor Lei Geng at The University of Iowa and received his Ph.D. in 2005. He is currently a postdoctoral researcher under the direction of Professor Isiah M. Warner at Louisiana State University. His research interests include fluorescence instrumentation, fluorescence imaging and image analysis, single-molecule imaging/spectroscopy, fluorescence correlation spectroscopy, and time-resolved fluorescence measurements.

Sheryl Ann Tucker is Associate Professor and Associate Chair for Graduate Studies of the Department of Chemistry at the University of Missouri–Columbia (MU). She received her B.S. in chemistry from Kent State University in 1990 and her Ph.D. in chemistry from the University of North Texas in 1994. She served as a postdoctoral research associate at Duke University (1994–1996) and then as an Assistant Professor of Chemistry at MU in 1996. She was promoted with tenure in 2002 and became an Associate Chair in 2005. She received a Presidential Award for Excellence in Science, Mathematics and Engineering Mentoring from the White House in 2005. Her current research interests include the analytical applications of organized media to chemical separations and chemical sensing and the use of fluorescence spectroscopy for understanding the intricacies of complex systems (dendritic and hyperbranched polymers; photosensitizers and squamous cell carcinomas; and supramolecular, selfassembling pyrogallol nanocapsules).

Sharon L. Neal received her B.S. in chemistry from Spelman College in 1980 and her Ph.D. in chemistry from Emory University in 1988. She joined the faculty at University of Delaware in 1998 where she currently holds the position of Associate Professor of Chemistry. She currently serves on the A-page Advisory Panel of Analytical Chemistry. Her current research interests include the development of multidimensional fluorescence methods for characterization of complex samples, development of chemometrics methods for analysis and interpretation of multidimensional fluorescence, characterization of biomembrane models, characterization of intrinsic protein fluorescence, photokinetic characterization of multiply emitting probes, drug or toxin/protein interactions, and drug or toxin/ lipid interactions.

Irene W. Kimaru received a B.S. in chemistry and biochemistry in 1998 at Kenyatta University, Nairobi, Kenya. She then pursued graduate studies under the direction of Dr. Matthew E. McCarroll at the Department of Chemistry and Biochemistry at Southern Illinois University Carbondale where she is currently a Ph.D. candidate in analytical chemistry. Current efforts are focused on the development fluorescent sensors and applications of fluorescence anisotropy in characterizing chiral stationary phases. Her research interests include studies of chiral recognition, characterization of chiral stationary phases for optimization of chiral separations, and development of fluorescent sensors.

Matthew E. McCarroll is an Assistant Professor in the Department of Chemistry and Biochemistry at Southern Illinois University. He received his B.A. and B.S. degrees in chemistry and interdisciplinary studies from Appalachian State University in 1994. He pursued graduate studies at the University of Idaho under the direction of Professor Ray von Wandruszka, where he received his Ph.D. in 1998. He then spent two years as a postdoctoral associate under the direction of Professor Isiah M. Warner at Louisiana State University. In 2000, he joined the faculty at Southern Illinois University as an Assistant Professor. His general research interests focus on the application of analytical chemistry to investigate complex chemical phenomena with an emphasis in the areas of molecular spectroscopy and organized media. Current efforts included studies of chiral and molecular recognition, the development of fluorescence sensors, and the development of new materials for chiral separations and drug delivery. **Gabor Patonay** is a professor of analytical chemistry in the Department of Chemistry at Georgia State University. He received his M.S. (1973) and Ph.D. (1979) degrees from the Faculty of Chemistry of the Technical University of Budapest, Hungary. After graduation he spent a brief period at the same school mostly developing new analytical instruments and techniques. In 1982, he joined Professor Isiah M. Warner's group at Emory University as a postdoctoral associate. He joined the faculty of Georgia State University in 1987. Dr. Patonay has been active in several areas of near-infrared (NIR) fluorescence spectroscopy, including development of new optical detection methods. NIR fluorescence has the highest utility in abblications where the interference is significant i.e., in biological sambles. applications where the interference is significant, i.e., in biological samples. During the last several years Dr. Patonay and his research group have developed new bioanalytical and biomedical applications using NIR probes and labels. Lately his research group has been active in developing new forensic analytical tools for presumptive trace evidence detection using NIR dyes.

Philip B. Oldham is a professor of analytical chemistry and currently serves as Dean of Arts & Sciences at Mississippi State University. He received his B.S. in chemistry from Freed-Hardeman University in Henderson, TN, in 1980 and his Ph.D. in analytical chemistry from Texas A&M University in 1985. He spent a year following his graduate work as a Wellcome Research Fellow at Burroughs Wellcome Company, Research Triangle Park, NC, before joining the faculty at Mississippi State University in 1986. Dr. Oldham's research interests involve the develop-ment of analytical techniques for the characterization of solute molecules ment of analytical techniques for the characterization of solute molecules resident is specific microenvironments such as biological membranes, micelles, and/or surface films. The current technique of interest is total internal reflection fluorescence (TIRF) with electrochemical control of the sensor surface. Much of his efforts are currently being directed toward application of TIRF in the design and microfabrication of novel biosensor devices.

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Robert M. Strongin is the Philip and Foymae Kelso West Distinguished Professor of Organic Chemistry in the Department of Chemistry at LSU. He received his B.A. degree in chemistry from Temple University in Philadelphia, PA, in 1988 and worked at FMC Corp. and SmithKline Beecham before pursuing his Ph.D. in 1990. He received his Ph.D. in organic chemistry from the University of Pennsylvania under the direction of Professor Amos B. Smith, III, in 1995 and has served on the faculty of SU ince that time. His recearch interest include the combesis and LSU since that time. His research interests include the synthesis and evaluation of novel redox and fluorophore materials as well as organic mechanisms relevant to sensing and biomedicine.

Isiah M. Warner is a Boyd Professor of the LSU System, Philip W. West Professor of Analytical and Environmental Chemistry in the Department of Chemistry at Louisiana State University (LSU). He received his B.S. degree in chemistry from Southern University in Baton Rouge, Louisiana, in 1968. He worked at Battelle Northwest in Richland, Washington, for five years before pursuing his Ph.D. in analytical chemistry from the University of Washington in 1973. He received his Ph.D. in analytical chemistry from the University for five years and on the faculty of Emory University for 10 years before joining the faculty of LSU in 1992. His research interests include fluorescence spectroscopy, studies in organized media, and separation science, with a focus on solving biomedical and environmental analytical problems.

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