

Fluorescent Bioprobes for Life Science Applications

ライフサイエンス分野に向けた蛍光バイオプローブの開発

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Bioprobes, and especially fluorescent bioprobes, have become essential tools for the detection and monitoring of important biological species and their related biological processes in live samples. Key applications of bioprobes fall into two main categories. The first one is bioimaging, from the cellular level, up to the organ and even small-animal level, *in-vitro* but also under *in-vivo* conditions. The second is biosensing, where probes are employed to detect various entities like ions, reactive oxygen species (ROS) or macromolecules, to help detect cancer, to observe drug internalization, and much more. This featured article will detail the typical characterization needs for the development and the use of the most common families of bioprobes: 1) determination of optical emission profiles; 2) measurement of size; 3) assessment of elemental composition or molecular signature. The article will showcase how HORIBA Scientific instruments are used by chemists all over the world to obtain rapid feedback on the best direction to follow to improve their synthesis processes.

key words

Fluorescence, Bioprobes, Sensing, Bioimaging

バイオプローブ、特に蛍光応答が得られる蛍光バイオプローブは、生体試料中の重要な生物種やその生体反応の検出と監視に不可欠な手法となっている。バイオプローブの主な適用分野は二つあり、まずバイオイメーjing(生体画像処理技術)分野では、細胞レベルから器官・小動物を対象に、インビトロ(試験管内)だけでなくインビボ(生体内)条件下でも用いられている。次にバイオセンシング(生体起源の分子認識機構を利用した化学的検出技術)分野があり、各種イオン、活性酸素(ROS)、高分子の検出に蛍光バイオプローブを用いることで、癌の検査や薬剤のインターナリゼーション(内部移行)の観察など多方面に応用されている。この特集論文では、広く用いられている何種類かのバイオプローブについて詳述し、分析ニーズの代表例について 1)発光特性の決定、2)大きさの計測、3)元素組成や分子シグネチャー(特徴的配列)評価 といった用途について紹介する。私たちHORIBAの科学分析機器が世界中の化学者の皆様にどのように使われて、皆様の分析プロセスの向上をもたらすベストの指針を提供しているかをお示したい。

キーワード

蛍光、バイオプローブ、センシング、バイオイメーjing

Introduction

Bioprobes are fluorophores, fluorescent chemical compounds that re-emit light upon light excitation, intended

for Life Sciences applications. Bioprobes can be called many things, tags, labels, sensors, reporters, or sensors, but they always refer to same thing. They are sometimes used alone, as tracers in fluids, as dyes for staining, as



Figure 1 General families of fluorescent bioprobes.

indicators (when their fluorescence is affected by environmental factors such as polarity, temperature, viscosity, or pH), or bound to macromolecules, serving as a marker for affinity or bioactive reagents.

Bioprobes are employed in a variety of domains from biomedical research to clinical applications and diagnostics. The first application is biosensing, which is the detection of biomolecules using an analytical device (or biosensor) combining a biological component with a detector. Small point-of-care devices used for diagnostics often rely on fluorescent bioprobes for their optical readout. This is also the case for devices intended for the monitoring of anthropogenic pollutants in various water matrices and aquatic environments.

Bioprobes are widely used to stain tissues, cells, or other biomaterials in a variety of analytical methods, including fluorescence imaging and spectroscopy. For instance, for *in-vivo* bioimaging in cancer research, to help with guided surgery in combination with other multimodalities. For that purpose, many groups are currently working on the development of near-infrared (NIR) emitting tags which are better adapted to deep-tissue imaging, taking advantage of reduced interference from photon absorption and scattering in this optical window.

High-content screening (HCS) systems used in the pharmaceutical domain for drug discovery also rely on fluorescent probes. The most common analysis workflow involves labeling proteins with fluorescent tags, while changes in cell phenotype are measured using automated image analysis in multiwell plates, in order to optimize synthesis processes or to confirm if a drug has the expected effect on its target.

Flow cytometry, which is used for biomarker detection, cell counting and sorting, or microorganism identification, is based on labels for measuring the physical properties of cells or particles. In most cases, fluorescent bioprobes with characteristic excitation and emissions peaks are designed to attach to a specific biological structure, for example a surface protein, to allow sorting cells

in a fluidics setup, based on the fluorescence measured in different detection channels.

A new and rapidly growing field is the clinical application of bioprobes for photodynamic therapy (PDT), which shows great promises for cancer treatment by killing cell with the heat produced locally by the particles upon light absorption.

Here, research focuses on finding dyes with properties like good biocompatibility, specific targeting, or strong two-photon absorption.

Bioprobes can be grouped into 3 main categories, as illustrated in Figure 1. Organic dyes (e.g., fluorescein, rhodamine), biological fluorophores (e.g., green fluorescent protein, phycoerythrin, allophycocyanin) and fluorescent nanostructures or nanomaterials (e.g. quantum dots, AIEgens).

Organic dyes are small molecules mostly synthesized through chemistry, but which can also be found in nature, like in plants. Common tags employed for confocal fluorescence microscopy are found in this category.

Biological fluorophores on the other hand are large molecules, typically fluorescent proteins, the most famous of all being GFP (Green Fluorescent Protein). DNA, which falls into this category does not have intrinsic fluorescence but can be used as biotags once coupled to a fluorophore.

Finally, we have nanomaterials that are engineered with specific chemical functions or tailored structures to generate unique light emission. This class of material encompasses a wide range of objects, like metallic nanoclusters, core-shell fluorophores, carbon dots, supramolecule assemblies and many more.

Characterization needs

Simultaneous Absorbance and Fluorescence

When studying novel bioprobes with unknown characteristics

or with emission properties impacted by solvent effects, having a complete fluorescence profile of a given material over a large range of wavelengths is key to avoid reperforming additional measurements at a later stage. An Excitation Emission Matrix (EEM), which is basically a three-dimensional scan that results in a contour plot of excitation wavelength vs. emission wavelength vs. fluorescence intensity, can provide such information (Figure 2).

Of importance as well when characterizing concentrated solutions (typically above 0.1 to 0.2 absorbance units), which is often the case when synthesizing bioprobes, is to account for the Inner Filter Effect (IFE). The IFE is a well-known phenomenon that distorts the measured fluorescence spectrum of a molecule due to absorption within the optical path length of the liquid sample, traditionally measured in a cuvette. IFE can significantly affect fluorescence emission spectral profiles, distorting their general shape, shifting the spectral position of the peak maximums, and decreasing the emission intensities, preventing from performing true quantitative analysis.

IFE can be corrected using absorbance data, and the HORIBA Duetta two-in-one spectrometer is unique in that it combines absorbance and fluorescence measurements on the same platform, allowing real-time correction for the IFE, on the same sample at the same time, as illustrated in Figure 2. It also takes advantage of an extended wavelength detection up to 1100 nm to cover the useful

NIR biological transparency window.^[1]

Quantum Yield

The fluorescence quantum yield (QY) is defined as the ratio of photons emitted to photons absorbed by a fluorophore, basically an indicator of its brightness. This parameter is crucial for fast bioimaging consideration, as samples are often light sensitive or present rapid time kinetics.

A reliable method for recording the QY is a comparative method which involves the use of well characterized reference standards with known properties.

A second approach compatible with liquid samples is based on lifetimes and different concentrations of a

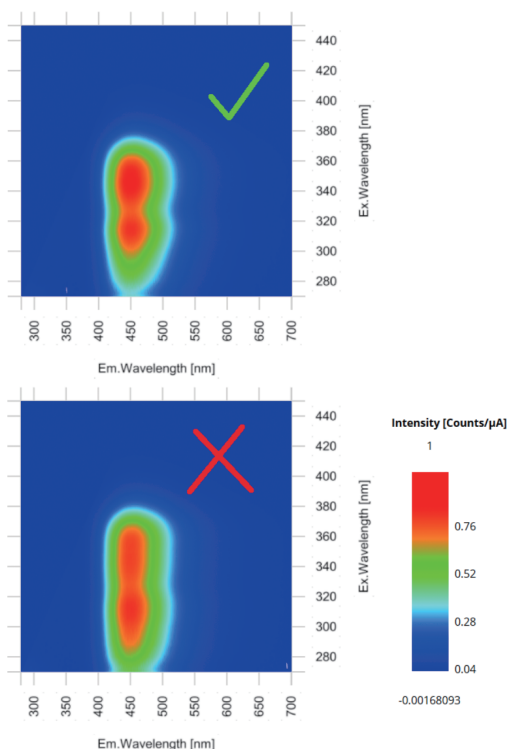


Figure 2 Fluorescence EEM profiles obtained with a HORIBA Duetta spectrometer, with (top) and without (bottom) inner filter effect correction.



Figure 3 Quanta-φ integrating sphere coupled to a FluoroMax-4 spectrometer by HORIBA for quantum field determination.

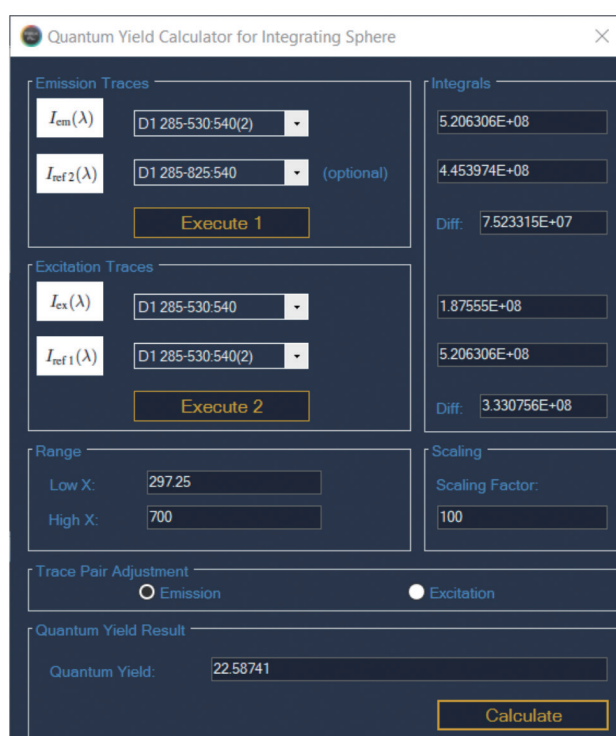


Figure 4 Interface window of the FelixFL software on the HORIBA Fluorolog-QM spectrometer with integrated quantum yield calculator.

quencher to calculate the QY of a molecule in accordance with the Stern-Volmer equation. HORIBA customers from the Swiss Federal Laboratories for Materials Science and Technology (EMPA) recently reported on this method for their work on polyimide-based photoemitters for oxygen biosensing.^[2]

However, the most accepted method to determine the absolute QY is to employ an integrating sphere (Figure 3), a sphere coated with an all-reflective surface to capture all the light going in and out.

A recent example of the use of the HORIBA Quanta- ϕ integrating sphere by scientists from the University of Chile explains the synthesis of an anthracene derivative dye with solvatochromic properties and highlights the importance of designing bright bioprobes for microscopy experiments on living cells.^[3] Accurate quantum yield determination can be made easier using a dedicated application embedded in most HORIBA software, as seen in Figure 4.

Lifetime

The fluorescence lifetime is a photophysical property of bioprobes, providing complementary information to the simple steady-state monitoring of fluorescence intensity. The technique has gained popularity thanks to its high sensitivity to microenvironmental parameters and changes in molecular conformation such as temperature, viscosity, pH, and ion concentration. At the same time, lifetime measurements are largely independent of fluorophore concentration and do not require calibration steps like intensity-based experiments.

Förster Resonance Energy Transfer (FRET) sensors,

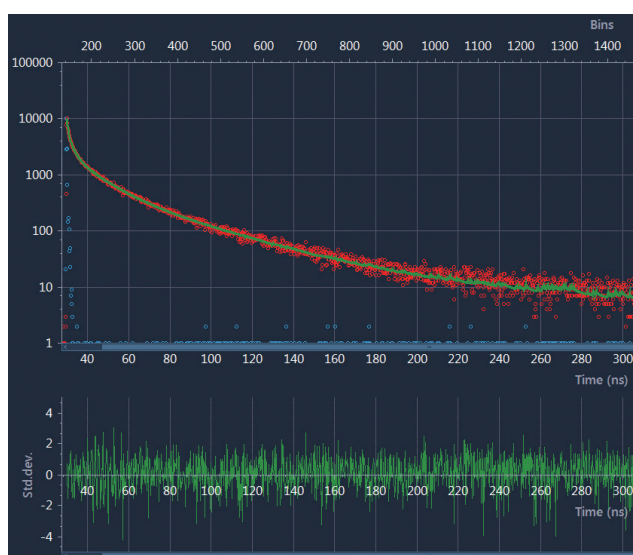


Figure 5 Lifetime decay (red) and fit (green) to a 5-exponential decay equation, expressed in photon counts. The lower graph shows the standard deviation error residuals from the fit to the fitted data.

which are widely used to monitor protein-protein interactions to elucidate cellular signaling, disease progression and drug efficacy, also benefit from a lifetime monitoring, especially for *in-vivo* applications. Figure 5 illustrates the lifetime decay for a solution of Qtracker 655 quantum dots, collected on a HORIBA DeltaPro spectrometer equipped with a photomultiplier and a DeltaDiode485L pulsed laser source.

Polarization

Fluorescence anisotropy or fluorescence polarization is a measurement of the changing orientation of a molecule in space, with respect to the time between the absorption and emission events. Anisotropy is an excellent tool for understanding changes in macromolecule shape, as well as molecular binding.

Figure 6. exemplifies the confirmation of effective binding between an antibody and the bronchodilator drug Theophylline labeled with a fluorescein dye and mixed with an antiserum in pH 7.2 buffer, then incubated for 3 minutes. The free and the complexed drug were distinguished through polarization measurements.

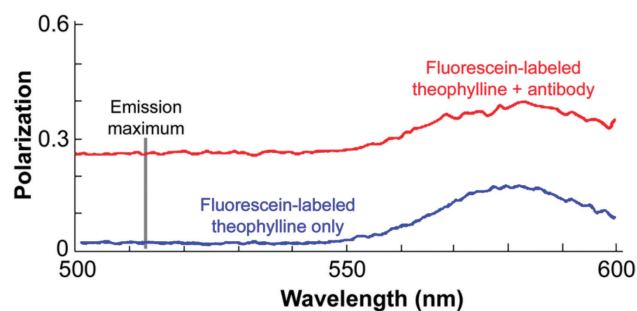


Figure 6 Polarization emission scan for labeled theophylline with (red) and without (blue) antibody ($\lambda_{exc} = 485$ nm). The emission maximum for fluorescein-labeled theophylline is marked by a gray line.

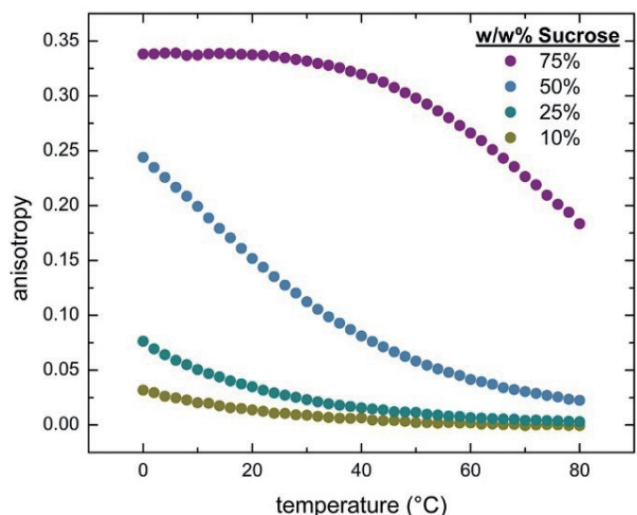


Figure 7 Fluorescein dissolved in sucrose solutions. With increased temperature, viscosity decreases, yielding faster rotation times and lower anisotropies.

Figure 7 shows additional polarization measurement of fluorescein dye dissolved in four aqueous solutions, performed with a HORIBA Fluorolog-QM spectrometer.

This indirect measure of the local viscosity gives information on sample aggregation, structural changes, molecular binding, and other mechanisms.

Bioimaging

Fluorescent bioprobes, especially small organic molecules, have become indispensable tools in modern biology. For research on living cells, these probes need to fulfill specific requirements like high permeability and good intracellular solubility, good affinity and specificity for the target, photostability, as well as low toxicity.

Microscopes with epifluorescence illumination are routinely used to observe the penetration and distribution of labelled targets within cell organelles to provide information on their structure and functions. Figure 8 illustrates

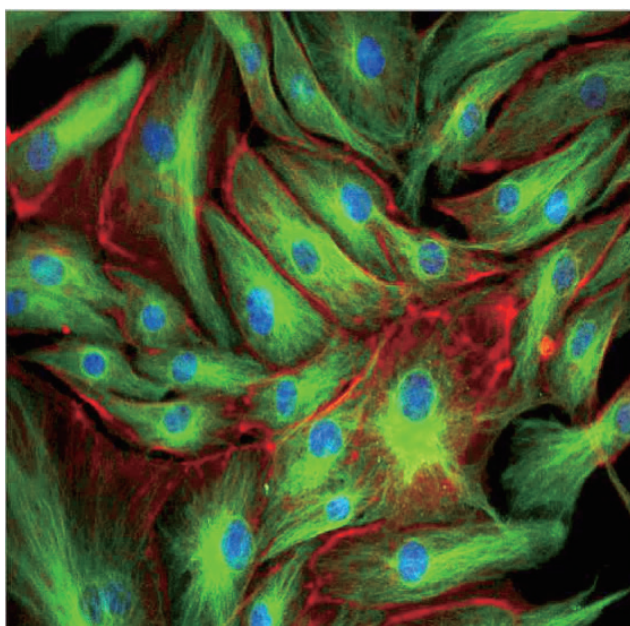


Figure 8 Epifluorescence imaging of Bovine Pulmonary Artery Endothelial cells (BPAE Line) with Anti- α -Tubulin antibody, stained with BODIPY, Texas Red and DAPI fluorophores, using a HORIBA XploRA microscope.

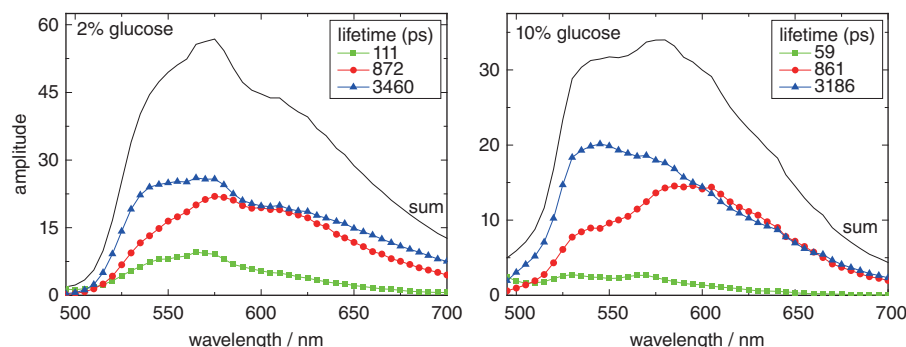


Figure 9 Left: Decay associated spectra for the FUN-1 dye in yeast cells grown in different growth media, also showing the sum spectrum that relates to the steady state emission. Right: FLIM measurement of DASPMI stained *Saccharomyces cerevisiae* in a polysaccharide polymer grown.

cell staining with multiple probes, Texas Red as a marker of proteins, DAPI as an indicator of membrane viability, and BODIPY to reveal neutral lipids.

The influence of environmental parameters on intra- and intermolecular interactions at the cellular level can also be obtained with microscopy instruments by monitoring the fluorescence lifetime of fluorophores instead of their intensity.

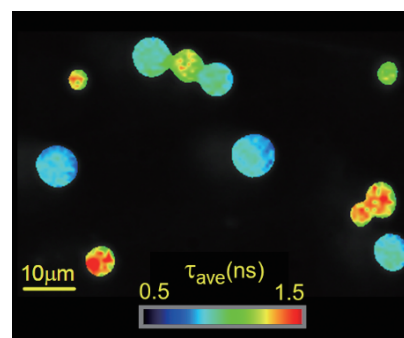
HORIBA now offers a new concept of wide field imaging, called FLIMera, capable of video-rate lifetime determination, based on time-correlated single photon counting technique (TCSPC) realized independently in each pixel.^[4]

Figure 9 highlights research done with the FLIMera camera by HORIBA application teams in collaboration with Glasgow Caledonian University (UK). *Saccharomyces cerevisiae*, a single-celled fungus microorganism, was stained with a viscosity sensitive mitochondrial fluorescent probe called DASPMI to assess its viability. The cells displaying a longer average lifetime for DASPMI are associated with cells having a higher microviscosity, which is indicative of cell death (Figure 9 Right).

In addition, the decay associated spectra were determined for yeast cells suspended in growth medium, to investigate the photophysics of the fluorescence emission (Figure 9 Left). The use of the two-color FUN-1 stain, which transports into cell vacuoles, revealed the condition of plasma membranes through the formation of cylindrical intravacuolar structures.^[5]

Research based on HORIBA instruments

HORIBA Scientific is proud to count world-leading research teams among its customers. This following section will highlight a selection, far from exhaustive, of their recent publications in the field of bioprobes.



Photodynamic therapy

Photodynamic therapy refers to treatments which involve light-sensitive therapeutics which can be switched “on” with a light source to destroy abnormal cells and treat various types of diseases.

The development of multifunctional bioprobes for simultaneous specific near-infrared (NIR) imaging and phototherapy of tumors being of great significance. For this purpose, our Fluoromax-4 spectrometer users at the City University of Hong Kong (China) reported on the integration of protoporphyrin IX (PpIX) into nanoscale metal-organic frameworks (NMOFs).^[6]

Another example can be found in a recent publication in the journal *Nanomedicine: Nanotechnology, Biology and Medicine*, where a fluorophore called indocyanine green was co-encapsulated with a CT (Computed Tomography) contrast agent within nanoliposomes for imaging guided treatment. For this work, a Fluorolog-3 instrument from HORIBA recorded the fluorescence spectra in the NIR range and helped with the formulation of the particles.^[7]

Bioluminescence

Recently, our European laboratory, based South of Paris (France) on the Paris-Saclay campus, was closely involved in research with the famous Institut Pasteur.^[8] Time-dependent chemiluminescence properties of core-modified coelenterazine luciferin analogues were recorded in the range of 250 to 800 nm using a HORIBA Aqualog spectrometer. These small molecules generated in bioluminescent organisms show great promise for bioimaging and therapeutics, by eliminating the need for external optical illumination and avoiding autofluorescence background in existing fluorescence techniques.

A recent publication highlights the application of such probes for the detection and imaging of peroxynitrite, a reactive nitrogen species (RNS), in live cells and mice *in-vivo*.^[9] A HORIBA Fluoromax-4 spectrofluorometer was used to measure the bioluminescence emission of the synthesized compounds.

Ion sensing

As detailed in the introduction, a major application for bioprobes is biosensing, especially to monitor inorganic ions which are essential for cellular activity.

Researchers from the College of Pharmacy at Seoul National University (Korea) have developed novel fluorescence cellular iron ions sensors to better understand cell death processes.^[10] Their work required advanced characterization tools. A HORIBA FluoroMax-4 spectrometer was used to study the fluorescence response of their che-

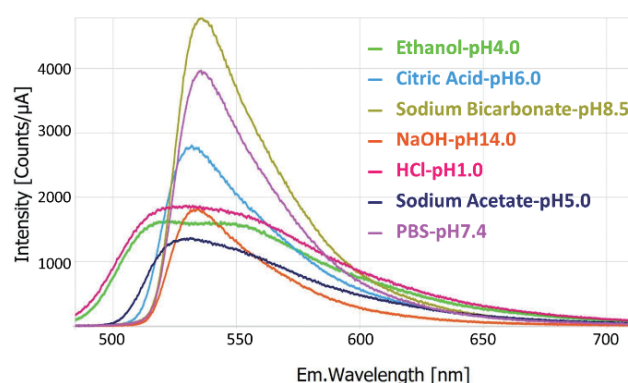


Figure 10 Fluorescence spectra of the widely used FITC dye (Fluorescein isothiocyanate) in different solvents and under varying pH conditions.

mosensors in the presence of various metal ions. The brightness of the probes was assessed by measuring the absolute quantum yield with an integrating sphere assembly. Finally, kinetics information was obtained through fluorescence decay curves and analyzed using the DAS6 decay analysis software.^[11]

Another group from the College of Chemistry and Materials Science from Nanning (China) proposed an efficient fluorescent sensor to detect Ag⁺ but also L-cysteine in complex biological fluids and living cells using nitrogen, boron, sulfur co-doped carbon dots. The fluorescence emission profile, quantum yield, as well as lifetime of those nanostructures were determined using a HORIBA QM-8075 QuantaMaster instrument.^[12]

Microenvironment

Bioprobe fluorescence spectra are very sensitive to local environment, including solvent polarity, pH and temperature. This sensitivity is often leveraged in cell physiology research as many processes (metabolism, cell proliferation, membrane potential, etc.) are affected by changes in these parameters. Figure 10 shows the evolution of FITC emission properties under different pH conditions.

Besides pH, other local environmental factors can be assessed with specific tailored probes. Scientists from the National Academy of Sciences of Belarus have for instance designed carboxyfluorescein bifluorophores as viscosity sensors in model lipid bilayers or internal membranes of live cells.^[13] Indeed, viscosity is one of the key parameters that controls the diffusion rate of molecular species and affects the reaction speeds of diffusion-controlled processes at the microscopic scale.

For their research, they have used a HORIBA Fluorolog-3 spectrometer to measure the fluorescence spectra and degree of polarization of the molecules.

A focus on quantum dots

An important class of fluorescent nanomaterials is nanoparticles of semiconductors called quantum dots or QDs. QDs are artificially designed nanostructures which display unique emission properties depending on their composition and shape. They offer several advantages over organic fluorophores, among them brighter emission, photostability, broader excitation spectra combined with sharper emission peaks to avoid signal overlap for multi-color labelling.

As quantum objects, QDs emission wavelengths directly relates to their dimension (Figure 11. a), therefore controlling their size at different steps of their production is key. Using a HORIBA SZ-100 analyzer relying on dynamic light scattering (DLS), CdTe QDs with a passivation coating layer were measured in aqueous solution in a microcuvette and revealed a monodisperse distribution centered around 9.9 nm with a standard deviation of 3.4 nm (Figure 11. d)

Because of their brightness, QDs emission profiles can readily be measured with any spectrofluorometer.

However, we have seen before that due to inner filter effects (IFE), the fluorescence of bioprobes may not coincide with the true fluorescence emission, even for low concentrations.^[14] This is visible in Figure 11.b where InP/ZnS QDs were measured with the HORIBA Duetta instrument capable of simultaneous IFE correction. Besides the obvious difference in fluorescence intensity, we see that the position of the peak maximum is blue shifted by 15 nm.

Apart from controlling the expected optical emission properties, verifying the elemental composition of synthesized or purchased QDs is important to validate chemical synthesis routes, but also for safety and ecological consideration. This information can be easily obtained by using X-Ray Fluorescence (XRF) spectroscopy. Figure 11. c. shows a XRF spectrum of commercially available CdTe QDs functionalized with an organosulfur compound, characterized under solid form with a HORIBA XGT-9000 analyzer.

Typical QDs have the disadvantage of showing potential cytotoxicity for biomedical applications, as their core-

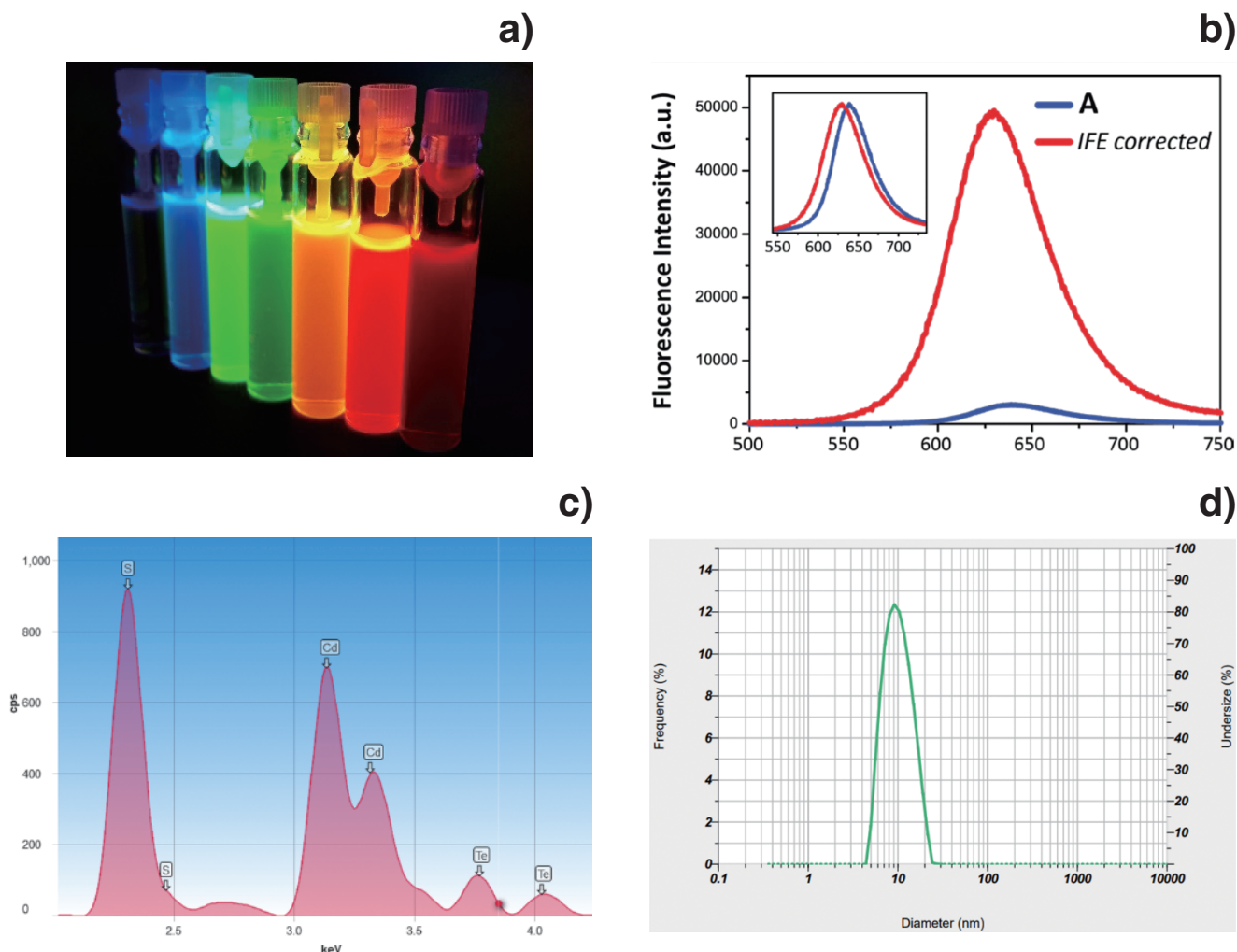


Figure 11 a) QDs of different sizes showing different emission colors. b) QDs fluorescence emission profile extracted from an Excitation Emission Matrix. c) QDs elemental composition determined by X-Ray Fluorescence spectroscopy. d) QDs size distribution obtained by dynamic light scattering.

shell structures often contain heavy metals in the form of binary chemical compounds such as lead sulfide (PbS), cadmium selenide (CdSe), gallium arsenide (GaAs), or zinc telluride (ZnTe), which can generate reactive oxygen species, which in turn damage cellular proteins, lipids, and DNA. Therefore, significant efforts have recently been made to find alternative biocompatible materials.

Such a structure, with low toxicity and chemical inertness can be found in the form of carbon dots. Researchers from the University of Madras (India) recently reported on carbon dot nanocomposites conjugated with chitosan, acting both as a nano-drug carrier (for dopamine encapsulation in this case) and as a delivery tracer through bioimaging.^[15]

Besides photoluminescence characterization of the probes with a HORIBA Fluorolog-3 spectrometer, the authors also employed Raman spectroscopy with a HORIBA LabRAM HR microscope to confirm the intensity ratio of the D band (1378 cm⁻¹) to the G band (1580 cm⁻¹) to control the presence of defects or amorphous carbon.

Another approach to avoid QDs toxicity is to encapsulate them in order to prevent their degradation when internalized, and to control their release at the right target. Our FluoroMax user at the University of Leeds (UK) proposed “sweet” QDs capped with mannose, (a sugar monomer important in human metabolism), to probe multivalent interactions of HIV/Ebola receptors using a sensitive, ratiometric FRET assay. The detection scheme is based on the fluorescence intensity ratio measured at wavelengths of 626 nm and 554 nm.^[16]

Conclusion

We have seen that fluorescence bioprobes have become tools of choice for various applications in bioimaging and biosensing.

HORIBA Scientific offers a comprehensive suite of analytical tools capable of covering the variety of characterization needs required for the design and the optimization of chemical synthesis routes of such novel probes.

* Editorial note: This content is based on HORIBA’s investigation at the year of issue unless otherwise stated.

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