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Advanced Fluorescence Reporters in Chemistry and Biology II

Molecular Constructions, Polymers and Nanoparticles

Volume Editor: Alexander P. Demchenko

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Aims and Scope

Fluorescence spectroscopy, fluorescence imaging and fluorescent probes are indispensible tools in numerous fields of modern medicine and science, including molecular biology, biophysics, biochemistry, clinical diagnosis and analytical and environmental chemistry. Applications stretch from spectroscopy and sensor technology to microscopy and imaging, to single molecule detection, to the development of novel fluorescent probes, and to proteomics and genomics. The *Springer Series on Fluorescence* aims at publishing state-of-the-art articles that can serve as invaluable tools for both practitioners and researchers being active in this highly interdisciplinary field. The carefully edited collection of papers in each volume will give continuous inspiration for new research and will point to exciting new trends.

Preface

A variety of fluorescent and luminescent materials in the form of molecules, their complexes, and nanoparticles are available for implementation as reporting units into sensing technologies. Increasing demands from these application areas require development of new fluorescence reporters based on association and aggregation of fluorescence dyes and on their incorporation into different nanostructures. Interactions between these dyes and their incorporating matrices lead to new spectroscopic effects that can be actively used for optimizing the sensor design. One of these effects is a spectacular formation of J-aggregates with distinct and very sharp excitation and emission bands. By incorporation into nanoparticles, organic dyes offer dramatically increased brightness together with improvement of chemical stability and photostability. Moreover, certain dyes can form nanoparticles themselves so that their spectroscopic properties are improved. Semiconductor quantum dots are the other type of nanoparticles that possess unique and very attractive photophysical and spectroscopic properties. Many interesting and not fully understood phenomena are observed in clusters composed of only several atoms of noble metals. In conjugated polymers, strong electronic conjugation between elementary chromophoric units results in dramatic effects in quenching and in conformation-dependent spectroscopic behavior.

Possessing such powerful and diverse arsenal of tools, we have to explore them in novel sensing and imaging technologies that combine increased brightness and sensitivity in analyte detection with simplicity and low cost of production. The present book overviews the pathways for achieving this goal. In line with the discussion on monomeric fluorescence reporters in the accompanying book (Vol. 8 of this series), an insightful analysis of photophysical mechanisms behind the fluorescence response of composed and nanostructured materials is made. Based on the progress in understanding these mechanisms, their realization in different chemical structures is overviewed.

Demonstrating the progress in an interdisciplinary field of research and development, this book is primarily addressed to specialists with different background – physicists, organic and analytical chemists, and photochemists – to those who develop and apply new fluorescence reporters. It will also be useful to specialists in bioanalysis and biomedical diagnostics.

Kyiv, Ukraine Alexander P. Demchenko
June 2010

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Part I
General Aspects

Nanocrystals and Nanoparticles Versus Molecular Fluorescent Labels as Reporters for Bioanalysis and the Life Sciences: A Critical Comparison

Ute Resch-Genger, Markus Grabolle, Roland Nitschke, and Thomas Nann

Abstract At the core of photoluminescence techniques are suitable fluorescent labels and reporters, the spectroscopic properties of which control the limit of detection, the dynamic range, and the potential for multiplexing. Many applications including recent developments in intracellular labeling rely on well established molecular chromophores such as small organic dyes or fluorescent proteins. However, one of the most exciting – but also controversial – advances in reporter technology, the emerging development and application of luminescent nanoparticles with unique optical properties, yet complicated surface chemistry paves new roads for fluorescence imaging and sensing as well as for in vitro and in vivo labeling. Here, we compare and evaluate the differences in physico-chemical properties of common fluorophores, focusing on traditional organic dyes and luminescent nanocrystals with size-dependent features. The ultimate goal is to provide a better understanding of the advantages and limitations of both classes of chromophores, facilitate fluorophore choice for users of fluorescence techniques, and address future challenges in the rational design and manipulation of nanoparticulate labels and probes.

Keywords Amplification · Fluorescent reporter · Fluorophore · FRET · In vitro · In vivo · Labeling · Lanthanide chelate · Multiplexing · Nanoparticle · Quantum dot · Transition metal complex

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Contents

1 Introduction

The investigation of many fundamental processes in the life sciences requires straightforward tools for the fast, sensitive, reliable, and reproducible detection of the interplay of biomolecules with one another and with various molecular or ionic species. One of the best suited and most popular methods to meet these challenges presents the use of photoluminescence or fluorescence techniques in conjunction with functional dyes and labels [1–3]. Advantages of fluorescence methods, which range from fluorescence spectroscopy over fluorescence microscopy and flow cytometry to in vivo fluorescence imaging, include the comparatively simple measurement of a number of unique experimental parameters (excitation wavelength, emission wavelength, intensity/quantum yield, fluorescence lifetime, and emission anisotropy) with nanometer scale resolution and possible sensitivity down to the single molecule level [4]. The potential of these methods, e.g., the achievable sensitivity (detection limit), the dynamic range, and the number of emissive species to be distinguished or detected simultaneously (multiplexing capability), is controlled by the physico-chemical properties of the fluorescent reporter(s) employed. Generally, a suitable label or reporter must be (1) conveniently excitable, without excitation of the (biological) matrix, and detectable with conventional instrumentation; (2) bright, i.e., possess a high molar absorption coefficient at the excitation wavelength and a high fluorescence quantum yield; (3) soluble in application-relevant media such as buffers, cell culture media, or body fluids; and (4) thermally and photochemically stable under relevant conditions. (5) For site-specific labeling, functional groups, often in conjunction with spacers, are beneficial. Depending on

the desired application, additional important considerations should include (6) the luminescence lifetime of the label, e.g., for suitability for time-gated emission, lifetime sensing or fluorescence lifetime multiplexing [5] (7) steric and size-related effects, (8) the sensitivity of the chromophore's optical properties to its microenvironment including the interplay between the chromophore and the biological unit, (9) the possibility of delivering the fluorophore into cells, and (10) potential toxicity and biocompatibility. Similarly relevant are (11) the suitability for multiplexing and (12) compatibility with signal amplification strategies such as Förster resonance energy transfer (FRET) [6] in antennae-type systems or controlled aggregation approaches [7]. Crucial for the eventually desired application for routine analysis is (13) the reproducibility of the reporter's synthesis and chemical modification (binding to biomolecules, surface functionalization in the case of particles, etc.) in conjunction with the availability of simple and evaluated characterization procedures [1]. In this respect, reported photophysics of the chromophore can also be beneficial.

There is an ever increasing toolbox of fluorescent labels and reporters to choose from: (1) molecular systems with a defined, yet versatility tunable chemical structure like small organic dyes [1, 2], metal–ligand complexes (MLC) such as $[Ru(bpy)_3]^{2+}$ [8, 9], and lanthanide chelates [10–12] as well as fluorophores of biological origin like phycobiliproteins and genetically encoded fluorescent proteins [3, 13], (2) nanocrystal labels with size-dependent optical and physico-chemical properties which includes quantum dots (QDs) made from II/VI and III/V semiconductors [1, 14], carbon [15] and silicon nanoparticles [16] as well as luminescent metal particles and clusters [17], self-luminescent organic nanoparticles [18], and (3) nanometer-sized upconversion phosphors as a new class of evolving inorganic nanocrystal labels with promising, partly size-dependent spectroscopic features composed of a crystalline host doped with emissive lanthanide ions (localized luminescent centers) [19]. (4) All these chromophores can be incorporated into nanometer- to micrometer-sized inorganic and organic polymeric particles, yielding multichromophoric particulate labels [20, 21].

In this chapter, we compare and evaluate the differences in physico-chemical properties and application-relevant features of organic dyes as the most versatile molecular labels and nanocrystal labels, thereby focusing on QDs made from II/VI and III/V semiconductors, which are the most frequently-used nanocrystal labels in bioanalytics or medical diagnostics. The discussion of many of the properties of organic dyes, such as their photophysics, is similarly relevant for fluorescent proteins. The spectroscopic properties of metal–ligand and lanthanide complexes, that are commonly employed only for specific applications, e.g., in fluoroimmunoassays or certain sensor systems as well as phosphorescence emitters and components in bio- and chemoluminescent systems, are only briefly reviewed, thereby providing the basis for judging their advantages and limitations in comparison to organic dyes and semiconductor QDs. Their applications are not further detailed here. This is similarly true for carbon and silicon nanoparticles, metal nanoparticles, and clusters, as well as for nanometer-sized upconverting phosphors, that are only currently becoming more prominent in the field of biological assays as well as

medical diagnosis and imaging. Increasingly used chromophore-doped particle labels (4) and materials based on conjugated polymers [22] are beyond the scope of this review. The optical properties of such chromophore-doped particles are controlled by the parent chromophores or dopants, and the surface modification and labeling strategies presented here for the QDs labels can also be typically applied to these systems.

2 Properties of Molecular and Nanoparticular Labels and Reporters

2.1 Spectroscopic Properties

The relevant spectroscopic features of a chromophore include the spectral position, width (FWHM: full width at half height of the maximum), and shape of its absorption and emission bands, the Stokes shift, the molar absorption coefficient (ε_M), and the photoluminescence efficiency or fluorescence quantum yield (Φ_F). The Stokes shift equals the (energetic) difference (in frequency units) between the spectral position of the maximum of the lowest energy absorption band (or the first excitonic absorption peak in the case of QDs) and the highest energy maximum of the luminescence band. This quantity determines the ease of separation of excitation from emission and the efficiency of emission signal collection. It can also affect the degree of spectral crosstalk in two- or multi-chromophore applications such as FRET or spectral multiplexing and the amount of homo-FRET (excitation energy transfer between chemically identical chromophores) occurring, e.g., in chromophore-labeled (bio)macromolecules that can result in fluorescence quenching at higher labeling densities [23, 24]. The product of ε_M at the excitation wavelength (λ_{ex}) and Φ_F, that is termed brightness (B), presents a frequently used measure for the intensity of the fluorescence signal obtainable upon excitation at a specific wavelength or wavelength interval and is thus often used for the comparison of different chromophores. A value of B below 5,000 M^{-1} cm^{-1} renders a label practically useless for most applications [25]. Further exploitable chromophore properties include the luminescence or fluorescence lifetime (τ_F), that determines, e.g., the suitability of a label for time-gated emission [4], time-resolved fluorescence immunoassays [26–28], and lifetime multiplexing [5], and the emission anisotropy or fluorescence polarization. The latter quantity, that presents a measure for the polarization of the emitted light, reflects the rotational freedom or mobility of a chromophore in the excited state and provides information on the orientation distributions of fluorescent moieties or on the size of molecules (hydrodynamic radius) via the measurement of the rotational correlation time [4]. This can be exploited, e.g., for the study of enzyme activity, protein–peptide and protein–DNA interactions, and ligand–receptor binding studies in homogeneous solution.

2.1.1 Luminescent Nanocrystals and Nanoparticles

The most prominent nanomaterials for bioanalysis at present are semiconductor QDs. Rare-earth doped upconverting nanocrystals and precious metal nanoparticles are becoming increasingly popular, yet they are still far from reaching the level of use of QDs. Other luminescent nanoparticles like carbon-based nanoparticles start to appear, but the synthesis and application of these materials are still in their infancy and not significant for practitioners in the field of bioanalysis.

The photoluminescence of these nanoparticles has very different causes, depending on the type of nanomaterial: semiconductor QDs luminescence by recombination of excitons, rare-earth doped nanoparticles photoluminescence by atom orbital (AO) transitions within the rare-earth ions acting as luminescent centers, and metallic nanoparticles emit light by various mechanisms. Consequently, the optical properties of luminescent nanoparticles can be very different, depending on the material they consist of.

The optical properties of semiconductor QDs (Fig. 1a–c, Tables 1 and 2) are controlled by the particle size, size distribution (dispersity), constituent material, shape, and surface chemistry. Accordingly, their physico-chemical properties depend to a considerable degree on particle synthesis and surface modification. Typical diameters of QDs range between 1 and 6 nm. The most prominent optical features of QDs are an absorption that gradually increases toward shorter

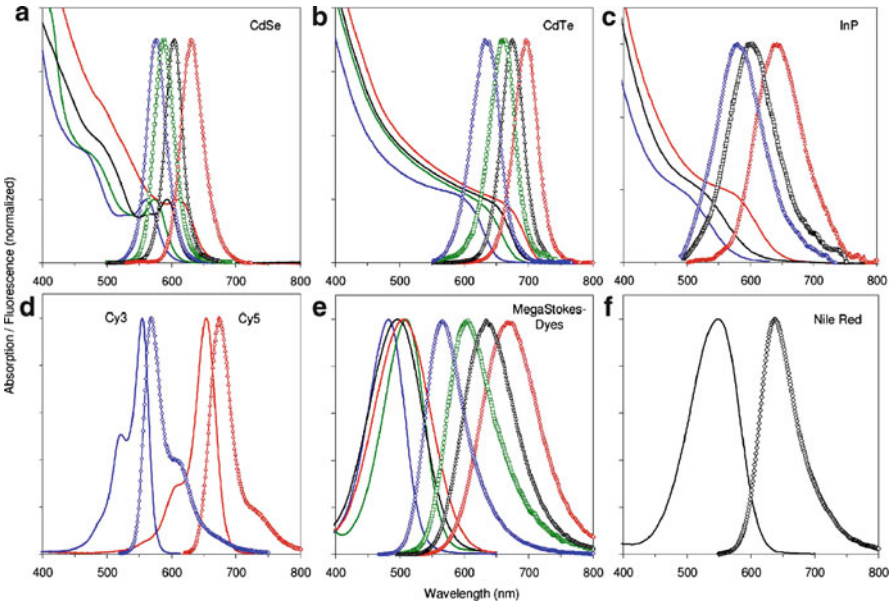


Fig. 1 Spectra of QDs and organic dyes. Absorption (*lines*) and emission (*symbols*) spectra of representative QDs (**a**–**c**) and organic dyes (**d**–**f**). Reprinted by permission from Macmillan Publishers Ltd: Nature Methods [1], copyright (2008)

Table 1 Spectroscopic properties of labels and reporters

	Organic dye	Semiconductor quantum dot
Absorption spectra	Discrete bands, FWHM[a] 35 nm[b] to 80–100 nm[c]	Steady increase toward UV starting from absorption onset, enables free selection of excitation wavelength
	Examples[d] (λ_{abs}/FWHM)	
	Nile Red: 552 nm/90 nm (MeOH)	*CdSe*: 450–640 nm/-
	Cy3: 550 nm/33 nm (phosphate buffer)	*CdTe*: 500–700 nm/-
	Alexa750: 749 nm/55 nm (phosphate buffer)	*PbSe*: 900–4000 nm/-
	IR125: 782 nm/62 nm (MeOH)	*$CuInS_2$*: 400–900 nm/-
Molar absorption coefficient	2.5×10^4–2.5×10^5 M^{-1} cm^{-1} (at long wavelength absorption maximum)	10^5–10^6 M^{-1} cm^{-1} at first exitonic absorption peak, increasing toward UV, larger (longer wavelength) QDs generally have higher absorption
	Examples	
	Nile Red: 4.5×10^4 M^{-1} cm^{-1}(MeOH)	*CdSe*: 1.0×10^5 (500 nm) –7.0×10^5 (630 nm) M^{-1} cm^{-1}
	Cy3: 1.5×10^5 M^{-1} cm^{-1} (phosphate buffer)	*CdTe*: 1.3×10^5 (570 nm) –6.0×10^5 (700 nm) M^{-1} cm^{-1}
	Alexa750: 2.4×10^5 M^{-1} cm^{-1}(phosphate buffer)	*PbSe*: 1.23×10^5 M^{-1} cm^{-1} (chloroform)
	IR125: 2.1×10^5 M^{-1} cm^{-1}(MeOH)	*$CuInS_2$*: *n. d.*
Emission spectra	Asymmetric, often tailing to long-wavelength side, FWHM 35 nm[b] to 70–100 nm[c]	Symmetric, Gaussian-profile, FWHM 30–90 nm
	Examples (λ_{em}/FWHM)	
	Nile Red: 636 nm/75 nm (MeOH)	*CdSe*: 470–660 nm/~30 nm
	Cy3: 565 nm/34 nm (phosphate buffer)	*CdTe*: 520–750 nm/35–45 nm
	Alexa750: 775 nm/49 nm (phosphate buffer)	*PbSe:* >1,000 nm/80–90 nm
	IR125: 528 nm/58 nm (MeOH)	$CuInS_2$: 500–1,000 nm/ 70–150 nm
Stokes shift	Normally <50 nm[b], up to >150 nm[c]	Typically <50 nm for vis-emitting QDs
	Examples	
	Nile red: 84 nm (MeOH)	*CdSe*: 15–20 nm
	Cy3: 15 nm (phosphate buffer)	*CdTe*: 30–40 nm
	Alexa: 26 nm (phosphate buffer)	*PbSe*: 60–80 nm
	IR125: 44 nm (MeOH)	$CuInS_2$: ~100 nm
Quantum yield	0.5–1.0 (vis), 0.05–0.25 (NIR)	0.1–0.8 (vis), 0.2–0.7 (NIR)
	Examples	
	Nile Red: 0.7 (dioxane)	*CdSe*: 0.65–0.85
	Cy3: 0.04 (phosphate buffer)	*CdTe*: 0.3–0.75
	Alexa: 0.12 (phosphate buffer)	*PbSe*: 0.12–0.81
	IR125: 0.04 (MeOH)	*$CuInS_2$*: 0.2–0.3
Fluorescence lifetimes	1–10 ns, monoexponential decay	10–100 ns, typically multiexponential decay
Solubility/ dispersibility	Control by substitution pattern	Control via surface chemistry (ligands)

(*continued*)

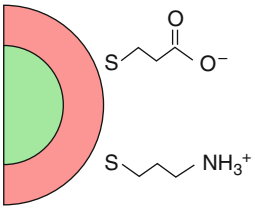
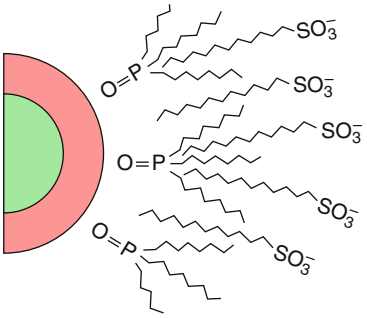
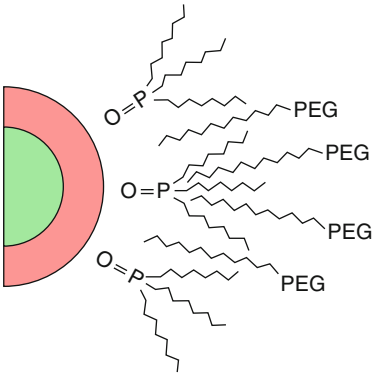
Table 1 (continued)

	Organic dye	Semiconductor quantum dot
Binding to biomolecules	Via functional groups following established protocols, often binding of several dyes to single biomolecule, labeling-induced effects on spectroscopic properties of reporter studied for many common dyes	Via ligand chemistry, only few protocols available, binding of several biomolecules to single QD, very little information on labeling-induced effects
Size	~0.5 nm	1–6 nm
Thermal stability	Dependent on dye class, can be critical for NIR-dyes	High, depends on shell/ligands
Photochemical stability	Sufficient for many applications (vis), but can be critical for high-light flux applications (e.g., fluorescence microscopy), often problematic for NIR dyes	High (vis and NIR), orders of magnitude that of organic dyes, can reveal photobrightening
Toxicity	From very low to high, dependent on dye	Little known yet (heavy metal leakage to be prevented, nanotoxicity)
Reproducibility of labels (optical, chemical properties)	Good, due to defined molecular structure and established methods of characterization, available from commercial sources	Limited by complex structure and surface chemistry, limited data available, few commercial systems available, often individual solutions
Single-molecule capability	Moderate, limited by photobleaching	Good, limited by blinking
FRET	Well described FRET pairs, mostly single donor–single acceptor configurations, enables optimization of reporter properties	Few examples, single donor–multiple acceptor configurations possible, limitation of FRET efficiency due to nanometer-size of QD-coating
Spectral multiplexing	Possible, 3 colors (MegaStokes dyes), 4 colors (energy-transfer cassettes)	Ideal for multicolor experiments, up to 5 colors demonstrated
Lifetime multiplexing	Possible	Possible
Signal amplification	Established techniques	Unsuitable for many enzyme-based techniques, other techniques remain to be adapted and/or established

[a]FWHM: full width at half height of the maximum
[b]Dyes with resonant emission like fluoresceins, rhodamines, cyanines (see section 3.3)
[c]CT dyes (see section optical properties, organic dyes)
[d]Spectroscopic data taken from [29–33]; data for Alexa750 provided by Invitrogen

wavelength below the first excitonic absorption band and a comparatively narrow luminescence band of typically Gaussian shape. Both the onset of absorption and the spectral position of the emission band shift to higher energies with decreasing particle size (Table 1 and Fig. 1a–c). This size dependence is caused by the alteration of the electronic properties of these materials (e.g., energetic position

Table 2 Methods for water transfer

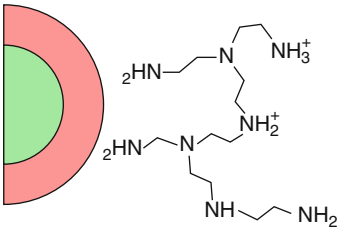
Method		Applications
Electrostatic stabilization	 Ligand exchange with small charged adsorbants, e.g., 3-mercaptopropionic acid (MPA) [34]	–Labeled with immuno-molecules, QDs recognized specific antigens/antibodies –DNA immobilization to QDs surfaces and possibility of hybrid assemblies [35] –Coupled to transferrin, QDs underwent receptor-mediated endocytosis in cultured HeLa cells
	 Intercalation with charged surfactants [36]	
Steric stabilization	 Intercalation with bulky, uncharged molecules, e.g., polyethyleneglycol [37]	–In vivo cancer targeting and imaging –Conjugation with DNA and in vivo imaging (embryogenesis) [36] –Encoding of cells [38] –Noninvasive in vivo imaging with localization depending on surface coating [39]

(*continued*)

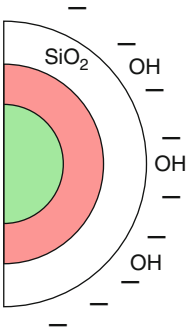
of the valence and conduction band etc.) if the dimensions of the relevant structural features interfere with the delocalized nature of the electronic states. For semiconductor QDs, such quantum-size effects occur typically for sizes in the range of a

Table 2 (continued)

Method	Applications
Hybrid methods	–Proteins can be directly coupled to PEI amine groups –Silica can be easily functionalized and then bioconjugated



Bulky, partially charged ligands (polyelectrolytes), e.g., polyethyleneimine (PEI) [40]



Additional inorganic shells, e.g., silica [41, 42]

few to 10 nm. The size of the photoluminescence quantum yield of QDs is primarily determined by the number of dangling bonds at the core particle's surface. Thus, the modification of the surfaces of bare QDs is very important for the realization of high fluorescence quantum yields. This can be achieved, e.g., by the deposition of a layer of inorganic, chemically inert material or by organic ligands. Accordingly, in the majority of cases, QDs present core–shell (e.g., CdSe core with a ZnS shell) or core-only (e.g., CdTe) structures capped with specific organic or polymeric ligand molecules. The most prominent materials for life science applications are currently CdSe and CdTe. III/V group or ternary semiconductors such as InP, InGaP, $CuInS_2$, and $AgInS_2$ – which lack cytotoxic cadmium ions – are possible alternatives that have been synthesized and used recently [43, 44]. At present, commercial products are available for CdSe (Sigma–Aldrich, Invitrogen, Evident, Plasmachem), CdTe (Plasmachem), and InP or InGaP (Evident).

Lanthanide (Ln) – or rare-earth-doped upconverting nanocrystals usually have similar optical properties as their bulk counterparts [45]. Upconversion is characterized by the successive absorption of two or more photons via intermediate

long-lived excited states followed by the emission of a photon of higher energy than each of the exciting photons. Accordingly, upconverting materials absorb light in the near infrared (NIR) part of the spectrum and emit comparatively sharp emission bands blue-shifted from the absorption in the visible region of the spectrum yielding large antiStokes shifts [46]. Nanoscale manipulation can lead to modifications of, e.g., the excited state dynamics, emission profiles, and upconversion efficiency [47]. For instance, the reduction in particle size can allow for the modification of the lifetime of intermediate states and the spatial confinement of the dopant ions can result in the enhancement of a particular emission. The most frequently used material for the design of upconverting nanocrystals is $NaYF_4$:Yb, Er. The attractiveness of upconverting nanocrystals lies in the fact that the NIR excitation light does not excite background fluorescence and can penetrate deep into tissue, in the large antiStokes shifted, narrow, and very characteristic emission, and in their long emission lifetimes. Despite their obvious potential as fluorescent reporters for the life sciences, upconverting nanoparticles are not commercially available yet. Moreover, in comparison to other longer existing fluorophores, many application-relevant properties have not been thoroughly investigated yet for nanometer-sized upconverting phosphors due to difficulties in preparing small particles (sub-50 nm), that exhibit high dispersibility and strong upconversion emission in aqueous solution.

Precious metal nanoparticles show strong absorption and scattering of visible (vis) light, which is due to collective oscillation of electrons (usually called localized surface plasmon resonance, LSPR) [48]. The cross section for light scattering scales with the sixth power of the particle diameter. Consequently, the amount of scattered light decreases significantly when the nanoparticles become very small. Fluorescence of metal nanoparticles was observed in the late 60s of the last century [49]. Even though this effect is often very small, it becomes increasingly interesting for small nanoparticles or clusters (the properties and applications of silver and gold nanoclusters are discussed in chapters of Diez and Ras [150] and of Muhammed and Pradeep [151] in this volume), since the absorption cross section scales only with the third power of the nanoparticle diameter. Quantum yields of Au_5 clusters as high as 0.7 have been reported [50]. At present, the major field of application of metal particles like gold involves Raman spectroscopy.

2.1.2 Organic Dyes

The optical properties of organic dyes (Fig. 1d–f, Table 1) are controlled by the nature of the electronic transition(s) involved [4]. The emission occurs either from an electronic state delocalized over the whole chromophore (the corresponding fluorophores are termed here as *resonant* or *mesomeric* dyes) or from a charge transfer (CT) state formed via intramolecular charge transfer (ICT) from the initially excited electronic state (the corresponding fluorophores are referred to as *CT dyes*) [4]. Bioanalytically relevant fluorophores like fluoresceins, rhodamines, most 4,4′-difluoro-4-bora-3a,4a-diaza-*s*-indacenes (BODIPY dyes), and cyanines (symmetric

cyanines in general and, depending on their substitution pattern, also asymmetric cyanines) present resonant dyes. Typical for these fluorophores are slightly structured, comparatively narrow absorption and emission bands, which often mirror each other, and a small, almost solvent polarity-insensitive Stokes shift (Fig. 1d) as well as high molar absorption coefficients. For example for the best cyanine dyes, ε_M values of 2–3 $\times$ 10^5 M^{-1} cm^{-1} can be found. Commonly associated with a small Stokes shift are high fluorescence quantum yields for dyes with rigid structures emitting in the visible region (Φ_F values of 0.80–1, e.g., rhodamines, fluoresceins, and BODIPY dyes) and, in the case of near-infrared (NIR) chromophores, moderate Φ_F values of 0.1–0.2 (Table 1). The small Stokes shift of these chromophores results in a considerable spectral overlap between absorption and emission, that can be disadvantageous for certain applications (see, e.g., Sects. 3.4 and 3.5). CT dyes such as coumarins or dansyl fluorophores are characterized by well-separated, broader, and structureless absorption and emission bands at least in polar solvents and a larger Stokes shift (Fig. 1f). The molar absorption coefficients of CT dyes, and in most cases, also their fluorescence quantum yields, are generally smaller than those of dyes with a resonant emission. CT dyes show a strong polarity dependence of their spectroscopic properties (e.g., spectral position and shape of the absorption and emission bands, Stokes shift, and fluorescence quantum yield). Moreover, in the majority of cases, NIR absorbing and emitting CT dyes reveal only low fluorescence quantum yields, especially in polar and protic solvents. The spectroscopic properties of resonant and CT dyes can be fine-tuned by elaborate design strategies if the structure–property relationship is known for the respective dye class. Selection within large synthetic chromophore library becomes popular. The chapter of Kim and Park within these series [152] addresses the comparison of rational design and library selection approaches.

2.1.3 Metal Ligand Complexes

The most prominent metal ligand complexes used in bioanalytics and life sciences are ruthenium(II) complexes with ligands such as bipyridyl- or 1,10-phenenthroline derivatives [8, 9] followed by platinum(II) and palladium(II) porphyrins [51]. Ru(II) coordination compounds absorb energy in the visible region of the spectrum (typically excitable at, e.g., 488 nm) or in the NIR depending on the ligand [52] populating a metal-to-ligand charge transfer (^{1}MLCT) state. Subsequent intersystem crossing leads to quantitative population of the ^{3}MLCT state, which can be deactivated via luminescence, nonradiative decay, or via population of a nonemissive metal- or ligand-centered state. The most characteristic spectroscopic features of this class of fluorescent reporters are broad, well-separated absorption and emission bands, moderate luminescence quantum yields, and comparatively long emission lifetimes in the order of a few 10 ns up to several hundred nanoseconds due to the forbidden nature of the electronic transitions involved [53]. Platinum (II) and palladium(II) porphyrins, that present, e.g., viable oxygen sensors, as well as other coordination compounds such as iridium(II) complexes are not further detailed here. The spectral features of

these Ru(II) complexes (as well as other MLC), their luminescence quantum yields and their lifetimes can be elegantly tuned via the ligand [52].

Luminescent lanthanide complexes (Tb^{3+}, Eu^{3+}, etc.) are of growing interest, e.g., as fluorescent reporters for biological applications. Since the lanthanide f–f transitions have low absorption coefficients (symmetry-forbidden transitions), typically sensitized emission is used to rationalize more intense luminescence, thereby exploiting energy transfer (via intersystem crossing) from the triplet state of the initially excited sensitizer or antenna (ligand with an integral or appended chromophore like phenanthroline) to the emissive lanthanide ion. Accordingly, application-relevant compounds present multicomponent systems, in which the active components, the metal cation, the antenna, and the coordination site are organized in a supramolecular structure. The ligand is commonly also chosen to protect the rare earth ion (chelates in the case of DELFIA and cryptates for the compounds from CISBio International) from potential quenching by the environment (water molecules in the coordination sphere etc.) [54]. The optical properties of luminescent lanthanide complexes are thus determined by the absorption properties of the antenna ligand, the efficiencies of intersystem crossing in the ligand within the complex, triplet-mediated energy transfer from the excited state of the ligand to the lanthanide ion yielding the excited lanthanide, and the quantum yield of the lanthanide emission [55]. The most remarkable features of luminescent lanthanide complexes, that are typically only excitable in the short wavelength region (commonly at ca. 365 nm, sometimes at longer wavelength like 405 nm or even longer), are their narrow and characteristic emission bands in the visible (Tb^{3+}: 490, 545 nm; Eu^{3+}: 580, 613, 690 nm; Sm^{3+}: 598, 643 nm; Dy^{3+}: 575 nm), in the NIR region (Yb^{3+}: 980 nm; Nd^{3+}: 880, 1,065 nm; Er^{3+}: 1,522 nm) and their long luminescence lifetimes (e.g., Eu^{3+}: 300–1,500 μs, Tb^{3+}: 100–1,500 μs; Sm^{3+}: 20–50 μs) [10, 56, 57]. Maximum luminescence quantum yields are in the order of 0.25 found for Eu^{3+} – and 0.15 for Tb^{3+}-complexes in aerated solution and decrease for all the other rare earth ions. Although criteria for the choice of the lanthanide ion and the antennae have been reviewed [11, 55, 58], the complicated mechanism of light generation renders the design of highly luminescent lanthanide reporters still a challenge.

2.1.4 Comparison of Chromophores

In comparison to organic dyes as well as metal–ligand and lanthanide complexes, nanocrystal labels offer a wide variety of spectroscopic properties which are often scalable, optically stable, and not achievable in these molecular fluorophores (e.g., size-controllable spectroscopic properties and continuous absorption below the first excitonic absorption band in the case of QDs, see Fig. 1a–c; upconversion luminescence). With values in the range of 100,000 to 1,000,000 M^{-1} cm^{-1}, the (size-dependent) molar absorption coefficients at the first excitonic absorption band of QDs are generally large as compared to organic fluorophores [33] (Table 1) and strongly excelling the ε_M values obtained for MLC (in the order of a few 10,000 M^{-1}

cm^{-1}) and lanthanide complexes (ε_M determined by the organic ligand with typical values in the order of 20,000–70,000 M^{-1} cm^{-1}) [58]. Fluorescence quantum yields of properly surface-passivated QDs are in the same order of magnitude that is found for vis-emitting organic dyes, [43, 59], thereby clearly exceeding the photoluminescence quantum yields of MLC and lanthanide complexes [58]. Moreover, QDs can have high quantum yields in the NIR above 700 nm in the range of about 0.3–0.8, found, e.g., for CdTe, HgCdTe, PbS, and PbSe [60, 61], whereas organic dyes are at maximum only moderately emissive above 750 nm, see Table 1. Compared to QDs and organic dyes emitting in the visible region, upconverting nanocrystals generally have a low absorption cross section and photoluminescence quantum yield, yet their narrow emission bands are rather characteristic and ideal for multiplexing. Other luminescent nanocrystals such as metal nanoclusters, silicon or carbon nanoparticles have comparatively low quantum yields and often broad emission bands.

Another favorable feature of QDs as compared to organic dyes are their typically very large two-photon (2P) action cross sections [62, 63] that are very attractive for two- (or multi) photon applications such as two- (or multi) photon microscopy and bioimaging [64]. The 2P action cross section equals the product of the two-photon absorption cross-section and the fluorescence quantum yield and describes the probability of simultaneous absorption of two photons and transition of the fluorophore to an excited state that differs energetically from the ground state by the energy of these two photons. The 2P action cross sections of organic fluorophores are commonly in the range of 1.0×10^{-52}–4.7×10^{-48} cm^4 $photon^{-1}$ [65].

The fluorescence decay kinetics of exemplary chosen QDs and small organic dyes are compared in Fig. 2. The size of the fluorescence parameter luminescence lifetime is determined by the electronic nature of the transitions involved. As a rule

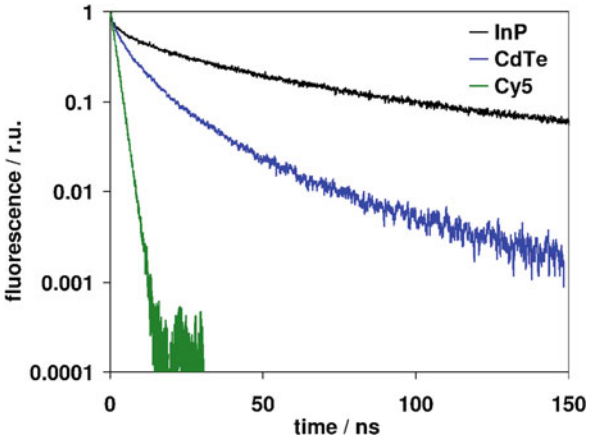


Fig. 2 Comparison of the luminescence decays of QDs and organic dyes. InP and CdTe QDs decay multiexponentially with a mean lifetime ($\tau_{1/e}$) of 17 and 6 ns, respectively. The organic dye Cy5 shows monoexponential decay with τ_F of 1.5 ns

of thumb, for molecular fluorophores, a high ε_M value does not allow obtaining a long emission lifetime. The fluorescence lifetimes of organic dyes, that typically display allowed transitions between singlet states, are in the order of about 5 ns for vis emitters and $\leq$ 1 ns for NIR fluorophores (Table 1). This is too short for efficient temporal discrimination of short-lived background fluorescence and scattered excitation light. The most prominent exceptions used for bioanalytical applications are the vis-emitting acridone dyes displaying fluorescence lifetimes in the order of 5–20 ns, that, however, require short-wavelength excitation (excitation, e.g., at 405 nm, emission at ca. 440–500 nm) [66] and the only recently reported UV-absorber and vis-emitter DBO (2,3-diazabicyclo[2.2.2]oct-2-ene) with a lifetime of ca. 300 ns in aerated water [67]. Due to the forbidden nature of the electronic transitions involved, in addition to its short wavelength absorption and emission (absorption and emission maximum at ca. 365 nm and ca. 430 nm, respectively, in water), DBO shows very low molar absorption coefficients which reduces the overall sensitivity. Nevertheless, advantageous for the vast majority of organic dyes can be their typically mono-exponential decay kinetics (in a homogeneous microenvironment), that can be exploited for the straightforward dye identification from measurements of fluorescence lifetimes [68].

In comparison to conventional organic dyes shown in Fig. 2, MLC like Ru(II) complexes and lanthanide complexes show attractive long emission lifetimes in conjunction with mono-exponential decay kinetics, that render them superior to organic chromophores in this respect [53]. This provides the basis for the straightforward temporal discrimination of shorter-lived autofluorescence and scattered excitation light from label emission with the aid of time-gated measurements, thereby enhancing the sensitivity [69], and enables lifetime-based sensing. Due to their long lifetimes in conjunction with the straightforward excitation and emission in the visible or rarely, even in the NIR, Ru(II) complexes are common probes and labels in lifetime-based assays and (bio)sensors and in fluorescence polarization assays [70]. As the emission lifetimes of Ru(II) complexes are typically oxygen-sensitive, these species present the most commonly used lifetime-based oxygen sensors [71, 72]. The exceptionally long luminescence lifetimes of the lanthanide chelates (typically monoexponential decay kinetics), detailed in the previous section, can, but must not necessarily be, oxygen-dependent [10, 58]. This, in combination with "shielding ligands" like certain chelates or cryptates and narrow emission bands makes these lanthanide fluorophores ideal candidates for all applications of time gated emission (e.g., DELFIA technology in fluoroimmunoassays) and as energy donors in homogeneous time-resolved fluorescence assays [10, 73]. Moreover, their distinct sharp emission bands can be exploited for spectral multiplexing applications [74].

Attractive for the use of QDs are their long lifetimes (typically 5 ns to hundreds of nanoseconds), compared to organic dyes, that are typically insensitive to the presence of oxygen. In conjunction with time-gated measurements, this provides the basis for enhanced sensitivity [69]. This property can be also favorable for time-resolved applications of FRET. The complicated size-, surface-, and wavelength-dependent, bi- or multi-exponential QD decay behavior (Fig. 2) can complicate

species identification from time-resolved fluorescence measurements. Nevertheless, for QD labels displaying a concentration-independent fluorescence decay behavior, the quantification of these multiexponentially decaying species could be recently demonstrated for mixtures of different chromophores [5]. The luminescence lifetimes of upconversion nanocrystals are in the long microsecond to millisecond time domain and are not sensitive to oxygen. Similarly as described for MLC and lanthanide chelates, this can be exploited, e.g., for time-gated emission and time-resolved FRET applications which have already been reported for micrometer-sized upconverting phosphors.

This comparison of the spectroscopic properties of the different types of fluorescent reporters underlines that semiconductor QDs and upconverting nanoparticles have no analogs in the field of organic dyes. Therefore, their unique features are unrivaled. The different molecular labels detailed here each display unique advantages that can compete with some of the favorable features of QDs and upconverting phosphors such as long lifetimes in the case of MLC systems and lanthanide chelates or very narrow emission bands for lanthanide chelates beneficial for spectral multiplexing.

2.2 Solubility and Aggregation

The solubility of a chromophore is one of the mayor factors governing its applicability. Suitable labels and probes should not aggregate or precipitate under application-relevant conditions. For bioanalysis and life sciences, this includes aqueous solutions, in vitro conditions (cell cultural media), on supports such as microarrays, in cells or in vivo conditions. Moreover, for many biological applications such as the specific labeling of cells and tissue, nonspecific binding to the cell surface and the extracellular matrix can also play a role. Organic molecules (dyes as well as ligands for MLC and lanthanide complexes) can be easily solubilized by derivatization with substituents such as sulfonic acid groups. Provided that the structure–property relationship is known for the respective dye class, the solubility can be tuned by substitution without considerably affecting the labels' optical properties and other application-relevant features. A whole range of organic dyes, that are soluble in relevant media, are commercially available.

Nanoparticle dispersibility is controlled by the chemical nature of the surface ligands (coating). Nanoparticles, which are prepared in aqueous solution, are inherently dispersible in water. However, with the exception of CdTe, high-quality nanocrystals with narrow size-distributions are typically synthesized in organic solvents and must be rendered water-dispersible (i.e., aggregation of nanoparticles in aqueous solution must be prevented). As summarized in Table 2, this can be accomplished electrostatically, by using small charged ligands such as mercaptopropionic acid [34], cystamine [75], or with charged surfactants that intercalate with the hydrophobic ligands present from synthesis [36]. Alternatively,

nanoparticle stabilization in aqueous solution can be accomplished by coating the particles with sterically demanding surface ligands such as polyethyleneglycol (PEG) [76].

Electrostatically stabilized nanoparticles are usually much smaller than sterically stabilized ones. Since this is favorable for most applications in the life sciences, electrostatic stabilization strategies are recommended if small nanoparticles in low ionic strength buffers are to be used. However, these particles tend to aggregate in solutions of high ionic strength such as biological matrices. Sterically stabilized nanoparticles are mostly too large to enter cells, but are less likely to aggregate. A compromise can be reached by using smaller, but nevertheless still bulky, charged polyelectrolytes such as polyethyleneimine (PEI) [40], or an additional amphiphilic inorganic shell like silica [41, 42] which can be further functionalized using standard silica chemistry.

It is difficult to predict the effect of surface functionalization on the optical properties of nanoparticles in general. Surface ligands have only minor influence on the spectroscopic properties of nanoparticles, the properties of which are primarily dominated by the crystal field of the host lattice (e.g., rare-earth doped nanocrystals) or by plasmon resonance (e.g., gold nanoparticles). In the case of QDs, the fluorescence quantum yield and decay behavior respond to surface functionalization and bioconjugation, whereas the spectral position and shape of the absorption and emission are barely affected.

2.3 Thermal and Photochemical Stability

Aside from spectroscopic considerations, one of the most important features of a fluorescent label or reporter is its stability under application-relevant conditions. This includes typically used solvents such as buffers, cell medium, or other supports, the presence of oxygen and typical reagents such as dithiothreitol (DTT), common temperatures as well as typical excitation wavelengths, and excitation light fluxes over routinely used detection times. The latter parameter is also linked to the detection method employed with certain fluorophores being suitable only for specific applications. In any case, chromophore stability is of crucial relevance for the achievable sensitivity and limit of detection, especially in single molecule experiments, and for contrast in fluorescence imaging. Blinking, that is the interruption of the photoluminescence of continuously illuminated QDs or organic dyes by dark periods, is relevant for single molecule applications and is briefly discussed in section 3.7.

Organic dyes like fluorescein and TRITC and the majority of NIR fluorophores suffer from poor photostability [77]. In addition, many NIR dyes, such as clinically approved indocyanine green (ICG) reveal poor thermal stability in aqueous solution [78]. Moreover, the presence of ozone can result in dye decomposition as observed for Cy5 [79]. In the last years, many organic dyes like the Alexa dyes have been

designed that display enhanced photostability in comparison to first generation fluorophores such as fluorescein. Simultaneously, due to technical improvements, readout times for many fluorescence techniques could be decreased. Despite these improvements, the nevertheless limited photostability of organic chromophores can still hamper microscopic applications requiring high excitation light intensities in the UV/vis region or long-term imaging. Thus, the search for brighter and especially more stable dyes is still going on. With respect to photochemical stability, lanthanide chelates can be superior to conventional organic chromophores.

In contrast, almost all types of luminescent nanoparticles display excellent thermal and photochemical stability. From the range of these nanocrystals, QDs are the ones most sensitive to photooxidation and photobleaching, but even these effects can be almost completely suppressed by epitaxical growth of a protective shell to shield the core material for relevant time intervals [80]. Moreover, the inorganic nature of the QDs makes them typically resistant to metabolic degradation in live cells and organism which is beneficial, e.g., for long-term imaging. This is a significant advantage over organic fluorophores for imaging applications, where excitation with intense lasers is employed for long periods of time [64]. A superior long-term stability compared to organic dyes has been demonstrated for example for CdSe/ZnS and rhodamine-labeled tubulin [42] CdSe and Texas Red [81] as well as for antibodies labeled with CdSe, FITC, R-phycoerythrin, and AlexaFluor 488 [77]. However, nanoparticles can show specific phenomena such as photobrightening [82] see also Sect. 3.7 on *Reproducibility, Quality Assurance, and Limitations*, and undesired aggregation of nanocrystals can contribute to reduced stability.

The thermal and photochemical stability of both organic dyes and nanocrystals are influenced by an extremely broad variety of conditions that need to be considered: excitation wavelength and intensity, matrix or microenvironment, label concentration, and, in the case of nanoparticles, surface chemistry. Therefore, the individual study of the stability of a chromophore under the conditions required can usually not be avoided.

2.4 Cyto- and Nanotoxicity

"All things are poison and nothing is without poison, only the dose permits something not to be poisonous (Paracelsus)." Although this property of molecular and nanoparticular reporters is not relevant for ex vivo applications such as immunoassays, it is critical for imaging in cells or in vivo. In general, toxicity of organic dyes is not often reported as a significant problem, with the exception of DNA intercalators. Despite the ever increasing interest in in vivo imaging applications and the obvious importance of cytotoxicity data of fluorescent reporters for in vivo applications, there are only very few data available on the cytotoxicity of NIR fluorophores at present [78, 83].

The only organic fluorophores approved by the Food and Drug Administration (FDA) for use in humans are fluorescein (e.g., for opthalometry), Nile Blue, and

ICG, a symmetric cyanine [83]. It is common sense that the expression of green fluorescent protein (GFP) or fluorescent proteins in general can increase or at least sensitize cells to undergo apoptosis induced by the generation of reactive oxygen species (ROS) or due to aggregation of *GFP-fusions* [84]. Therefore, expression levels of fusion or reporter proteins have to be kept as low as possible. Organic dyes used as reporters in live cells can be loaded by incubation in their lipophilic acetoxymethyl-ester form, which achieves high intracellular dye concentrations, but can also result in toxic concentrations preferably in the mitochondria or other organelles with high esterase activity. Moreover, it has to be kept in mind that during continuous imaging, bleached dye species and/or ROS are formed, which can be toxic to live cells in contrast to the initially used fluorophore.

Toxicity of nanoparticles is a much more complicated issue as compared with organic fluorophores: Nanoparticles may be nanotoxic, they may contain cytotoxic elements or compounds, or their surface ligands/coating may contain toxic species. Nanotoxicity refers to the ability of a substance to be intrinsically cytotoxic due to its size (and independent of its constituent materials). The most prominent example of nanotoxicity is asbestos. Even though there are no systematic studies on the nanotoxicity of different nanocrystals available the results from several cytotoxicity studies suggest that nanotoxicity is not dominating for nanoparticular reporters [85, 86].

The QD toxicity depends on multiple factors derived from both physico-chemical properties and environmental conditions like QD material, size, charge, concentration, and outer coating material (capping material and functional groups) as well as oxidative, photolytic, and mechanical stability [87]. Many of these factors also govern the cytotoxicity of other inorganic or organic fluorophore-doped nanoparticles [88]. The cytotoxicity of heavy metals or rare-earth elements, which are present in many nanocrystals as core and shell materials, is well known. Thus, it is critical to know whether these cytotoxic substances can leak out of the nanocrystals over time. This may happen upon illumination or oxidation [89]. Furthermore, toxic ligands or coatings might be released into solution [85]. Some groups found that CdSe-based QDs were cytotoxic to cells [90], other did not detect cytotoxic reactions [91]. In cases where cytotoxicity was observed, it was attributed to leaking of cytotoxic elements, cytotoxic surface ligands, and/or nanoparticle aggregation. Moreover, e.g., for unmodified cadmium telluride QDs, the induction of the formation of ROS formation leading to multiple organelle damage and cell death has been reported [92].

The preparation of both, the particles themselves and the protective surface layer, has direct influence on their cytotoxicity. It is common belief that in the case of core/shell nanoparticles, properly prepared, close shell or multiple shells such as ZnS/SiO_2-shells prevents the leakage of toxic elements and thus makes cytotoxicity unlikely. Naturally, a better solution is to avoid cytotoxic materials in the first place. QDs, for example, can be synthesized without utilization of any class A or B elements: InP/ZnS QDs have photophysical properties comparable to those of CdSe-based systems [43, 93]. Principally, whenever a new approach for QD synthesis or coating is used or if the QDs are applied in an extreme environment that could compromise their integrity, it is recommended to assess their cytotoxicity.

The work on the toxicity of nanoparticular reporters is still in its infancy. The clear evaluation of cytotoxicity will require verified data using at least two or more independent test systems, standardization in the experimental set-up and exposure conditions in order to be reliable. In addition, the involvement of toxicologists in the systematic assessment of QD toxicity would be beneficial.

3 Application of Molecular and Nanoparticulate Fluorophores

The fast, sensitive, reliable, and reproducible detection of (bio)molecules including quantification as well as biomolecule localization, the measurement of their interplay with one another or with other species, and the assessment of biomolecule function in bioassays as well as in vitro and in vivo plays an ever increasing role in the life sciences. The vast majority of applications exploit extrinsic fluorophores like organic dyes, fluorescent proteins, and also increasingly QDs, as the number of bright intrinsic fluorophores emitting in the visible and NIR is limited. In the near future, the use of fluorophore-doped nanoparticles is also expected to constantly increase, with their applicability in vivo being closely linked to the intensively discussed issue of size-related nanotoxicity [88].

Suitable fluorescent labels and reporters must typically indicate the presence of a given target in the analyzed medium and must often also provide a quantitative measure for this species. Depending on the desired application, these chromophores can be chosen to retain their spectroscopic properties (dyes for labeling without real "reporting" function as, e.g., many dyes in fluorophore–biomolecule conjugates or so-called targeted optical probes for fluorescence in vivo imaging) or change their spectroscopic features on interacting with the target, typically in the broadest possible range of variation (i.e., affecting as many fluorescence parameters as possible). The latter type of chromophore is often termed dyes with reporting function or probe or sensor [24, 51]. In the following, we do not attempt to distinguish between both types of chromophores.

3.1 Coupling Chromophores to Biomolecules

In many cases, the application of fluorophores includes the covalent or noncovalent attachment of at least one fluorescent label to biomolecules like proteins, peptides, or oligonucleotides. Prerequisite for chromophore labeling of biomolecules are reactive or functional groups at the fluorophore. The great advantage of organic dyes in this respect is the commercial availability of a unique toolbox of functionalized chromophores, in conjunction with established labeling protocols, purification, and characterization techniques for dye-bioconjugates, as well as information on the site-specificity of the labeling procedure [1]. Also, many

metal ligand complexes and lanthanide chelates equipped with functional groups are commercially available. Furthermore, the small size of organic dyes minimizes possible steric hindrance, which can interfere with biomolecule function in the case of larger chromophores and allows attachment of several fluorophores to a single biomolecule to maximize the fluorescence signal [1]. Nevertheless, with regard to retaining biomolecule function, the dye-to-biomolecule ratio (*D*/*P* ratio) should not be too high and labeling of the biomolecule's binding sites is to be avoided. Moreover, high label densities can result in fluorescence quenching, with the *D*/*P* ratio where such effects become prominent being dependent on dye structure (e.g., planarity favoring π–π-interactions), charge (electrostatic repulsion of neighboring molecules), and hydrophilicity [30, 78, 94] as well as spectral overlap [24]. This is, e.g., an advantage of lanthanide labels where no fluorescence self-quenching as a function of label density is observed due to their strongly Stokes shifted emission. Also site-specificity can be problematic even for small organic dyes with the development of strategies for site-specific label attachment (often of a single label), that should be ideally generalizable and applicable to many different types of fluorophores - currently being an active area of research.

For nanoparticles, there is no consensus method for the labeling of biomolecules [95]. The most critical steps for labeling of biomolecules with QDs are ligand exchange to overcome the inherently hydrophilic nature of the QDs prior to bioconjugation, control of the number of linkers attached to a single QD (control of QD valency), and purification of the bioconjugated QDs. The general principle for biofunctionalization of nanoparticles is that, at first, the particles are made water-soluble and then bound to biomolecules (Table 2). This can be done electrostatically, by a biological immuno- or other key/lock reaction, by covalent linking (for example, carbodiimide-activated coupling between amine and carboxylic groups), or by nickel-based histidine tagging [96]. Biomolecules that bear surface active groups can replace ligands on nanoparticles directly [97]. Currently, only few standard protocols for labeling biomolecules with nanoparticles are available [64] and the choice of suitable coupling chemistries depends on the surface functionalization of the particles. It is difficult to define and employ general principles because nanoparticle surfaces may be very different, depending on their chemical nature and method used for their synthesis. Accordingly, for users of commercial nanoparticles, knowledge of surface functionalization is very important.

Most of the challenges in organic dye biofunctionalization also apply to nanoparticles, with the exception of fluorescence quenching at high label density. A problem which arises with nanoparticles is aggregation due to nonoptimal surface chemistry. Moreover, contrary to labeling with small organic fluorophores, several biomolecules are typically attached to a single nanocrystal due to the multivalency of QDs and control of biomolecule orientation is difficult. This can affect the spectroscopic properties and colloidal stability of the nanoparticles as well as biomolecule function. Similar drawbacks arise for all types of fluorophore-doped nanoparticles. Only recently, methods have been developed to optimize the 1:1 stochiometry of QD-biomolecule conjugates [98].

3.2 Extra- and Intracellular Targeting of Biomolecules

The location and dynamics of biomolecules like proteins play an important role in cell signal transduction. Similarly relevant are issues like the assessment of molecular function of biomolecules, e.g., for cancer research and target quantification. A prerequisite, e.g., for monitoring molecular function in vivo is the ability to track biomolecules within their native environment, i.e., on the cell surface or inside cells, and needs to be met by any fluorescent label suitable for this purpose. The challenges here include intracellular delivery of the chromophore as well as selective labeling of the target biomolecule within its native setting without affecting its function. The latter is the prerequisite for assessing changes in the local environment or the distances between labeling sites using hetero-FRET (chemically different chromophores) or homo-FRET (chemically identical chromophores). Successful experiments require the selection of labels that are matched with the biological system, for instance, the location of the target (cell surface, intracellular, or vascular compartments), the expression level of the target, or whether the target is within a reducing versus an oxidizing environment.

The report of several established and recent methods for extracellular and intracellular labeling of biomolecules, in conjunction with some commercial tools for these applications [99] is mainly advantageous for organic fluorophores. This includes several strategies for site-specific covalent and noncovalent labeling of biomolecules, typically proteins, in living cells. Examples are enzyme-catalyzed labeling by posttranslational modification, as in biotin ligase-catalyzed introduction of biotin into biotin acceptor peptides, which may be used to label proteins at the cell surface. Both intracellular and surface labeling have also been achieved by specific chelation of membrane-permeant fluorescent ligands (biarsenical dyes such as FIAsH or ReAsH bind to the tetracysteine motif, Ni-nitriloacetic acid (NTA) conjugates bind to the hexahistidine motif, and Zn conjugates), or by self-labeling, in which proteins fused to O6-alkylguanine-DNA alkyltransferase are combined with enzymatic substrate derivatives (O6-alkylguanine-DNA alkyltransferase (AGT) or SNAP-tags) [1, 99]. Other alternatives present the HaloTag technology, exploiting a modified haloalkane dehalogenase designed to covalently bind to synthetic ligands which can be used for the highly specific labeling of fusion proteins in living or chemically fixed cells and irreversible capture of these proteins onto solid supports [100] or the use of 2,4-diamino-5-(3,4,5-trimethoxybenzyl) pyrimidine (trimethoprim or TMP). For organic labels, also several methods are well established for fluorophore delivery into cells. This includes acetomethoxymethyl (AM) ester derivatization as well as simple microinjection, gene guns, cationic liposomes, controlled cell volume or cell membrane manipulation, and endocytosis [101] or electroporation [102]. In particular the first strategy which renders the dyes cell permeable, presents a huge advantage for this class of labels.

Meanwhile, extracellular targeting with QDs has been frequently reported [103]. Moreover, strategies have been described to reduce nonspecific QD binding and uptake as a prerequisite for applications, where specific cell–chromophore

interactions are to be investigated and the distinct, specific, and nonspecific pathways of QDs into cells as well as their intracellular fate have been studied [104]. Extracellular targeting is typically accomplished through QD functionalization with specific antibodies to image cell–surface receptors [39] or via biotin ligase-catalyzed biotinylation in conjunction with streptavidin-functionalized QDs [105]. The HaloTag method has just recently been combined with QDs allowing much simplified protocols for cell surface labeling [106]. Due to their larger size, the intracellular delivery of QDs is much more challenging compared to small organic dyes, and accordingly, the state-of-the-art of delivery of QDs into cells and internal labeling strategies are far behind. Although there exists no general protocol to achieve this so far, individual solutions have been reported, that, however, need to be empirically established in each case. Moreover, there are reports on successful cell labeling via microinjection [36], electroporation [107], nanoinjection [108], mechanochemical [109], or nonspecific or receptor-mediated endocytosis [1, 86]. As has been recently shown, the labeling specificity and efficiency can be improved with specifically functionalized QDs [98]. More sophisticated tools are needed for labeling of specific intracellular structures outside endocytosed vesicles or imaging of cellular reactions in the cytoplasm or the nucleus with QDs. Only a few successful studies have been published with QDs targeted to specific cellular locations so far [110]. More research is required in this respect to establish suitable strategies. Here, ligand design also plays a crucial role for the design of stable and small hydrophilic QDs, to minimize undesired nonspecific interactions, and to provide the basis for further functionalization [111]. Positively charged peptide transduction domains (PTDs) such as TAT (Tat peptide from the cationic domain HIV-1 Tat), polyarginine, polylysine, and other specifically designed cell penetrating peptides (CPPs), can be coated onto QDs to effect their delivery into cells [112]. It remains to be shown whether other recently developed cell penetrating agents like a synthetic ligand based on an *N*-alkyl derivative of 3β-cholesterylamine termed streptaphage designed for efficient uptake of streptavidin conjugates by mammalian cells [113] or polyproline systems equipped with cationic and hydrophobic moieties [114] can be adapted for QD delivery.

3.3 Interactions Between Chromophores and their Microenvironment

One of the unique features of fluorophores is the general sensitivity of their spectroscopic properties to temperature and dye local environment, i.e., matrix polarity and proticity (hydrogen bonding ability), viscosity, pH, and ionic strength, and also to the presence of, e.g., surfactants or serum proteins in the case of in vivo studies as well as fluorescence quenchers such as oxygen or conjugated (bio) molecules. Such factors need to be considered for most applications of fluorescence ranging from analyte sensing to the characterization of cell function and behavior. Absolute quantification from measured fluorescence signals typically requires the

signal-relevant optical properties of fluorophores to be ideally insensitive to environmental factors [115]. This renders the assessment of the sensitivity of chromophores to their application-relevant environment increasingly important. In addition, the photochemical stability of fluorophores also responds to dye microenvironment.

The chromophore environment can affect the spectral position of the absorption and emission bands, the absorption and emission intensity (ε_M, Φ_f), and the fluorescence lifetime as well as the emission anisotropy, e.g., in the case of rigid matrices or hydrogen bonding. Changes in temperature typically result only in small spectral shifts, yet in considerable changes in the fluorescence quantum yield and lifetime. This sensitivity can be favorably exploited for the design of fluorescent sensors and probes [24, 51], though it can unfortunately also hamper quantification from simple measurements of fluorescence intensity [116]. The latter can be, e.g., circumvented by ratiometric measurements [24, 115].

The microenvironment dependence of the optical properties of organic fluorophores is controlled by dye class, nature of the emitting state(s), excited state redox potential, charge, and hydrophilicity. Dyes with resonant emission such as fluoresceins, rhodamines, and cyanines typically show only moderate changes in their spectral characteristics, yet can change considerably in fluorescence quantum yield and lifetime. Moreover, they are prone to aggregation-induced fluorescence quenching (due to, e.g., homo-FRET and static quenching [24, 117]. CT dyes with an emission from an excited state that has a considerable dipole moment like coumarins respond with notable spectral changes to changes in microenvironment polarity as well as with changes in absorption and emission intensity. These dyes can also be sensitive to solvent proticity. CT dyes, that are occasionally termed solvatochromic dyes, can be thus exploited for the design of fluorescence probes for microenvironment polarity [118].

In the case of QDs, the chromophore microenvironment mainly affects the fluorescence quantum yield and fluorescence decay behavior. These effects are governed by a whole range of factors: the nature of the nanocrystals, their ligands, shells, and the accessibility of the core surface [119]. Typically, properly shelled/ligated nanocrystals are minimally sensitive to microenvironment polarity provided that no ligand desorption occurs [5]. Also, the emission and absorption properties of most nanoparticles are barely responsive to viscosity, contrary to that of many organic dyes. All nanoparticles are colloids and thus susceptible to changes in ionic strength: electrostatically stabilized particles tend to aggregate upon increasing ionic strength. Some nanoparticles (e.g., gold nanoparticles) are prone to aggregation-induced optical changes that can be exploited as signal amplification strategy.

For both organic dyes and QDs, bioconjugation often leads to a decrease in fluorescence quantum yield and thus typically also in emission lifetime. Parameters that can affect label fluorescence are the chemical nature and the length of the spacer and, at least for organic dyes, the type of neighboring biomolecules like oligonucleotides or amino acids in the bioconjugated form.

Generally, the knowledge of microenvironment effects greatly simplifies label choice. This is an advantage of organic dyes as the spectroscopic properties of many

common labels have been investigated in a broad variety of environments including dye–biomolecule conjugates, whereas only few systematic studies have yet been performed on the microenvironment effect on QD spectroscopic properties. Moreover, the generalization of such effects is hampered by the broad variety of QD coatings used, matrix-dependent ligand adsorption–desorption equilibria, and the interplay between proper core shielding and microenvironment effects.

3.4 Exploitation of Förster Resonance Energy Transfer

FRET is an interaction between the electronic states of two chromophores, in which excitation energy is transferred from a donor fluorophore to an emissive or nonemissive acceptor chromophore. FRET is commonly exploited as a basis for tuning the Stokes shift (see also Sect. 3.5), to measure the distance between donor and acceptor chromophores (spectroscopic ruler, monitoring of conformational changes), for the design of ratiometric probes and sensors as well as signal amplification strategy [117, 120]. Typically, donor and acceptor chromophores are chemically different (hetero-FRET or donor–acceptor energy transfer (DAET). More recently, chemically identical, yet photophysically different chromophores (homo-FRET or donor–donor energy migration (DDEM); measurement of the rate of energy migration) are also used for this purpose, e.g., to sense the protein aggregation state based on steady state and time-resolved measurements of the fluorescence anisotropy [117]. FRET applications thus require labeling of biomolecules or other targets with one donor and one acceptor group (hetero-FRET) or with a single class of chromophores (homo-FRET). Typically, challenging site-specific labeling is desired for hetero-FRET, whereas for homo-FRET, this can be circumvented by the performance of polarization-dependent measurements that, however, require sophisticated instrumentation. A measure of the efficiency and comparison of FRET pairs provides the Förster distance or radius (R_0) equaling the distance at which the energy transfer is 50% efficient.

There exists an ever increasing toolbox of commercial functionalized organic fluorophores with extensively described FRET properties [6]. For many FRET applications that do not need very small molecules, organic chromophores have been increasingly replaced by fluorescent proteins [121]. Numerous FRET probes based on fluorescent proteins for intracellular ion and second messenger measurements (calcium, pH, cAMP, cGMP, kinases) are established [122, 123]. For commonly used organic dyes, R_0 reaches values of 2–10 nm. Limitations of organic dyes and fluorescent proteins for FRET applications are related to crosstalk in excitation and emission. This can result from direct acceptor excitation due to the relatively broad absorption bands of these fluorophores. Moreover, the spectral discrimination of the fluorescence emission from the donor and acceptor can be difficult in the case of emissive acceptors, due to the relatively broad emission bands of organic fluorophores. In the case of dyes like fluoresceins, rhodamines, BODIPYS, and cyanines, that display a resonant emission (Fig. 1a), this is further complicated by the small

Stokes shifts and the "red" tails of the emission spectra of these chromophores. Thus, often tedious corrections of measured signals are mandatory.

Meanwhile, there are numerous examples for the successful use of QDs as FRET-donors in conjunction with organic dyes as acceptors, with the QD emission being size-tuned to match the absorption band of the acceptor dye [124]. There are also few examples of QD-only FRET pairs. In the case of QDs as donors and organic dyes as acceptors, excitation crosstalk can be easily circumvented due to the QD-inherent free choice of the excitation wavelength. Moreover, the longer lifetime of QDs can be exploited for time-resolved FRET. A QD-specific limitation for FRET applications presents both the bigger size of the QD itself and the size of the surface coating. This typically renders distance-dependent FRET with QD donors less efficient as compared to organic dyes. This limitation can be only partly overcome by using donor–acceptor ensembles where a single QD-donor is linked to several organic acceptor dyes. Due to the broad absorption bands of QDs favoring excitation crosstalk, use of QDs as FRET acceptors is not recommended [125]. Generally, FRET applications of QDs should only be considered if there is another QD-specific advantage for the system in question, such as the possibility of avoiding excitation crosstalk, their longer fluorescence lifetimes, their very large 2P action cross sections, or multiplexing FRET applications. In most cases, fluorescent proteins or organic dyes are to be favored for FRET. This is similarly true for metal ligand complexes and lanthanide chelates, the application of which in FRET pairs is not further detailed here. Despite their low molar absorption coefficients, lanthanide chelates are especially interesting FRET donors due to their strongly Stokes shifted narrow emission and long lifetime, that is often exploited for time-resolved FRET immunoassays (e.g., TR-FRET assays) [10, 54].

3.5 Multiplexing Detection Schemes

Current security and health concerns require robust, cost-effective, and efficient tools and strategies for the simultaneous analysis, detection, and often even quantification of multiple analytes or events in parallel. The ability to screen for and quantify multiple targets in a single assay or measurement is termed multiplexing.

3.5.1 Spectral Multiplexing

Spectral multiplexing or multicolor detection is typically performed at a single excitation wavelength, and relies on the discrimination between different fluorescent labels by their emission wavelength. Desirable optical properties of suitable fluorophores are a tunable Stokes shift and very narrow, preferably well-separated emission bands of simple shape.

The suitability of organic dyes for multicolor signaling at single wavelength excitation is limited due to their optical properties (Fig. 1d, f and Table 1). With

respect to small fluorescent labels and reporters, here, lanthanide chelates are to be favored, yet depending on the respective application, they may encounter problems with respect to accomplishable sensitivity. In the case of organic dyes, an increasingly common multiplexing approach implies the use of donor–acceptor dye combinations (so-called tandem dyes or energy-transfer cassettes) that exploit FRET to increase the spectral separation of absorption and emission and thus to tune the Stokes shift [6]. A typical example of a four color label system consists of a 5-carboxyfluorescein (FAM) donor attached to four different fluorescein- and rhodamine-type acceptors (e.g., JOE, TAMRA, ROX) via a spacer such as an oligonucleotide. FRET dye-labeled primers and FRET-based multiplexing strategies are the backbone of modern DNA analysis enabling e.g. automated high speed and high throughput DNA sequencing and the development of robust multiplex diagnostic methods for the detection of polymerase chain reaction (PCR) products. With suitably designed systems, even intracellular dual FRET measurements using a single excitation wavelength were described [123]. Although broadly used, the limitations of organic dyes for FRET applications discussed in the previous section nevertheless also hamper the efficiency of these FRET-based multiplexing systems. This can be overcome by multiwavelength excitation using different lasers, which is becoming affordable due to progress in laser technology. This approach has been already successfully used in flow cytometry with the independent detection of 12 different analytes being reported using organic labels and state-of-the art cytometers [126].

The unique flexibility in excitation and the very narrow and symmetric emission bands simplifying color discrimination render QDs ideal candidates for spectral multiplexing at a single excitation wavelength. Accordingly, there are many reports of the use of QDs as labels in multiplexed assays or immunohistochemistry or imaging applications requiring multiplexing [6, 39]. Although rarely discussed, despite their very attractive spectroscopic features, the simultaneous detection and quantification of several different analytes with QD labels can also require spectral decomposition procedures of measured signals, as has been recently demonstrated for a multiplexed fluoroimmunoassay for four different toxins [127]. The importance of spectral unmixing for QD multiplexing was recently evaluated and demonstrated [128].

3.5.2 Lifetime Multiplexing

Multiplexing can also be performed by making use of the fluorophore-specific decay behavior, measured at a single excitation and single emission wavelength, to discriminate between different fluorophores. This approach requires sufficiently different lifetimes of the chromophores. With a single exception, lifetime multiplexing, as well as a combined spectral and lifetime discrimination have only been realized with organic chromophores [129]. This is most likely, related to the fact that the need for monoexponential decay kinetics was often assumed for this application. Meanwhile, successful lifetime multiplexing has been also reported both for a mixture of a QD and an organic dye and for a mixture of two different QDs [5] despite the multiexponential decay kinetics of the QDs. This may pave the road for future

applications of QDs for combined spectral and lifetime multiplexing, thereby further increasing the number of species to be discriminated.

3.6 Strategies for Signal Amplification

Signal enhancement is one of the major challenges not only in the improvement of luminescent sensors, but also for many luminescence-based methods used for the analysis of samples available only in very small quantities. This can help to improve the signal-to-noise ratio and to minimize the influence of background fluorescence or ambient light. Moreover, it paves the road for increasingly desired miniaturization and simple readout devices and helps to reduce costs. Fluorescence amplification strategies include enzymatic amplification, avidin–biotin or antibody–hapten secondary detection techniques, nucleic acid amplification, controlled aggregation, chromophore–metal interactions (metal-enhanced fluorescence or MEF, observed for the metals silver and gold), and multiple-fluorophore labels (e.g., phycobiliproteins or particle labels including systems with releasable fluorophores, dendrimeric systems, and FRET-based light harvesting systems). Such amplification strategies have been established for organic dyes and can often be used only for certain applications, such as fluoroimmunoassays. These approaches can be transferred to QDs only to certain degrees. For instance, methods involving the use of a fluorogenic enzyme substrate cannot be transferred to QD technology. However, enzymatic amplification has been combined with QDs in the past [130]. Approaches such as controlled aggregation or the construction of multichromophoric systems like chromophore-doped particle labels are similarly suited for both organic dyes and QDs. MEF, that exploits the coupling of the chromophore's transition dipole moment to metal plasmons, can provide emission enhancement factors of typically ca. 10 up to a few hundred for organic chromophores, depending on the fluorescence quantum yield of the respective dyes, in conjunction with reduction in fluorescence lifetime and increased photostability [131]. The enhancement factors, however, depend on the type, shape, and size of the metal, on the type of chromophore, and on geometrical parameters (metal–fluorophore distance, orientation) and thus require sophisticated dye–metal nanoparticle systems or (dye-doped) core/shell-nanostructures. In the case of QDs, only moderate amplification effects (e.g., fivefold fluorescence enhancement for a CdTe–Au-system) have been observed [132, 133]. The potential of this and other signal amplification approaches to optimize QD properties and to enable new sensor applications still needs to be thoroughly investigated.

3.7 Reproducibility, Quality Assurance and Limitations

Aside from instrument-specific contributions that can be corrected for, target quantification from measurements of fluorescence is affected to a nonnegligible

extent by both the sensitivity of the chromophore's spectroscopic properties to the environment and fluorophore photochemical and thermal stability [116]. Organic dyes have been successfully applied for quantification in a broad variety of in vitro fluorescence applications, but reports of analyte quantification with QD labels are still rare. In the case of organic dyes, dye stability can be critical for all fluorescence applications using intense light sources such as fluorescence microscopy or for methods like in vivo fluorescence imaging, where lasers are used as excitation light sources and measurements are performed over several days. This long term known stability issue has been partly overcome by the synthesis of more stable dyes, see section on thermal and photochemical stability [94, 134]. Nevertheless, there is still considerable interest in the development of brighter and more stable dyes. Of interest are also comparative stability studies of bioanalytically relevant dyes and labels under application-relevant conditions providing all the experimental parameters used including the excitation intensity or light flux reaching the sample as a prerequisite for data reliability and comparability. In the case of generally more photostable QDs, the recently reviewed problems still arise like photobrightening, blinking, bluing, and also bleaching [82]. QD photobrightening, i.e., the increase in emission efficiency with continuous illumination, can hamper direct quantification and may render the use of reference standards necessary [135]. This QD-specific effect is most likely related to light-induced surface passivation. The size of this phenomenon, that often reveals a dependence on excitation wavelength and is typically most pronounced for UV excitation [136], is expected to depend on the quality of the initial QD surface passivation (i.e., the saturation of surface defects by ligands or a passivating shell), and also on shell quality, thereby principally reflecting the accessibility of the QD core. This can be thus exploited as a screening test for QD quality [80]. In addition, the luminescence quantum yield of QDs can be concentration-dependent [5], thereby yielding concentration-dependent signal fluctuations, that hamper quantification. This effect depends on the bonding nature of organic ligands to the surface atoms of nanocrystals and the related ligand- and matrix-dependent adsorption–desorption equilibria which have been only marginally investigated [137–139]. This can be critical for all applications where the initially applied concentration of QD labels and probes changes during analysis, especially in the case of QDs capped and stabilized with weakly bound ligands such as many monodentate compounds. The latter processes can also result in concentration-dependent fluorescence quantum yields, especially for weakly bound ligands.

For single molecule spectroscopic applications, chromophore blinking (see Table 1) can be problematic. This phenomenon, that is often related exclusively with QDs, but also occurs for organic dyes, implies that a continuously illuminated chromophore emit detectable emission only for limited times, interrupted by dark periods during which no emission occurs. This can be a significant disadvantage of otherwise very attractive QDs as can be the blinking of organic dyes [140]. For example, QD blinking has been reported to affect the results from bioaffinity studies [141]. Another aspect that might influence the usability of QDs for quantification lies in the fact that not all QDs in a set of QDs luminesce [142]. For

ensembles of QDs, accurate quantification thus requires the ratio of emissive to nonemissive QDs to be constant.

Generally, reliable and comparable fluorescence measurements require fluorescent labels with reproducible physico-chemical properties and established tools to evaluate this. This is a unique advantage of organic dyes. These compounds can be synthesized on a large scale and characterized according to their structure and purity using well-established analytical techniques. This is more challenging for dye–biomolecule conjugates, such as fluorophore-labeled antibodies or proteins, due to batch-to-batch variations in label density and label density distribution and the lack of methods to reliably and accurately determine label density. Nevertheless, this is manageable in principle. In the case of QDs, the colloidal nature of these chromophores, in conjunction with the broad variety of synthetic strategies and surface functionalities, renders chromophore characterization more challenging compared to organic dyes. For commercial QDs, this is often further complicated by the fact that commercial distributors usually refrain from providing any information about the ligand(s). For instance, at present, there are no established methods available to determine the surface coverage and number of ligands attached to the surface of a QD. Even more challenging is the characterization of QD–biomolecule conjugates, e.g., the measurement of the QD-to-biomolecule ratio [143].

4 Applications of Nanoparticles: State-of-the-Art and Future Trends

Organic molecules are well established as fluorescent labels and reporters for in vitro assays and in vivo imaging, despite their nonoptimum spectroscopic features and photochemical instability. Due to their availability from many commercial sources, established functionalization protocols, and extensively studied properties organic dyes present a simple, safe, and comparatively inexpensive option. This holds similarly true for metal ligand complexes and lanthanide chelates. To further improve the reliability of the data obtained with these labels and reporters, e.g., the fluorescence quantum yields of typical chromophores under commonly used measurement conditions should be reevaluated and comparative photostability studies could be beneficial. With respect to the ever increasing number of in vivo applications of chromophores, reliable data on the cytotoxicity of these chromophores are also needed, preferably obtained under standardized measurement conditions. Generally, there is an increasing need for bright and stable NIR chromophores [144]. Whether this can be met with the rational design of organic dyes, metal ligand complexes, and lanthanide chelates or whether the use of established NIR chromophores encapsulated into organic or inorganic nanoparticles is a more straightforward approach to tune the spectroscopic properties and the stability of such NIR fluorophores [145] remains to be seen in the coming years. Here, particulate labels and reporters are expected to have a bright future if the

nanotoxicity issue is resolved. There also exist many different instances where QDs have been applied to biological systems. Although most of these studies are proof-of-principle, they underline the growing potential of these reagents. QDs are very attractive candidates for bioanalytical applications that can either exploit their potential for spectral multiplexing, do not require strong signal amplification or that rely on NIR fluorescence.

Apart from the advantageous properties discussed above, QDs could have a bright future especially in the field of near infrared fluorescence imaging (NIRF), because they show high fluorescence quantum yields in the 650–900 nm window, may have adequate stability, good water solubility as well as large 2P action cross sections as desired for deep tissue imaging. The only clinically approved organic NIR fluorophore ICG (Table 1) suffers from a very low fluorescence quantum yield [31, 78], limited stability, and binding to plasma proteins. Other organic fluorophores for the NIR range (with pending approval like, e.g., Cy5.5, $\Phi_F = 0.28$ in phosphate buffer solution) still possess small quantum yields compared to NIR-emitting QDs such as CdTe (Table 1). In addition, QDs are attractive candidates for the development of multifunctional composite reporters for the combination of two or more bioanalytical imaging techniques, such as NIRF/magnetic resonance imaging (MRI) [146].

Despite the promising possibilities offered by the different types of nanoparticles, their routine use is still strongly limited by the very small number of commercially available systems and the limited amount of data on their reproducibility (in preparation, spectroscopic properties, and application) and comparability (e.g., fluorescence quantum yields, stability) as well as on their potential for quantification. To date, no attempt has yet been published comparing differently functionalized nanoparticles from various sources (industrial and academic) in a Round Robin test, to evaluate achievable fluorescence quantum yields, and batch-to-batch variations for different materials and surface chemistries (including typical ligands and bioconjugates). Such data would be very helpful for practitioners and would present the first step to derive and establish quality criteria for these materials.

In addition to the practical questions linked to the application of nanoparticles, fundamental questions such as the elucidation of quantum dot lifetime characteristics, e.g., for lifetime multiplexing [147] and combined lifetime and spectral multiplexing in conjunction with the development of suitable algorithms for data analysis and for time resolved FRET have to be addressed. Other current limitations include the comparatively large size of nanoparticles. The ligand-controlled size of nanoparticles does not only affect their FRET efficiency but could also sterically hamper access to cellular targets and could affect the function of labeled biomolecules. So far, nanoparticles for bioanalytical applications can only be prepared on a very small scale. Commercialization of, e.g., NIR QDs requires more systematic studies of nanoparticle nucleation and growth. This involves the control of nanoparticle surface chemistry, and the establishment of functionalization protocols. A first useful step in this direction would be the design of a reliable and reproducible test for the quality of surface coatings, i.e., the degree of perfection of the surface ligand shell, as this is the most crucial parameter affecting the spectroscopic and

toxicity properties of nanoparticles [80]. Eventually, the cytotoxicity of differently functionalized nanoparticles (including typical ligands) should be systematically assessed using previously standardized procedures.

Even though nanoparticles have extremely promising and advantageous (optical) properties, at present, they cannot be recommended for routine applications, due to the problems discussed in this review. In very specific cases, such as single molecule/single particle imaging and tracking applications, QDs are superior to most luminescent dyes due to their photostability, in principle allowing single-particle tracking for a much longer time span compared with organic fluorophores. However, blinking that is observed for all QDs is a major drawback even for these specialized applications. Nevertheless, there is hope that quantum dot blinking can be overcome, making them eventually the ideal labels for all applications in need of exceptional photostability [148]. On the other hand, blinking, as well as other QD-specific features, may be even exploited for advanced techniques such as superresolution microscopy [82, 149]. Here, further exciting potential applications of QDs are expected to appear in the near future.

5 Conclusions

Nanocrystals have been exploited in several areas of biosensing and -imaging, including immunohistochemistry, microarray technologies as well as advanced fluorescence techniques such as FISH, and in vivo fluorescence imaging using conventional techniques and multiphoton microscopy. Despite many superior optical properties of these particles, such as tunable absorption and emission bands and extremely broad and intense absorption, high fluorescence quantum yields even in the NIR region, and large two-photon action cross sections as well as unique spectroscopic prerequisites for spectral multiplexing in the case of QDs, or sophisticated optical effects such as upconversion luminescence in the case of rare-earth doped nanocrystals, until now, nanocrystals failed to be routinely used on a large scale. The fact that these materials behave like colloids but not like molecules complicates their application in biological environments. Practitioners must consider the costs of finding a solution to the challenges of their particular experimental system against the benefits of their advanced spectroscopic features. However, it is anticipated that advances in nanosciences combined with the attractive features of many nanoparticle systems will render these particles increasingly attractive for bioanalytical applications in the future.

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