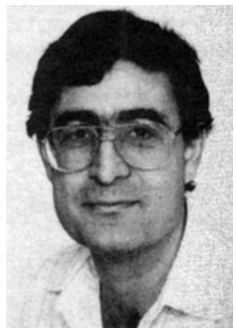
Fluorescence Spectroscopy

Eleftherios P. Diamandis
Department of Clinical Biochemistry, The Toronto Hospital, Toronto Western Division, 399 Bathurst Street, Toronto, Ontario M5T 2S8, Canada

Fluorometry is usually 10–1000-fold more sensitive and inherently more specific than spectrophotometry. It is thus not surprising that researchers usually turn to fluorescence when they want to quantify analytes below 10^{-6} mol/L. The fluorometric measurement is versatile and specific because it can combine several parameters simultaneously such as excitation and emission wavelengths, fluorescence lifetime, or fluorescence polarization. Because some of the above parameters are affected by the microenvironment of the fluorescence compound, fluorescence spectroscopy frequently allows the direct study of molecular processes such as antigen–antibody interaction without prior separation of the bound and free fractions of the fluorescent probe. This forms the basis of the homogeneous fluorescence immunoassays.

Fluorescence spectroscopy is not frequently used in the routine clinical chemistry laboratory. Most of the well-established biochemical tests are performed with automated analyzers equipped with spectrophotometric and potentiometric readers. The reason is that sensitivity is not an issue and the fluorometric reading is more demanding in terms of reagent purity and expense of instrumentation. However, in specialized areas such as therapeutic drug monitoring and endocrinology, many analytes are now measured using fluorescence-based immunoassays. Fluorometry is also used widely for research and new method development applications. In this review, which mainly covers the literature between January 1991 and October 1992, I will describe applications and some important new developments in the field of fluorescence spectroscopy.



Eleftherios P. Diamandis is presently Deputy Biochemist-in-Chief, Toronto Western Division, The Toronto Hospital, and Associate Professor of Clinical Biochemistry at the University of Toronto, Faculty of Medicine. He received his B.Sc. and Ph.D. degrees in chemistry and analytical chemistry from the University of Athens, Greece, in 1976 and 1979. He also received an M.D. degree from the same university in 1986 and a Diploma in clinical biochemistry from the University of Toronto in 1984. Dr. Diamandis is a Diplomate of the American Board of Clinical Chemistry and certified clinical biochemist by the Canadian Society of Clinical Chemists. He is on the Board of Editors of *Clinical Chemistry News* and has published over 100 papers. He has won many awards including the Young Investigator Award of the American Association of Clinical Chemistry (1985). His current interests include the development of ultrasensitive nonisotopic methodologies for immunological and molecular biology techniques and the development of new testing for the diagnosis and monitoring of cancer.

REVIEWS

The field of time-resolved fluorescence spectroscopy has been repeatedly reviewed especially as it applies to immunological assays (*Q1–Q9*). Other reviews on time-resolved fluorescence (TRF) deal with general applications in biology and medicine, e.g., the study of macromolecular structure and dynamics (*Q10*), of calmodulin (*Q11*), and of organic molecules tethered to biological membranes (*Q12*). The principles and applications of TRF to clinical microbiology and DNA probe technology were also reviewed (*Q13*). General reviews covering applications of fluorescence in immunoassay (*Q14–Q16*), studies of protein structure (*Q17*), histamine determination (*Q18*), interleukin-2 gene expression (*Q19*), catecholamine analysis by HPLC (*Q20*), and DNA in-situ hybridization (*Q21*) have also been published. Other reviews deal with general applications of fluorescence in clinical chemistry, biology, and biochemistry (*Q22–Q27*). Another review covers labeling protocols with fluorescein derivatives (*Q28*).

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) WITH FLUOROMETRIC DETECTION

This is one of the most rapidly growing areas of application of fluorescence spectroscopy. This technique combines the powerful separation capabilities of HPLC with the excellent sensitivity and specificity of the fluorometric detection. With suitable instrumentation design (cuvette shape and volume capacity, laser excitation, optical system configuration) this technique can detect single fluorescent molecules (*Q29, Q30*).

HPLC with fluorometric detection is sometimes applied without any sample pretreatment by taking advantage of the native fluorescence of the analyte of interest. However, in most cases, cleanup procedures including protein precipitation and liquid–liquid or solid-phase extractions with disposable cartridges are applied before analysis. When the analyte does not exhibit native fluorescence or for improved sensitivity, pre- or postcolumn derivatization is also applied.

Many drugs have been analyzed by HPLC with fluorometric detection for therapeutic drug monitoring and/or pharmacokinetic studies. The sample is usually serum or plasma but urine and whole blood are also used in selected cases. Drugs analyzed include miloxacin (*Q31*) fluoxetine and norfluoxetine (*Q32, Q33*), doxorubicin and metabolites (*Q34*) theophylline (*Q35*), CL-275,838 (*Q36*), diclofenac (*Q37*), indomethacin (*Q38*), valproic acid (*Q39*), methotrexate (*Q40*), ethacrynic acid (*Q41*), etoposide (*Q42*), furosemide and amiloride (*Q43*), josamycin and rokitamycin (*Q44*), fluvoxamine (*Q45*), nadolol (*Q46*), diphenhydramine (*Q47*), isoniazid and metabolites (*Q48*), atenolol (*Q49*), nicotinic acid and nicotinamide (*Q50*), terfenadine (*Q51*), buprenorphine (*Q52*), succinylcholine (*Q53*), morphine and metabolites (*Q54*), 4-hydroxycoumarin (*Q55*), mexiletine (*Q56*), alentamol (*Q57*), amidepin (*Q58*), mefloquine (*Q59*), vancomycin (*Q60*), 4-amino-1-hydroxybutane-1,1-bisphosphonic acid (*Q61*), vinplastine derivatives (*Q62*), prednisone and prednisolone (*Q63*), mianserin (*Q64*), narciclasine (*Q65*), betaxolol (*Q66*), amsulosin (*Q67*), loxoprofen (*Q68*) albuterol (*Q69*), metolazone (*Q70*), and quinidine and quinine (*Q71*). Using the same technique, other analytes measured were as follows: sialic acids (*Q72*) and fucose (*Q73*) for tumor diagnosis, zinc coproporphyrin in maternal serum for the diagnosis of amnotic fluid embolism (*Q74*), D-mannose in urine for diagnosing candida infection (*Q75*), 3-hydroxypyridinium derivatives in urine as markers of bone resorption (*Q76*), malondialdehyde in plasma as an indicator of lipid peroxidation (*Q77*), 25-hydroxyvitamin D_3 for diagnosing bone disease (*Q78*), catecholamines in plasma for the differential diagnosis of hypertension (*Q79, Q80*) and of free fatty acids (*Q81*), selenium (*Q82*), adenosine (*Q83*), and pyridoxal 5′-phosphate for physiological studies (*Q84*).

GENERAL APPLICATIONS OF FLUORESCENCE IN CLINICAL CHEMISTRY

Fluorescence spectroscopy has been used extensively for the analysis of different classes of compounds useful for physiological or diagnostic investigations. For example, trace metals like selenium (*Q85*), europium, terbium, dysprosium (*Q86*), and aluminium (*Q87*) were measured fluorometrically in serum, urine, or tissues for assessing nutritional status or for physiological studies, respectively. The free cytosolic Ca^{2+} concentration, monitored by specialized fluorescent probes in single cells, has attracted a lot of interest due to the diverse activities of this ion in signal transduction pathways (*Q88, Q89*). Various drugs have also been quantified in biological fluids using fluorometric techniques. Among these are carbamazepine (*Q90*), acetylsalicylate and metabolites (*Q91, Q92*) fludarabine (*Q93*), and ampicillin (*Q94*). Enzymes have also been analyzed using fluorogenic substrates. For example, bacterial proteases were measured using fluorescein isothiocyanate-labeled proteins as substrates (*Q95*); human immunodeficiency virus (HIV) protease with a peptide carrying napthalenesulfonic acid (*Q96*) or using a homogenous assay based on fluorescence energy transfer (*Q97*); succinic semi-

aldehyde dehydrogenase in extracts of cultured human lymphoblasts for diagnosis of 4-hydroxybutyric aciduria (*Q98*); β-*N*-acetyl-β-D-glucosaminidnidase for investigating renal tubular function (*Q99, Q100*) and creatine kinase (*Q101*), alcohol dehydrogenase isozymes (*Q102*), and liver and bone alkaline phosphatase (*Q103*) were also measured.

Physiological metabolites have been fluorometrically measured for diagnostic applications. These include Vitamin D and metabolites (*Q104, Q105*), phenylalanine, branched-chain amino acids, homocysteine and histidine (*Q106*), urobilinogen (*Q107*), urea (*Q108*), prostaglandin E_2 (*Q109*), pyridoxal 5′-phosphate (*Q110*), amino acids (*Q111*), malonaldehyde (*Q112*), phospholipids (*Q113*), porphyrins (*Q114*), β-hydroxybutyrate and acetoacetate (*Q115*), L-alanine (*Q116*), carnitine (*Q117*), and bile acids (*Q118*).

FLUORESCENCE IMMUNOASSAY

This subject is also covered in detail in other parts of this review. One of the most successful homogeneous fluorescence immunoassay (FIA) methods, fluorescence polarization immunoassay (FPIA), is still used widely for measuring drugs, for therapeutic drug monitoring (*Q119*). More recently, hormones are being measured as well. Two other technologies, fluorescence excitation transfer immunoassay (FETI) (*Q120*) and substrate-labeled fluoroimmunoassay (SLFIA) (*Q121*) are also used for drugs, hormones, and proteins in biological fluids.

The heterogeneous fluorescence immunoassays are progressively replacing radioactivity-based assays. Particle concentration fluorescence immunoassay (PCFIA) (*Q122*) is mostly used for antibody screening. The time-resolved fluorescence immunoassay field is separately discussed below. The enzyme-linked fluorescence immunoassay (ELFIA), in which an enzyme is used to liberate a fluorescent moiety from a nonfluorescent substrate, is the method of choice for many new automated immunoassay analyzers. These systems are attractive because they combine the enzyme amplification with the excellent sensitivity and specificity of fluorescence measurement. When enzymes are used in combination with colorimetric substrates, the methods are generally 10–100-fold less sensitive (*Q123*). The most widely used enzymes are alkaline phosphatase (ALP), horseradish peroxidase (HRP) and β-galactosidase (β-DG). With ALP, 4-methylumbelliferyl phosphate (4MUP) is the preferred substrate in many automated analyzers including the IMx and Stratus (*Q124, Q125*). With HRP, (hydroxyphenyl)propionic acid is the most sensitive substrate (*Q126*). The β-DG enzyme is usually applied using β-D-galactopyranoside as the fluorogenic substrate.

The most recent significant advancement in fluorescence immunoassay is the advent of fully automated random access analyzers which are capable of analyzing samples in a fashion similar to the contemporary clinical chemistry analyzers (*Q127–Q133*).

Some recent advancements include the introduction of the principle of evanescent wave sensing and of fluoroimmunosensors for developing highly sensitive fluoroimmunoassays (*Q134–Q138*).

From the diagnostic point of view, a fluoroimmunoassay for measuring an Alzheimer's disease antigen has recently been patented (*Q139*). A universal fluorescent streptavidin-based reagent for immunoassays has also been prepared (*Q140*).

TIME-RESOLVED FLUOROMETRY

Time-resolved fluorometry is primarily used for nonisotopic immunoassays. This technique was introduced after the realization that the sensitivity of the conventional fluorescence measurement is restricted by the high background signals; the achievable detection limits are around 10^{-9} M. The rationale behind time-resolved fluorometry is the elimination of backround signals for the purpose of improving the signal-to-noise ratio. This is achieved by using the fluorescent lanthanide chelates, which have very long fluorescence lifetimes (10–1000 μs in comparison to <100 ns for conventional probes). The time-resolved fluorometric measurement involves sample excitation with a short pulse of light and monitoring fluorescence after an initial delay of 100–200 μs, during which any short-lived fluorescence dissipates to zero. This type of measurement assures very low backround signals and detectabilities in the 10^{-12}–10^{-13} M range.

Time-resolved fluorescence has been repeatedly reviewed (*Q1–Q9, Q141–Q144*). In general, two systems are commercially available which are based on the measurement of Eu^{3+} or a Eu^{3+}–chelate at the end of the immunological reaction. Both systems measure low and high molecular weight analytes using "competitive-type" and "noncompetitive-type" assays, respectively. The latest advance in time-resolved fluorometric immunoassay is the introduction of substrates which can be hydrolyzed by enzymes to products that form highly fluorescent complexes with lanthanides like Tb^{3+} and Eu^{3+} (*Q145*). These substrates were used for the highly sensitive determination of α-fetoprotein and thyrotorpin in serum (*Q146, Q147*). Time-resolved fluorometry has been used successfully for multianalyte immunoassays in two different formats (*Q148–Q151*). Multianalyte immunoassay shows promise for further future developments (*Q152*). Some other new advances in TR-FIA include the development of the "idiometric assay" for measuring low molecular weight analytes by noncompetitive techniques (*Q153, Q154*), the development of streptavidin-based universal detection reagents (*Q155, Q156*), the use of time-resolved fluorometry in HPLC (*Q157*), the application of liposomes as amplifier moieties (*Q158*), use of cofluorescence phenomena to improve sensitivity (*Q9, Q159–Q161*), the application of time-resolved fluorescence in nucleic acid hybridization assays (*Q162, Q163*), immunohistochemistry (*Q164*), target amplification assays (*Q165*), Western blot (*Q166*), PCR (*Q167*), antibody testing (*Q168*), and development of new chelators (*Q169*) and conjugates for competitive assays (*Q170*). Recently, time-resolved fluorometry was used in immunoassays with enzyme substrates that do not involve lanthanides (*Q171*).

Some recent applications of TR-FIA include the assay of lipoprotein a (*Q172*), triiodothyronine (*Q173*), progesterone (*Q174*), IgM rheumatoid factor (*Q175*), gonadotropins in amphibian species (*Q176*), vascular permeability factor (*Q177*), 17a-hydroxyprogesterone (*Q178*), fibronectin (*Q179*), 17-β-hydroxysteroid dehydrogenase (*Q180*), tetanus antitoxin (*Q181*), growth hormone (*Q182*), sex steroids (*Q183*), $β_2$-microglobulin (*Q184*), and lutropin (*Q185*). A new fluorescent enhancement solution has also been described (*Q186*). The sensitivity and specificity of enzyme immunoassays and time-resolved fluorometric immunoassays has been compared (*Q187*).

CYCLOSPORINE

The monitoring of cyclosporine A (CyA) levels in whole blood and serum has become a routine procedure for the posttransplant management of immunosuppression. The original methods for cyclosporin correlated poorly with the specific technique of HPLC because the polyclonal antibodies used were cross-reacting with CyA metabolites. Thus, values by RIA or fluorescence polarization immunoassay were 2–3 times higher in comparison to HPLC (*Q188*). More recently, a highly specific monoclonal antibody has become available with low cross-reactivity with CyA metabolites (*Q189–Q194*). This antibody was used to develop FPIA as well as RIA and enzyme-multiplied immunoassay technique (EMIT) procedures for CyA. The three methods correlate well but FPIA gives about 15–30% higher readings than RIA, EMIT, and HPLC (*Q190–Q192*). However, precision was excellent at 2–3%, making the FPIA assay for cyclosporine a very useful procedure for monitoring transplant patients. A very recent paper (*Q195*) reported positive bases of 23–40%, 12–31%, and 3–9% for FPIA, RIA, and EMIT, respectively, in comparison to HPLC, suggesting that EMIT is the most specific of the three immunological procedures.

APPLICATIONS OF FLUORESCENCE IN STUDIES INVOLVING NUCLEIC ACIDS

One of the fastest growing fields of laboratory testing is nucleic acid diagnostics. In these assays nucleic acids are used as targets and they are studied by using complementary DNA sequences labeled with a detectable moiety, e.g., a fluorochrome. When the base sequence of target and probe are complementary, perfect hybrids are formed which are

then detected through the label in the probe. In fluorescence in-situ hybridization (FISH), multiple fluorochromes have been used to visualize chromosomes 13, 18, 21, X, and Y with digital imaging microscopy (*Q196*). This approach allows evaluation of the most frequent aberrations in numbers of chromosomes, for diagnostic purposes. FISH has also been used to study the loss of a tumor suppressor gene in patients with breast cancer (*Q197*). Other applications dealing with localization, mapping, and distribution of DNA in situ are discussed in recent reviews with emphasis on tumor cytogenetics (*Q198–Q202*). FISH with probes labeled with biotin, digoxigenin, and fluorescein in the same hybridization experiment has been described (*Q203*). Fluorescence hybridization of chromosomes followed by flow cytometry was used for on-line screening of specific chromosomes and chromosome translocation (*Q204*). Two-color FISH was also used successfully to order sequences separated by >50 kilobase pairs for the construction of long-range maps of mammalian genomes (*Q205*).

Nucleic acid sequencing is an important technique which allows the elucitation of the base sequences of a nucleic acid of interest. Until recently, sequencing was exclusively performed using radioactive nuclides. In the last few years, powerful machines have emerged which can perform automated sequencing with use of fluorescent labels as probes (*Q206*). Currently, template preparation is usually done using variations of the polymerase chain reaction (*Q207*). A powerful combination of capillary gel electrophoresis with laser-induced fluorescence detection was used for sequencing DNA (*Q208*).

Double-stranded DNA has affinity for cationic fluorescent probes which are very useful for staining and quantification of DNA (*Q209*). Such methods have been used to quantify the DNA content of cell cultures (*Q210, Q211*) or in preliminary physical mapping experiments (*Q212*).

Southern blotting is a very useful method for studying nucleic acids, but until recently only radioactive nuclides were employed and the method was slow and cumbersome. Recently, efforts to devise alternative assays have focused on automated electrophoresis with fluorescence detection (*Q213, Q214*). The advent of polymerase chain reaction (PCR) simplified the Southern blot and alternative procedures by making available sufficient amounts of the DNA target (*Q215*). PCR products can be quantified by fluorescent techniques (*Q216*).

Fluorescence has also been used to visualize a plasmid DNA in bacterial cells (*Q217*), to study the expression of c-erbB-2 protein, a prognostic marker in breast and ovarian cancers (*Q218*), and for the study of the GTPase activity of the oncogene p21 *ras* (*Q219*).

In conclusion, it is clear that fluorescence will play a major role in nucleic acid diagnostics and especially in the areas of fluorescence in-situ hybridization, sequencing, Southern blot, and related techniques and in applications involving PCR and the quantification of PCR products.

DIAGNOSTIC APPLICATIONS OF FLUORESCENCE

Salmonella detection in food products or for clinical diagnosis was achieved by using particle concentration fluorescence immunoassay, a method claimed to be superior in comparison to enzyme immunoassay (*Q220*). Protein cross-linking due to nonenzymatic glycation produces compounds with characteristic fluorescence. These compounds, advanced glycation end products (AGEs), accumulate over many years and may be useful for determining the biological age of tissues (*Q221*). Studies with collagen from blood vessels have shown an increase in fluorescence of approximately 3.7% per year. The native fluorescence of protein tryptophans was used to quantify the degree of macromolecular vascular leakage (*Q222*). Chronically ischemic patients were successfully discriminated from normals by injecting fluorescein and monitoring its level with a dermofluorometer attached to the arm. In ischemic patients, fluorescein peak values occurred at 42 min as compared to 15 min in normals (*Q223*). The stone fluorescence spectrum proved to be useful in identifying the nature of gallstones in vitro (*Q224*). The incubation of blood samples with various (aminoacyl)-2-naphthylamines and determination of released 2-naphthylamine was used to determine the nature of the aminopeptidase present for diagnosing cirrhosis, pancreatitis, and hepatitis (*Q225*). The measurement of phospholipase A2 activity by a fluorometric technique was claimed to be useful for diagnosing schizophrenia (*Q226*). Retrovirus-related antibodies were detected in the serum of 60–80% of patients with leukemia, breast cancer, and other malignant tumors by using an immunofluorometric technique (*Q227*). The amplification of the erb-B-2 oncogene in breast cancer was detected by fluorescene in-situ hybridization (*Q228*). This assay may be a useful prognostic indicator in breast cancer. The pH of the interstitial fluid of malignant tumors tends to be lower than that of normal tissue and is depressed by glucose administration. Dual-wavelength fluorometry with a pH-dependent indicator was used to detect tumor area in vivo (*Q229*), based on differences in fluorescence intensity after glucose administration. Laser-induced fluorescence shows an interesting potential for clinical real-time tissue diagnostics (*Q230*). In cancer management, the localization of otherwise occult small tumors is an attractive possibility which could help in ensuring radical surgical tumor resection (*Q231*). Methods based on differences in tissue autofluorescence alone or in differences in uptake of systematically injected fluorophores are currently under investigation (*Q232–Q239*). Excitation and emission are sometimes monitored close to the tissue under study by using flexible catheters which exhibit other abilities as well, e.g., laser therapy of occluded coronaries due to arteriosclerosis. Multicolor fluorescence imaging techniques are currently under intense investigation (*Q240*). Photodynamic therapy is based on the selective accumulation of an injected agent in the tumor which is then destroyed by light irradiation (*Q241*). This technique can be combined with laser-induced fluorescence to achieve tumor localization and destruction.

Cancer detection by analysis in serum of protein p100, a neu-oncogene-related protein, using two monoclonal antibodies has also been proposed (*Q242*).

CONCLUSIONS

Flurorescence spectroscopy is growing rapidly and expanding to new areas of applications and research. Traditionally, fluorescence is used for analytical measurements, and various examples have been given which deal with clinically interesting analytes. Many new drugs have been measured for therapeutic drug monitoring and pharmacokinetic studies with HPLC coupled to fluorometric detection. Fluorescence immunoassay is one of a few technologies which is now used routinely as an alternative to radioisotopic immunoassay. Where sensitivity is not an issue, homogeneous techniques like fluorescence polarization immunoassay are highly successful. For assays demanding high sensitivity, heterogeneous time-resolved fluorescence immunoassay is used. This technique is among the most sensitive methodologies available today and is increasingly used in nucleic acid diagnostics as well. Cyclosporine assays, which are very important for monitoring immunosuppressed patients, are now carried out routinely with fluorescence polarization immunoassays. The use of fluorescence in nucleic acid-related applications is rapidly increasing. Of special interest are applications in fluorescence in-situ hybridization, automated sequencing, and nonisotopic Southern blot analysis and in methods involving the polymerase chain reaction. Another area of major importance is fluorescence-based diagnostics and especially tumor detection and tumor localization during surgery and fluorescence-based imaging. Clearly, fluorescence spectroscopy is expanding rapidly in traditional analytical areas and in new areas with great potential and practical usefulness.

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