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Luminescent Lanthanide Cryptates: from the Bench to the Bedside

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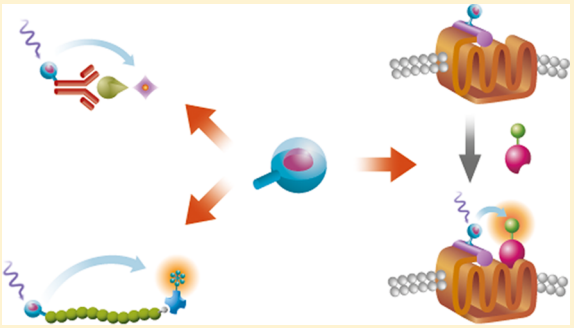
Supporting Information

ABSTRACT: The design and application of luminescent lanthanide cryptates for sensing biological interactions is highlighted through the review of the work performed in our laboratory and with academic collaborations. The path from the initial applications probing biochemical interaction in vitro to "state-of-the-art" cellular assays toward clinical applications using homogeneous time-resolved fluorescence technology is described. An overview of the luminescent lanthanide macrocyclic compounds developed at Cisbio in the recent past is given with an emphasis on specific constraints required by specific applications. Recent assays for drug-discovery and diagnostic purposes using both antibody-based and suicide-enzyme-based technology are illustrated. New perspectives in the field of molecular medicine and time-resolved microscopy are discussed.



INTRODUCTION

In the early 1980s, rare-earth chelates were used as long-lived tracers in heterogeneous immunoassays.[1] In the same era, Ullman et al.[2] pioneered homogeneous immunoassay methods particularly by using fluorescence resonance energy transfer.

Shortly after, following evolution of the immunological and biological analytical field toward automation and high-throughput screening, we launched a long-term research program for developing technologies and reagents suited for the monitoring and quantification of biomolecular interactions in biological fluids.

The following elements that we thought would be mandatory for the development of such technologies were identified: (1) a highly sensitive analytical detection technology using a tracer stable in biological media, (2) a modulation process of the tracer signal connected to the biomolecular interaction of interest, and (3) a measurement process that should be unaffected by the variations of the optical properties of the biological media.

This original global approach led us to identify at an early stage the interest of homogeneous technologies (no separation between bound and free tracer in an immunoassay) and particularly those using the modulation of the sensitive fluorescence signal.

However, in a homogeneous assay, the measurement takes place directly in the biological fluid where the intrinsic fluorescence of compounds and proteins can lead to serious limitations of sensitivity because of a high background obscuring the signal from conventional fluorophores in a steady-state fluorescent measurement.

We recognized the need of long-lived luminescent tracers, which as pointed out by Wieder[3] using time-resolved fluorescence detection, should eliminate most of the short-lived protein and diffusion background contributions.

Therefore, because of their unique photophysical and spectral properties, we identified the rare-earth complexes as being the key elements that could contribute to the design of a new homogeneous technology for simple and automated immunoassays. At this time, rare-earth chelates were already known as tracers in separation immunoassays. The luminescent chelate is formed in an extra step, after separation of the biological or blood sample, in controlled media containing surfactants and organic ligands.[4,5]

Rare-Earth Cryptates. When rare-earth chelates are used as tracers in homogeneous assays, incubation and measurement occur in biological fluids that suppose a high stability of the chelate in the presence of a high concentration of endogenous proteins and ions; otherwise, a loss of the signal is expected. The stability of the existing chelates in biological media did not fit the more stringent constraints of homogeneous assays. Instead of the well-accepted criteria of high thermodynamic stability, which drove the synthesis of new rare-earth chelates at that time, we stressed the key importance for a high kinetic stability in biological media for these new applications.[6]

This new paradigm opened the way for a new class of rare-earth chelates based on macrocyclic backbones and chemistry: the cryptates. A fruitful collaboration with Lehn starting in 1983 allowed us to illustrate the pertinence of our analysis and to synthesize a series of new europium and terbium rare-earth cryptates with tuned photophysical properties. Their conjugation with a diversity of biomolecules such as proteins, peptides, oligonucleotides, etc., was instrumental in the development of homogeneous fluorescent technologies.[7−12]

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Time-Resolved Förster Resonance Energy Transfer (TR-FRET). At the same time, at Cisbio, we explored the photophysical processes of rare-earth cryptates and cryptates that could be used in the design of assaying a real biological environment in a homogeneous way.

We discovered that, despite a low global luminescence quantum yield, rare-earth cryptates could be used as efficient fluorescent donors in resonance energy transfer (the rare-earth ion acting as the donor in this case), leading to long-lived emission of the fluorescent acceptor. Moreover, when an acceptor that emits between the characteristic emission lines of the rare-earth ions is chosen, a simultaneous detection of donor and acceptor emissions allows to us use the rare-earth emission as an internal probe to correct the acceptor emission from variations of the optical quality of the biological media.[10]

In the early 1990s, by combining these elements, we developed homogeneous time-resolved fluorescence (HTRF) and pioneered the TR-FRET technology. The power of the technology was illustrated, using the specific fluorescent properties of rare-earth cryptates, to probe molecular interaction mechanisms in biology and showed the following unique features: (1) the use of rare-earth cryptate emission with their atomic-like spectra allowing simultaneous detection of donor and acceptor signals; (2) rare-earth cryptates allowing high Förster distances to probe most of biological interactions; (3) homogeneous assays leading to a very simple mix and measure format easy for automation; (4) homogeneous assays allowing kinetic measurements; (5) ratiometric measurement, which allows one to avoid measurement issues due to optical quality variation between samples.

This allowed us to decipher biomolecular interactions in the real world of biology with applications most notably in clinical diagnostics and drug discovery.

Clinical Diagnostics. The set of unique properties offered by this fluorescent homogeneous technology **specific to the usage of kinetically stable rare-earth cryptates** allowed our team to pioneer the field and develop original instruments and immunoassays for the measurement of important blood circulating haptens, hormones, proteins, and cancer biomarkers.[13] The Kryptor technology[14] was introduced to the market in the late 1990s, showing high robustness and convenience, particularly for the measurement of tumor markers. It is the only example of immunometric homogeneous technology on the market[14] in the field of clinical diagnostics.[15]

Drug Discovery. Starting in the mid-1990s, we turned our interests toward the needs of the research community involved in drug discovery.[16]

Using rare-earth cryptates and TR-FRET, we have investigated directly or through collaborations most of the biological mechanisms that can be of interest as models to identify new chemical entities for important pathologies [DNA, RNA hybridization and mutation, nuclear helicases, telomerases, caspases, kinases, phosphatases, proteases, inhibitors or activators, P53 and other protein−protein interactions, epidermal growth factor receptor (EGFR), and other receptor ligand interactions, all mechanisms involved in important pathologies such as cancer, metabolic disorders, and degeneracy].[17]

The demonstrated effectiveness of the technology, to answer specific needs of the researchers in this field, makes HTRF nowadays a standard for drug-discovery processes in the pharmaceutical industry.

The rapidly growing knowledge in cellular biology opens up new fields of investigation with additional constraints and challenges such as working with living cells and native tissues. Nowadays, the bridge is being made between the discovery of these biological mechanisms and the clinic, requiring new optimizations and developments.

Besides a review of the basics of the chemistry of rare-earth cryptates and HTRF technology, the purpose of this Forum Article is also to illustrate recent research to further develop and adapt the technology to these new constraints and environments for answering new challenges in the field of molecular medicine.

PRINCIPLES OF LANTHANIDE-BASED TR-FRET AND HTRF

The interesting luminescent properties of lanthanides have been used throughout the scientific world and discussed extensively in reviews.[18−24] Briefly, the long lifetime of the excited state of the lanthanide ion, which is linked to its low absorption cross section, is its main feature. Because of the low absorption cross section, direct excitation of the lanthanide is cumbersome. Because organic chromophores are quenched by lanthanide ions (see ref 24 and references cited therein), it became clear that, to achieve increased excitation and emission, organic chromophores should be coordinated to the lanthanide ions. Our strategy to develop new lanthanide complexes is driven by the needs of the pharmacologic and diagnostic industries and the availability of affordable instrumentation to measure biological interactions. Detection systems in the near-IR are relative expensive and for economic reasons not an option to incorporate into plate readers. Therefore only europium and terbium complexes with strong emission bands in the visible are of direct interest. Furthermore, excitation sources in plate readers are usually limited to 320−340 nm excitation wavelengths; hence, complexes need sufficient absorption cross sections in this spectral region to be of interest for our technology. The europium cryptates developed by Lehn, Mathis, and co-workers in the 1980s[8,25] proved to be excellently suited,[10,16] and using these compounds led to development of the HTRF technology described for the first time in the early 1990s by Mathis.[10,16] Basically, the lanthanide cryptates are used as donors in a FRET system using compatible acceptor fluorophores such as the modified fluorescent protein allophycocyanine (XL665)[10,26] or the d2[27] dye, a polysulfonated cyanine, emitting strongly in the 665 nm region. When the donor and acceptor are in the proximity and FRET occurs, because of the millisecond lifetime of the donor excited state, a delayed acceptor emission is observed that can be discriminated from the fluorescence of the medium and direct excitation of the acceptor by using time gating, which is typically set at 50 μs for plate-based assays (Figure 1).

A second important feature due to the large pseudo Stokes shift and to the atomic-like emission spectra of the lanthanide complexes is that the donor intensity in the acceptor channel (at 665 nm) is very low compared to FRET with typical organic fluorophores. The third major parameter in HTRF is the use of an acceptor over the donor intensity ratio. The ratiometric treatment of the 620 and 665 nm intensity measurement allows real-time correction of the optical properties of the assay medium. This solves the problem of performing unbiased fluorescence measurement in the absorbing or turbid medium often observed either in clinical analysis or in drug discovery.

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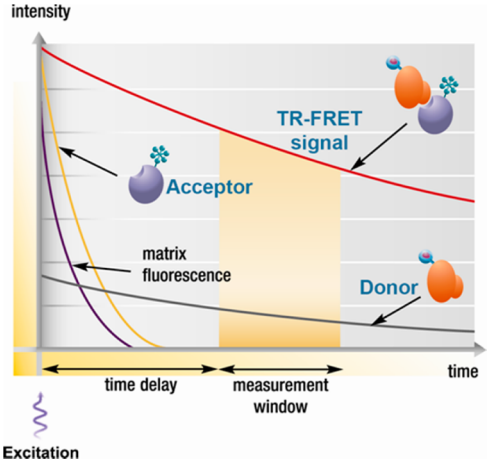

Figure 1. Schematic view of timing in HTRF assays. After excitation by either a flash lamp or a laser source, the donor (black curve, 620 nm) and the delayed acceptor (red curve, 665 nm) emissions are long-lived. Direct emission from the acceptor or compounds, proteins, and medium (orange curve) present in the well and the background emission/scattering from the matrix (purple curve) are suppressed by applying a delay of about 50 μs. Typically, a measurement window between 50 and 450 μs after excitation is applied in HTRF assays.

Taken together, this measurement strategy is of prime importance in the development of highly efficient and robust immunoassays.

LANTHANIDE COMPLEXES

Opposed to the main trends, we pointed out early on[6] that the key parameter to design an efficient fluorescent probe for immunoassays was driven by kinetic considerations. Indeed, we focus our research on lanthanide macrocycles that display high kinetic stability in biological media, and the logical choice was to investigate cryptates such as **1**[8] and their derivatives. More recently, we also started to investigate macrocycles bearing pendant arms (see Charts 1 and 2) to stabilize the complex that might be interesting enough to be incorporated into an immunoassay. Because the lanthanide atoms possess a very low extinction coefficient, sensitization through organic chromophores is mandatory to obtain a lanthanide complex displaying high brightness (quantum yield × extinction coefficient). Criteria to design a suitable lanthanide (europium and terbium) complex have been extensively reviewed[18,21,24] and can be summarized as follows: a high absorption cross section above 330 nm and especially around 337 nm (nitrogen laser); the highest quantum yield possible; no quenching in biological media, strong kinetic stability, high solubility in an aqueous buffer, a strong emission spectral band around 620 nm corresponding either for europium to the transition $^5D_0 \rightarrow {}^7F_2$ or for terbium to $^5D_4 \rightarrow {}^7F_3$ and an excited-state lifetime above 1 ms. Furthermore, an orthogonal functional group allowing bioconjugation is necessary to set up robust immunoassays. Cryptate **1** based on a bipyridine subunit (EuCtris-bipyridine) containing two amine functionalities for bioconjugation was among the first lanthanide complexes that fulfilled the requirements. In the EuCtris-bipyridines **1**, the rigidity afforded by the bipyridine moiety tends to increase the activation parameters, and a ΔG^* of approximately 25−30 kcal·mol^{-1} can be reached. On the contrary, the transition state of europium chelates is easily reached with a ΔG^* of 0−10 kcal·mol^{-1}, for instance, by protonation of the carboxylate groups.[6] Thus, this cryptate has been used extensively until now for HTRF assays. Although it displays both good photophysical and chemical properties, this cryptate has several drawbacks. It needs the addition of potassium fluoride to prevent quenching (deactivation of the luminescent excited state) because of the presence of two water molecules in the europium coordination sphere.[28] Second, the excited-state properties are not suitable for the corresponding terbium cryptate, and the absorption spectrum is not optimal at 337 nm. To circumvent these points, we developed new lanthanide macrocyclic complexes. Because of the very stringent set of physicochemical properties needed for real-world applications, the main difficulty was to tune one parameter without affecting the other factors, which contribute also to the overall luminescent properties.

To shift the maximum of the excitation wavelength bathochromically, several antennas have been studied in the cryptate framework.[29−31] The best compromise was obtained

Chart 1. Europium Macrocycles Studied in This Paper

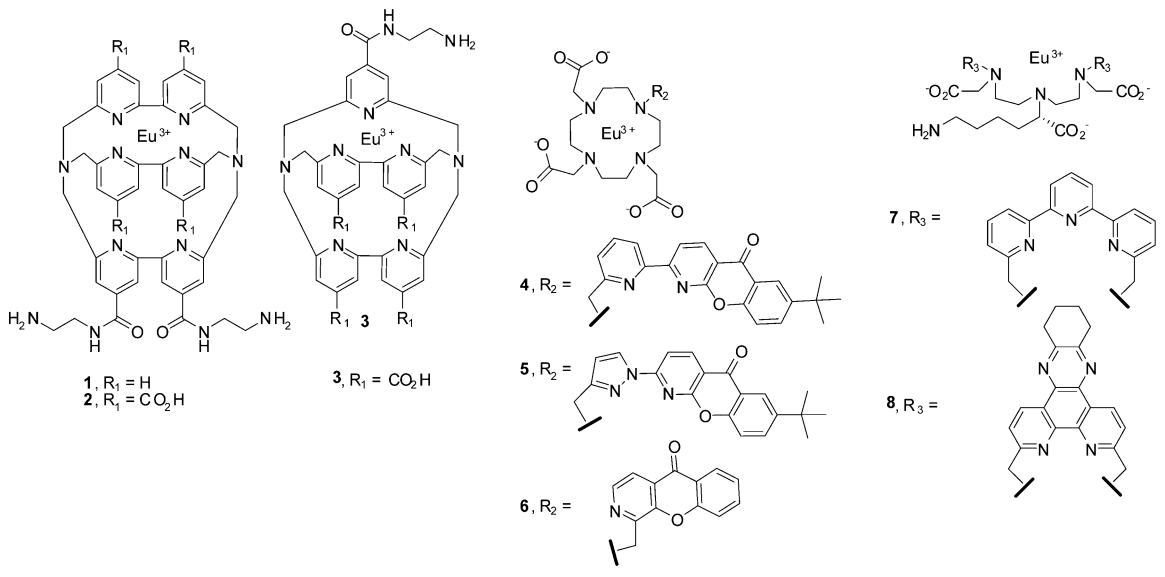

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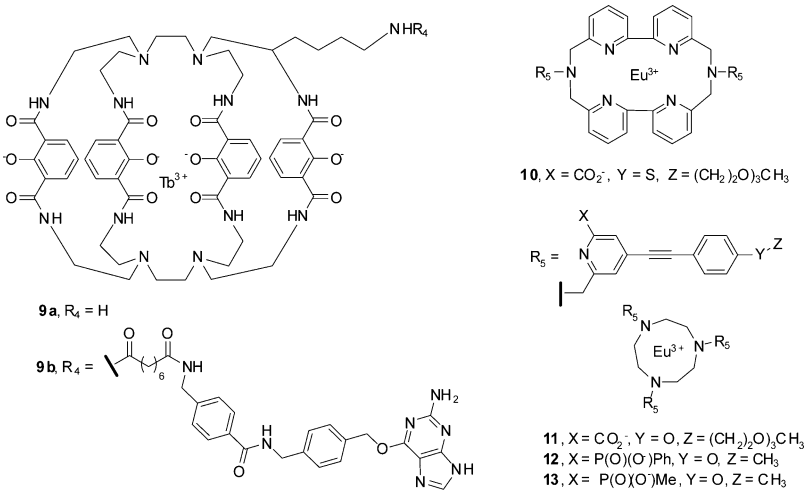
Chart 2. Recently Developed Terbium and Europium Macrocycles Discussed in This Paper



with Eu⊂tris-bipyridine·4CO_2H·cryptate **2** because the additional carboxylate function shifts the maximum of the excitation wavelength through extended conjugation and does not affect the solubility in the biological buffer.[28] One of the europium excited-state quenching processes is assigned to ligand-to-metal charge transfer or photoinduced electron transfer enabling reduction of Eu^{3+} (millisecond luminescent lifetime) to Eu^{2+} (nanosecond luminescent lifetime in solution[32]).[28,33] Eu⊂pyridinebipyridine cryptate **3** has been synthesized to prevent this quenching by reducing the size of the cavity. Although the goal to increase the luminescence quantum yield was achieved, the environment around europium changed and therefore shifted the $^5D_0 \rightarrow {}^7F_4$ emission band within the acceptor measurement channel, which dramatically affected the immunoassay sensitivity.[28]

Complexes based on DOTA macrocycles have been designed and assessed in collaboration with the Parker group.[34,35] Several complexes have been designed including 2-pyridylazaxanthone **4**, 2-pyrazoylazaxanthone **5**, or the less sterically hindered 3-azaxanthone **6** antennae, allowing sensitization of either europium or terbium. These complexes were not suitable for our applications because they proved to be quenched or partially quenched in the presence of divalent cations such as Mn^{2+} possibly through an electron-transfer mechanism.[35] In lanthanide chelates, divalent cations present at high concentrations in biological fluids can displace the lanthanide ions, destroying its luminescent properties.[21]

Some new complexes whose skeleton has been initially developed by Picard and co-workers[36] were synthesized with an extra-cyclic functional group, allowing bioconjugation.[37] The new synthetic approach generated flexible framework useful to screen chromophore efficiency. To validate the strategy, Eu⊂tpy (2,2′:6′,2″-terpyridine) **7** and Eu⊂dpqc (dipyrido-6,7,8,9-tetrahydrophenazine) **8** have been synthesized, and we demonstrated that the conjugates maintain the photophysical properties of the complex itself.[37]

Considering terbium complexes, spectacular improvements have been reported by the group of Raymond with the so-called Lumi4-Tb **9**.[38] In our hands, we confirmed the exceptional brightness of the complex based on the 2-hydroxyisophthalamide moiety, and after bioconjugation, it is now broadly used in HTRF applications. The benzylguanine derivative **9b** (see the Supporting Information for the syntheses; so far unpublished data) proved to be excellently suited for studies on cell surface receptors (vide infra).

Taking advantage of the results reported by Maury and co-workers,[39] where an electron-rich arylalkynylpyridyl chromophore was used, we recently developed new europium complexes involving an intramolecular charge-transfer mechanism. Initially, we designed the new europium complex **10**, which displayed poor luminescent properties because of unexpected nonradiative processes induced by an internal isomerization equilibrium.[40] We circumvented these issues by incorporating the latest chromophore on a 1,4,9-triazacyclononane macrocycle. Indeed, these complexes **11**−**13** (Chart 2)[41] display exceptional brightness, optimal maxima of the excitation wavelength (between 335 and 345 nm), high stability in the presence of divalent cations, a very intense 620 nm emission band (symmetry around europium is of the C_3 type), the absence of water molecules in the inner sphere, and excited-state lifetimes around 1 ms. This new family of europium macrocycles is probably the brightest europium complex we have had in our hands, which allows us to envisage in the future very broad applications in terms of immunoassay sensitivity but also in the fluorescent microscopy field.[41,42]

■ BIOCHEMICAL APPLICATIONS

Luminescent lanthanide crypates, as illustrated in the previous section, are developed and optimized in order to be applicable for industrial applications. This translation is not evident and needs continuous reevaluation depending on the demands. It is interesting to observe the changes over a long period of time not only in requirements but also of trends in the assay design.

The development of these applications was driven by the naissance in the early 1990s of a new paradigm in the pharmaceutical research based on the availability of large chemical libraries and the belief that the possibility to screen as many compounds as possible would increase the probability to identify new active chemical entities. Therefore, technologies like HTRF, allowing homogeneous (no separation steps) testing of compounds on biochemical targets with high-throughput rate, were introduced in the discovery process of a major pharmaceutical company.

We have therefore developed a large panel of biochemical assays for this purpose, exploring many different biological interactions using TR-FRET technology. The first HTRF

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applications of europium cryptates such as **1**−**3** are exemplified below. A sensor to detect prolactin in serum is one of the first examples of an HTRF assay.[10] Two antibodies, labeled with **1** as the FRET donor and a cross-linked allophycocyanine as the FRET acceptor, were used to design a homogeneous sandwich immunoassay (proximity assay).

The versatility of the HTRF technique was further exemplified with various models representative of molecular and cellular processes chosen from the signaling pathways involved in cellular communication and expression. Assays such as epidermal growth factor receptor (EGFR)−ligand interaction, EGFR kinase activity, and Jun/Fos protein−protein interaction were developed as proof of concept.[16]

These early examples of HTRF assays[43] paved the way for a whole range of assays such as kinase,[27,44] biomarker[27] and bioprocess assays,[27] to name a few.

Time-Resolved Fluorescence Using Europium Tris-bipyridine Cryptate Labeled DNA Probes. The earliest application of **1** was performed using the corresponding streptavidin conjugate (**1**−SA) for the indirect labeling of either short oligodeoxynucleotide (ODN) or long DNA probes.[9] Such labeled probes were used for the detection of DNA sequences by hybridization on membranes (dot blot); in this assay, time-resolved detection could improve the signal/noise ratio, decreasing dramatically the fluorescence background arising from the membrane. The sensitivity thus obtained was of the same order as the colorimetric protocol based on enzymatic signal amplification. The clear advantage is that, as in radioisotopic labeling, one can perform a real-time measurement in contrast with colorimetric methods, which are typical end-point measurements.

A similar fluorescent-detection procedure was used to detect amplified DNA combining the sensitivity and specificity of a nested polymerase chain reaction (PCR)[45] with the advantages of time-resolved fluorescent detection.[46] After a first PCR amplification and dilution of these PCR products, a second amplification was carried out, using a pair of labeled oligonucleotide primers bearing biotin and dinitrophenyl (DNP) groups, respectively. After affinity collection of the double-labeled PCR amplicons on a SA-coated microtiter plate, fluorescence detection was carried out using an anti-DNP antibody labeled with **1** and time-resolved measurement using a laser source. Interestingly, this protocol could be used with either cryptate **1** or a ^{125}I-labeled anti-DNP antibody to compare the sensitivity of time-resolved fluorescence detection versus radioisotopic detection.

Europium Tris-bipyridine Cryptate Labeled Nucleoside Triphosphates. The 5-(aminoallyl)deoxyuridine derivative was used as a thymidine triphosphate analogue and is labeled with **1**. This labeled triphosphate (K-11-dUTP) could be enzymatically incorporated in the 3′ position of an ODN using the terminal deoxynucleotide transferase and in DNA fragments using DNA polymerases.[47] K-11-dUTP was also used to monitor the extension reaction of a biotinylated oligonucleotide as the substrate for telomerase in a telomeric repeat amplification protocol, as described in Figure 2. After the addition of an allophycocyanine−SA conjugate, the extension products give rise to FRET with the incorporated cryptate.[48]

1-labeled uridine triphosphate (K-11-UTP) was obtained similarly by coupling the **1** moiety to a 5-aminoallyl-functionalized UTP analogue. K-11-UTP can be directly incorporated into RNA strands during enzymatic synthesis.[49] This was demonstrated in an in vitro transcription reaction

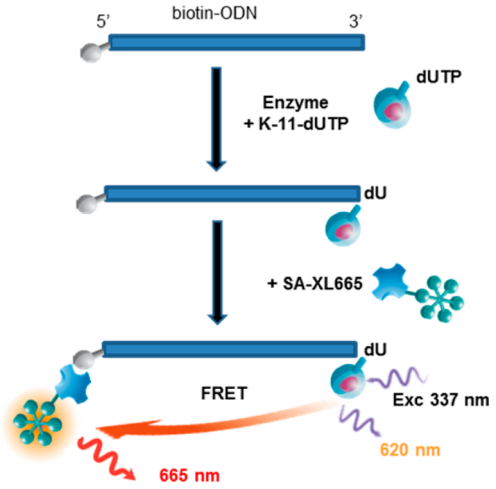

Figure 2. HTRF extension assay by the incorporation of a cryptate-labeled nucleotide into a biotinylated oligonuleotide primer. This format is exemplified by the telomerase-mediated incorporation of K-11-dUTP. The cryptate-labeled K-11-dUTP analogue is incorporated during enzymatic elongation of the biotin−ODN primer. After the addition of a SA−XL665 conjugate, a FRET signal is detected between the incorporated cryptate donor and the XL665 acceptor. A similar assay format can be used to monitor the incorporation of K-11-UTP in RNA.

promoted by T(7) RNA polymerase. The reaction was performed in the presence of K-11-UTP and biotin-labeled cytidine triphosphate (biotin-16-CTP). After the addition of the SA−XL665 conjugate, a FRET signal between the europium cryptate and the SA−XL665 acceptor bound to the biotin residues thus incorporated is observed. The study of this FRET detection assay format showed that such doubly labeled RNA can be easily detected even when a very low percentage of K-11-UTP is used (less than 1% of the total UTP concentration).

Detection of Single-Nucleotide Polymorphisms (SNPs) Using an Oligonuleotide Extention Assay. Because solid tumors contain a mixture of cancer cells representing SNPs (or mutations) and normal cells, a mutation present in the tumor sample may represent only a minor fraction of the total DNA. Therefore, tumor sample analysis requires detection strategies ensuring high sensitivity. Detection of known mutations can be broadly classified as either hybridization- or enzyme-based methods; the latter are inherently more specific because they rely on discrimination by an enzyme.

This enzymatic reaction can be a ligation, as in oligonuleotide ligation assay,[50] or a polymerase, as in minisequencing, also known as SNP extension (SNuPE) or template-directed dye terminator incorporation.[51]

In SNuPE assays, a DNA polymerase is used to label a specific primer hybridized to the target sequence by incorporating a single-labeled dideoxynucleotide (ddNTP). Such incorporation can be conveniently monitored using HTRF detection, as described in Figure 3.[52] The ODN primer was labeled on the 5′ end by coupling with **1**. In the presence of polymerase (sequenase) and of the biotin-labeled dideoxynucleoside triphosphate, complementary to the base next to the primer 3′ end, this ddNTP was incorporated only if the primer sequence was fully matching the sequence to be analyzed. This was exemplified using a portion of the coding sequence of the exon 7 p53 gene, CCG represented the wild-type codon 248

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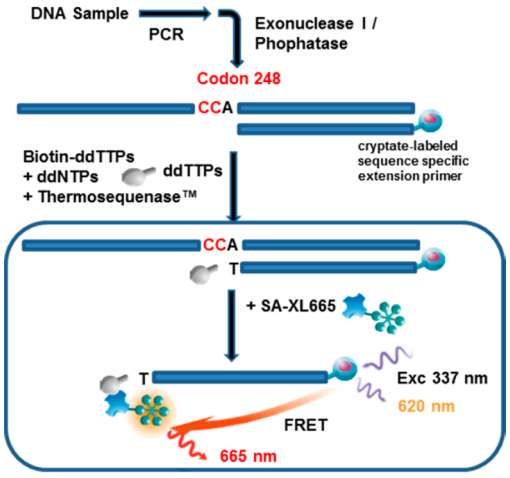

Figure 3. Detection of SNPs using an oligonuleotide extension assay based on HTRF detection. This figure describes the procedure used for codon 248 minisequencing in the p53 tumor suppressor gene. It exemplifies HTRF detection after the enzymatic incorporation of a labeled nucleotide onto a cryptate-labeled oligonucleotide primer. The DNA containing the SNP to analyze is first PCR-amplified and then purified by enzymatic digestions. A cryptate-labeled sequence-specific extension primer hybridizing to the antisense strand of the amplicon is annealed immediately adjacent to the polymorphic site. Extension in the liquid phase is performed in the presence of three nonlabeled ddNTPs and one biotin-labeled ddNTP (here using biotin−ddTTP as an example, leading to incorporation). Following the extension reaction, SA−XL665 is added and a TR-FRET signal is observed.

and CCA the corresponding mutated sequence. Biotin−ddCTP was incorporated by the enzyme on the primer 3′ end only in the presence of the wild-type codon; conversely, biotin−ddGTP was incorporated only in the presence of the CCG-mutated codon. Neither the corresponding ddATP nor ddTTP was incorporated. After the addition of SA−XL665, the incorporation of biotin−ddNTP was monitored by the FRET appearing between the europium cryptate donor and the SA−XL665 acceptor interacting with the biotin residue incorporated.

Mutation Detection Using DNA−Protein Interaction. The detection of mutation based on hybridization alone suffers from high false-positive rates, the use of a protein as auxiliary can improve the specificity, and this was exemplified using mutS, which is a protein involved in DNA mismatch repair processes. An indirect HTRF assay format was designed using 6-His-tagged mutS and XL665-labeled anti-(6-His) mAb in association with **1**−SA conjugate and biotinylated 21-mer DNA duplexes.[53] This assay was used to analyze the interactions between mutS and mismatched DNA or DNA containing the most common lesion of the anticancer drug cisplatin. A semidirect DNA/mutS HTRF assay using a **1**−mutS conjugate was also tested. Interestingly, a mobility shift assays showed that the **1** labeled mutS retains its DNA mismatch binding affinity.

CELL-BASED ASSAYS

In the previous section, we have shown that HTRF assays can address various biological interactions. The incredible progress in the knowledge of cell function and the discovery of new biological mechanisms were identified as possible important targets for drug discovery. This opened up a new field for development of the technology, however with new constraints because of the need to work with living cells. A selection of the cell-based applications developed is discussed in this section.

In Vitro Assays To Detect Stimulation of Membrane Receptors. In drug discovery, there is a need to use native and more physiological models; hence, cell-based assays are nowadays key technology to validate drug candidates. The ultimate goal is to monitor interactions on unmodified cell lines and even native tissues, but expression levels of the protein of interest are often low. To circumvent this problem, the DNA of the protein of interest is inserted into standard cell lines, leading to its expression, and can be used to monitor cellular responses of interest for drug discovery. Such responses can be signaling events within the cell after agonist stimulation of the receptor of interest. The changes of the concentrations of molecules involved in these reactions can be measured with HTRF-based biosensors.

Our main focus in recent years is the so-called G-protein-coupled receptor (GPCR)[54,55] or seven-transmembrane receptor (7TM) family, which is one of the most important drugable families[56] with up to 600 different proteins present in the human body. Basically, after stimulation of a GPCR with an agonist, the G-protein(s) are activated, which involves the displacement of guanosine diphosphate by guanosine triphosphate (GTP), and consequently signaling cascades within the cells are triggered. In the past decade, it also became evident that GPCR can also respond independently from G-proteins;[57,58] hence, the 7TM abbreviation has become more popular. For pharmaceutical companies, it is of prime interest to know the response of their libraries on the protein/receptor of interest. Primary screening is done by monitoring concentration changes of the second messengers involved in the signaling cascades such as Ca^{2+} ions or 3′−5′ cyclic adenosine monophosphate (cAMP). After analysis of the results, counterscreens and/or secondary screening on a subset of the initial library can be performed. This can involve ligand-binding studies, receptor internalization, protein phosphorylation, or β-arrestin recruitment as examples.

We have developed such functional assays or biosensors based on HTRF to monitor these signaling cascades. Two examples will be treated that form the basis of our GPCR portfolio: cAMP[27] and IP-One[59] assays. cAMP has been identified as a secondary messenger because its concentration is dependent on the up or down regulation of adenylate cyclase, which is involved in the signaling pathway of the G-proteins $G_{\alpha s}$- and $G_{\alpha i}$-coupled GPCR, respectively. The assay principle is shown in Figure 4. A monoclonal antibody labeled with **1** recognizes a cAMP molecule conjugated with a red-emissive acceptor dye (d2). Upon formation of cAMP, the generated HTRF signal will disappear because of competition of the unlabeled cAMP with the d2-labeled cAMP. The G_q-coupled receptors, which activate the phospholipase C pathway, have traditionally been monitored by measuring the $[Ca^{2+}]$ increase.[60] However, this signal, triggered by D-*myo*-1,4,5-inositol triphosphate, is transient, and thus for high-throughput screening (HTS) is not an ideal indicator. However, D-*myo*-1-inositol monophosphate (IP1), a further metabolite in the cascade, can be accumulated by inhibiting the enzyme inositol monophosphatase by the Li^+ ion. By using this propensity, the accumulation of IP1 can be measured[59,61] using a biosensor similar to the cAMP assay described above. The key to developing these assays is not just choosing the right donor and acceptors but also developing high-affinity antibodies. Both of these assays are now routinely used in HTS.[27,62] Current

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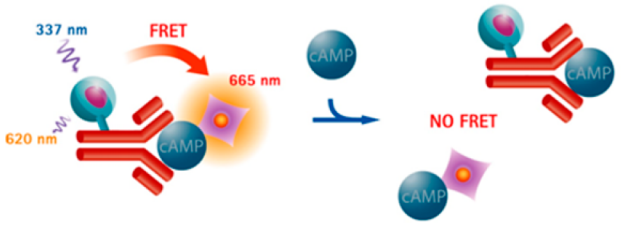

Figure 4. cAMP HTRF assay principle. In this competitive assay, cAMP is covalently labeled with the d2 dye, and an anti-cAMP antibody is labeled with a cryptate donor. Upon stimulation of the receptor of interest in living cells, cAMP is generated. After cell lyses and the addition of two labeled compounds, unlabeled cAMP will compete with cAMP−d2, and thus a decrease of FRET is observed. The concentration of cAMP can be determined as well using this cAMP sensor. The detection of inositol monophosphate (IP-One assay[59]) operates through a similar strategy.

ongoing efforts are made to develop sensors for later signaling events in these cascades and G-protein-independent signaling such as the detection of mitogen-activated protein kinases, tyrosine kinases, and phosphorylation. An example is an assay to detect and quantify ERK1/2 phosphorylation.[63]

TR-FRET Assays on Living Cells To Determine the Membrane Receptor Structure, Conformation, and Ligand Binding. Performing TR-FRET assays on living cells has supplementary requirements of the lanthanide complexes used.

(1) Because high-ionic-strength buffers disturb the cell structure, using concentrated KF solutions to enhance the luminescence of **1** is cumbersome.

(2) Nonspecific interaction of the lanthanide with the cellular environment needs to be negligible to reduce the signal from events not of interest to the interaction under study.

(3) Studying interactions at the cell surface requires that the lanthanide conjugate in question does not adhere to the cell membrane and should not internalize.

In the last 2 decades, receptor pharmacology has been revealed to be much more complex than previously thought. One of these complexities arises from the notion that GPCRs are not just occurring as entities on themselves but can form complexes at the plasma membrane with other GPCRs, which can modify the pharmacology of these receptors.[64] Compounds are discovered that can bind preferably to such homo- or heterooligomers, and hence target-specific assays will become more important. Antibody-based strategies are the most straightforward way to proceed, and, indeed, upon using a EuCpyridinebipyridine cryptate (**3**)-labeled anti-HA antibody and an Alexafluor-labeled antimyc antibody, it could be validated that $GABA_{B1}$−$GABA_{B2}$ exists as heterooligomeric species in living cells.[65] Rondard et al.[66] used the anti-HA- and anti-FLAG-labeled antibodies to show that *N*-glycan at the $GABA_{B2}$−venus fly trap (VFT) domain interface prevents dimerization with $GABA_{B1}$. To address the need for target-specific assays at the cell surface and to reduce the possible steric effects of antibodies, we developed a strategy based on the so-called "suicide enzymes", such as the SNAP,[67] CLIP,[68] or HALO tag.[69] Because of their nature, these proteins can only react once toward their substrate, forming a covalent bond. Although many protein-tagging systems exist,[70] a covalent strategy is needed because we would like to monitor interactions at low concentrations (picomolar to nanomolar range) and noncovalent techniques are more prone to dissociation, which will lead to artifacts in biological systems. To develop assays at the outer surface of the cell membrane, it would be necessary to tag a protein with a lanthanide cryptate. One of the first requirements is that labeling would only occur at the cell surface. If substrates would penetrate the cell membranes easily, labeling of immature proteins and proteins unavailable for response could increase nonspecific interactions on the assay and reduce the efficacy and dynamic range of the assay. Second, nonspecific labeling of the substrate to the cells or plates should be minimal. Third, the reactivity of the substrate should be elevated to reduce the costs and minimize the chance for the first two requirements to occur. Although it is often argued that protein tags might alter the functionality of the protein, with the SNAP tag,[67] we have encountered very few examples where this was indeed the case.

The first case study, in collaboration with the Pin laboratory at the Institut de Génomique Fonctionnelle in Montpellier, proved the power of the combination of the SNAP tag and antibody labeling, and TR-FRET technology was done on oligomerization studies of the $GABA_B$ and mGluR1 receptors,[71] GPCRs, which are involved in brain signaling of the neurotransmitters γ-aminobutyric acid and glutamate, respectively. Tagging these receptors using the SNAP tag and labeling with a combination of benzylguanine (BG)−europium cryptate and a red fluorescent acceptor proved for the first time that tetramers (or dimers of dimers of $GABA\text{-}_{B1}$−$GABA\text{-}_{B2}$)[71] of the GABA receptor exist in living cells. A further improvement of the system was achieved by designing the BG derivative **9b** including the very bright Lumi4-Tb.[38] This compound proves to be very useful not only because of its brightness but also because of its high reactivity toward SNAP-tag labeling and its low nonspecific labeling of cells not expressing SNAP-tagged proteins. Another advantage of the Tb^{3+} donor is that TR-FRET can be measured at several spectral windows using fluorophores emitting, for instance, in the green or red regions of the spectrum. This opens the possibilities for multiplexing (examples of terbium multiplexing are given by Geißler et al.[72] in this Forum issue).

With this so-called Tag-lite technology in hand, we have developed target-specific homogeneous ligand-binding assays on living cells using in-house-developed pharmacologically active fluorescent ligands.[73,74] Traditionally, binding assays[75] are done using radioactive compounds and have for a long time been the only way to address receptor pharmacology. It is, however, not practical for high-throughput screening purposes. Fluorescent ligand-binding assays have appeared since,[75,76] but still washing steps are often required and nonspecific binding involved often cannot be ruled out. In the Tag-lite binding assay, TR-FRET only occurs when a true binding event is measured because it is measured by a proximity interaction between the two binding partners. In Figure 5, an example of the fluorescent ligand binding and competition binding Tag-lite assay on the cholecystokinin-2 (CCK2) receptor is shown. Tag-lite assays for 18 GPCRs have been described by Zwier et al.[74] Assays for more GPCRs such as the Bombesin, Serotonin, Muscarinic, and Glucagon receptors have been addressed using this technology.[77] Currently (June 2013), there are 56 different assays available.[77]

Inhibition constants measured in these Tag-lite assays are in the same range as radioactive assays, and signal-to-noise ratios are elevated. Furthermore, these assays can be done on cell suspensions and can be measured in a HTS setting using 384 and 1536 well plates. Furthermore, after cells are labeled with

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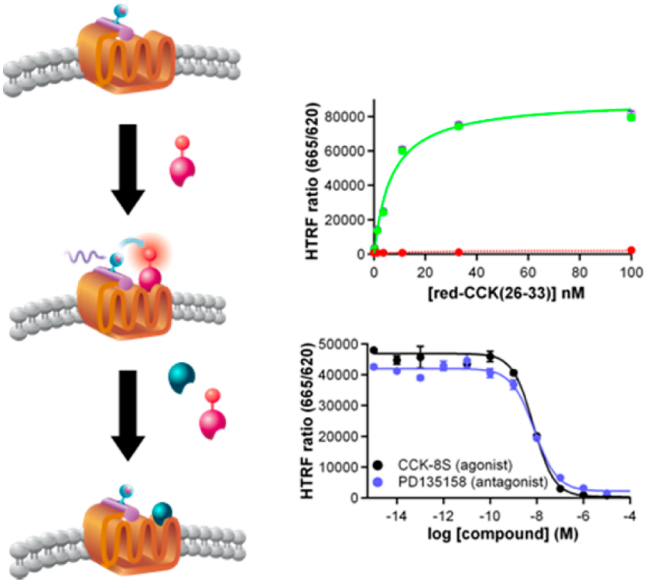

Figure 5. Tag-lite binding assay for the CCK2 receptor.[74] SNAP-tagged CCK2 receptors expressed in HEK293 cells are covalently labeled with **9b**. Binding of the fluorescent CCK(26−33) ligand to the receptor (green curve) can be followed by measuring the 665/620 HTRF ratio. Nonspecific binding (red dots) is measured by adding 10 μM CCK8S to these wells. A competition binding assay is done by adding 10 nM red-CCK(26−33) to the SNAP-Lumi4-Tb-labeled cells, which leads to a decrease in the HTRF ratio upon the addition of increasing amounts of a competitor ligand. In this case, an assay window (or dynamic range) of 67 is observed.

9b, the cells can be frozen and after shipment defrosted for use in assays. For the ghrelin receptor GSH-R1a,[73] 14 different ligands were tested in parallel with a radioactive assay on 384 well plates using prelabeled cells in suspension. It is shown that the ghrelin Tag-lite assay is more sensitive than the radioactive assay and that they were equally well able to properly characterize the ligands. A first opening toward HTS of the pleiotropic cytokine hormone leptin receptor OB-R has also been shown by labeling leptin with the d2 acceptor and monitoring its binding and competition using the Tag-lite format.[78] Recently, by using three time-resolved ligand-binding protocols using a d2 acceptor or **9**-labeled substrates/ligands, specific antibodies or both show that these techniques all are capable of measuring similar binding constants with high signal-to-noise ratios.[79]

Homo- and Heteromerization of GPCRs. Although oligomerization (or the formation of higher order protein complexes) of GPCRs has influenced the field for over a decade now,[80] assays to address this changed pharmacological landscape have been sparse.[81] The formation of homooligomers can be addressed using the Tag-lite technology using the SNAP tag. Basically, a standard concentration of **9b** is set while varying the concentration of the red or green acceptor BG derivative. If there is oligomeric organization, one will see a peak of the TR-FRET emission when there is an equivalent concentration of donor- and acceptor-labeled receptors, as shown for the $GABA_B$ and MgluR1 receptors.[71] On the M3 receptor, this and other strategies have allowed to show that this receptor is oligomeric and that its organization is regulated by ligand binding.[82,83] To address heterodimers, orthogonal protein-labeling methods are necessary. In order to address the dimeric landscape of mGluRs, such a strategy was developed by Doumazane et al.[84] All eight mGluRs were tagged with either SNAP or CLIP tags and labeled with **9b** and green fluorescent SNAP or CLIP substrates. This strategy was used to demonstrate that members of the mGluR family can form heterodimers but that this is restricted to strict dimers and to only certain groups within this receptor family. Using these SNAP-tagged mgluRs, a very powerful TR-FRET sensor

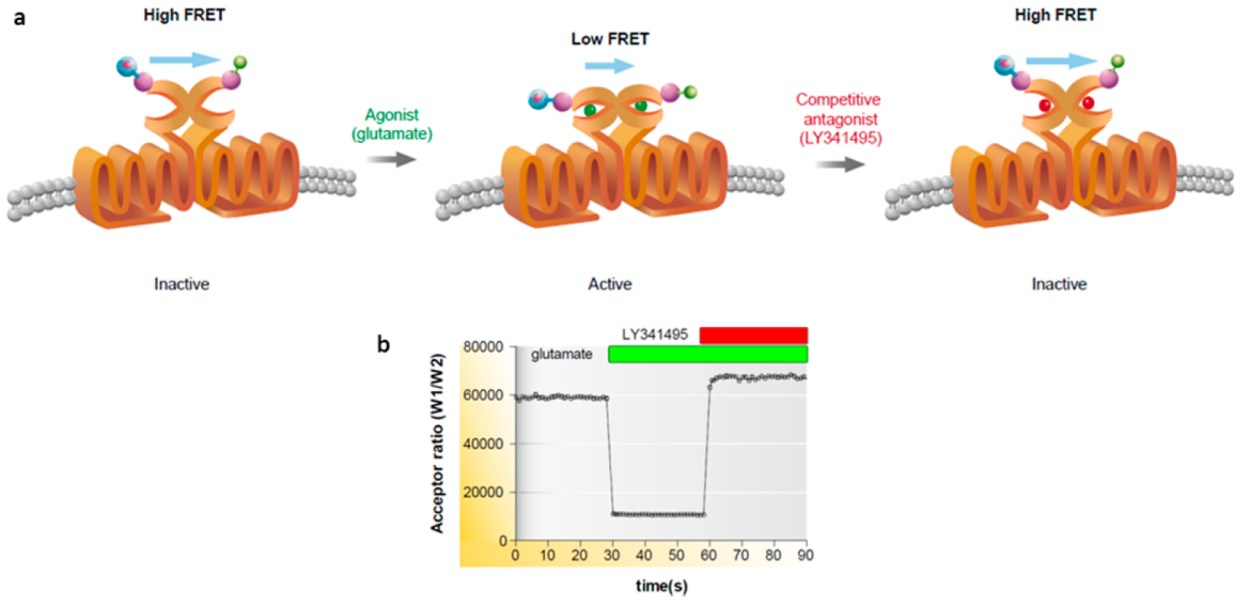

Figure 6. Metabotropic glutamate receptor 2 (mGluR2) TR-FRET activation sensor on living cells.[85] (a) Cartoon illustrating the assay principle. The SNAP-tagged mGluR2 dimer is labeled with **9b** and SNAP green, which leads to a green delayed emission signal at 520 nm upon excitation of the donor. Upon addition of the natural agonist glutamate, it is bound to the VFT binding domain, activating the receptor. This increases the distance between the chromophores, reducing the FRET intensity. Adding the antagonist LY341495 reverses the system. (b) TR-FRET output monitored in time. After 30 s, 100 μM glutamate is added, decreasing the FRET ratio. At 60 s, LY341495 is added, restoring the original signal. See Doumazane et al.[85] for more details.

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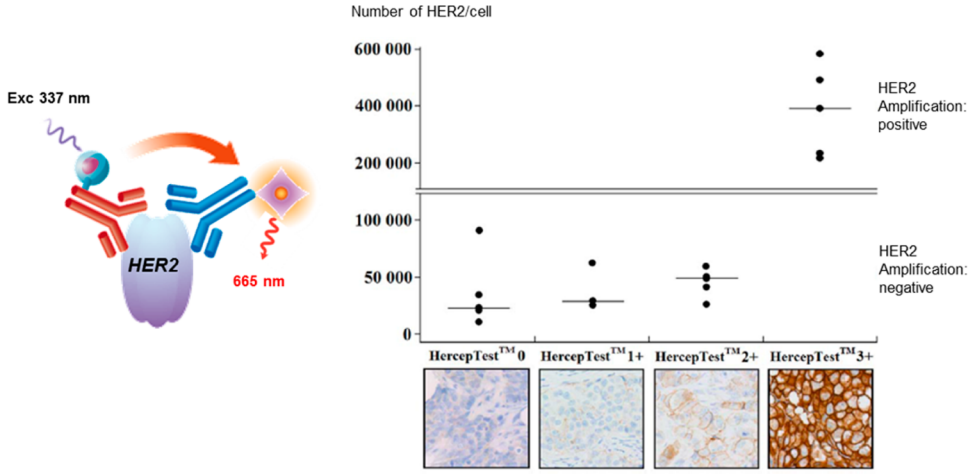

Figure 7. TR-FRET assay format (left) to determine the expression levels of HER2 receptors in breast tumor samples. Trastuzumab labeled with **9** and d2-labeled FRP5 antibodies, which recognize different epitopes of the HER2 receptor, give rise to a TR-FRET signal.[92] A comparison of the TR-FRET assay with standard techniques for assessment of the HER2 status is depicted on the right (reproduced from Ho-Pun-Cheung et al.,[92] an open access source). The number of HER2/cells was evaluated by TR-FRET in breast tumors stratified according to their Herceptest score and the HER2 gene amplification status. A Herceptest score of 3+ is considered positive, and patients with this score are considered for Trastuzumap treatment.

technology was developed to study the conformational changes of the extracellular ligand-binding domains of these receptors in living cells.[85] After stimulation of mGluR with agonist, the labeled dimer changes conformation, resulting in an increased distance between the fluorophores and reducing the TR-FRET (see Figure 6). Adding antagonist reversibly increases the FRET efficiency again, leading to a sensor with a dynamic range of up to 10, which is exceptional for fluorescent protein-based sensors. With this method, it is possible to study activation processes of mGluR in a relatively straightforward way and to identify the actions of partial agonists, positive- and negative-allosteric modulators.

The dynamics of the formation and stability of $GABA_{B1}$–$GABA_{B2}$ oligomers on living cells have equally been studied using a TR-FRET multiplex detection strategy using SNAP/CLIP-Lumi4-Tb and red- and green-emitting acceptors.[86] On the same receptor, Comps-Agrar et al.[87] have used a TR-FRET strategy with Lumi4-Tb- and d2-labeled antibodies to monitor the existence of higher-ordered oligomers of $GABA_{B1}$–$GABA_{B2}$ in the brain and evaluated the cooperativity of these oligomers using a mixed SNAP/antibody TR-FRET strategy.

TR-FRET ON NATIVE TISSUES

Finally, we turn toward practical applications at the bedside. To support the development of molecular medicine, there is a need to bridge the gap between cellular models and native tissues from a patient in order to stratify the responsive population and/or provide biomarkers for treatment efficacy. One of the new paradigms in this field is the importance of kinase receptor expression and oligomerization. This is the new playground where we focus our efforts in the development of the technology. As a proof of concept, we are exploring EGF receptor family receptors that are known as key proteins in the development of cancer.[88,89]

GPCR Oligomerization in Native Tissue. Although the evidence is growing that oligomers of GPCR play a significant pharmacologic role,[64] sensor technology to monitor the protein–protein interactions on native tissue is still lacking. A first example of TR-FRET assays between high-affinity fluorescent ligands on native tissue is described by Albizu et al.[90] using an approach based on labeling with **3** or **9** and fluorescent acceptors of agonists and antagonists of the vasopressin and oxytocin receptors. They succeeded in demonstrating the presence of oxytocin receptor dimers or oligomers in mammary glands of lactating rats.

Ex Vivo HER1 and HER2 TR-FRET Assays on Tumor Tissue. The development of cancer immunotherapy using monoclonal antibodies such as Cetuximab (Erbitux) targeting EGFR (HER1) for colorectal cancer treatment or Trastuzumab (Herceptin) targeting HER2 for treatment of breast cancer are associated with a companion test predicting response to therapy. For instance, treatment with Trastuzumab is indicated in cases where HER2 expression has a score of 3+ determined by immunohistochemistry (IHC) in association with either chromogenic or fluorescent in situ hybridization (CISH/FISH) for the ambiguous IHC scores. Efforts have been made to standardize such IHC analysis and improve the accuracy and reproducibility;[91] however, the technique remains semiquantitative.

In this respect, a quantitative HER2 assay on tumor tissue is of interest.[93] The development of new therapeutic monoclonal antibody programs would also benefit from cellular or ex vivo assay able to quantify receptor expressions and analyze receptor associations (homo- and heterodimers). A TR-FRET protocol has been developed to quantify EGFR/HER2 heterodimers on the cell surface in order to predict the efficacy and explain the mechanisms of action of therapeutic mAbs.[94] Cancer cells cultured in microtiter plates were incubated with various anticancer agents, and after a short fixation step, **9**-labeled anti-HER2 and d2-labeled anti-EGFR antibodies were added. After incubation and washing, the time-resolved fluorescence of the Lumi4-Tb donor and d2 acceptor was measured respectively at 620 and 665 nm; the augmentation of a long-lived 665 nm signal was correlated with the presence of EGFR/HER2 dimers. The use of two anti-EGFR antibodies targeting distinct epitopes and respectively labeled with **9** and d2 allowed quantification of the EGFR expression. Similarly, the use of two labeled anti-HER2 antibodies allowed quantification of the HER2 expression in tumor cells, which is illustrated in Figure 7. The use of a single antibody labeled with either Lumi4-Tb or

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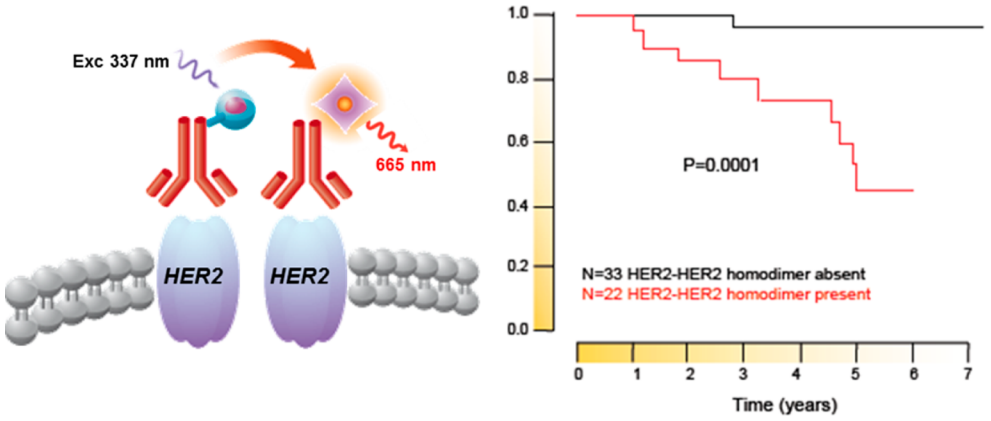

Figure 8. TR-FRET assay principle to monitor HER2 dimerization on tumor samples. Using an antibody labeled with either **9** or d2, only a positive signal will occur when there is a proximity interaction between the two units. The corrected TR-FRET intensity is proportional to the dimer concentration. Using different labeled antibodies, EGFR−EGFR or EGFR−HER2 dimer concentrations can also be obtained. On the right, a Kaplan−Meier curve for disease-free survival according to the presence or absence of HER2−HER2 homodimer assessed by TR-FRET in IHC−HER2 negative/ER positive patients. Note: these data were previously published at a conference.[97]

d2 allowed quantification of receptor homodimers (EGFR/EGFR or HER2/HER2). The assay principle is illustrated in Figure 8. Similarly, this protocol was used to quantify EGFR/HER2 dimers and study the efficiency of various anticancer treatments on the EGFR/HER2 dimer disruption.[95] Recently, an adapted version was also used to quantify HER2/HER3 heterodimers and study the effect of an anti-HER3 and anti-HER2 (trastuzumab) combination on HER2 low cancer cell lines, for which trastuzumab alone shows no or moderate efficiency.[96] This protocol was extended to the ex vivo TR-FRET detection of EGFR, HER2, and homo- and heterodimers in breast tumors cryosections.[92] In this ex vivo assay, with the cell number present in the sample being unknown, the 665 nm TR-FRET signal was normalized with the prompt fluorescence of the Hoechst-stained DNA measured simultaneously; the normalized signal is proportional to the receptor expression per cell. The EGFR expression determined by TR-FRET was significantly correlated with a EGFR mRNA copy number, and HER2 overexpression was confirmed by the standard IHC, FISH, and qPCR analyses.

Analysis of the quantity of HER2 homodimers measured by the normalized TR-FRET assay predicts a disease outcome in IHC−HER2 negative/ER positive breast cancer patients for up to 10 years of followup.[97] Using Cox proportional hazard analyses, the presence of a HER2 dimer was significantly associated with reduced disease-free survival (p = 0.0001; Figure 8).

LANTHANIDE-BASED TIME-RESOLVED IMAGING: TOWARD TR-FRET IMAGING

The HTRF and TR-FRET assays described above are all performed in plate-based formats and have been proven to be very successful, giving sensitive and quantitative data. However, detailed information on the cellular structure such as expression levels at different compartments of the cell cannot be retrieved from such data. Afterall, they are "bulk" measurements, not evaluating individual cells. Imaging of biological interactions or expression of proteins at different locations is of particular interest in the clinical field. Time-resolved (or time-gated) microscopy using lanthanide complexes might be suited to obtain better insight in cellular processes especially on native tissue. Time-resolved microscopy has the big advantage of suppressing background fluorescence, as has been demonstrated to increase detection limits significantly.[18,42] Ghose et al.[98] showed that using antibodies labeled with the pyridinebipyridine cryptate **3** it was possible to monitor $GABA_{B1}$−$GABA_{B2}$ heterodimers using a time-gated setup as a basis for future TR-FRET imaging, which was indeed recently described using derivatives of **9** by Rajapakse et al.[99] Using the SNAP-tag system, it proved also possible[100] to monitor lanthanide-based time-resolved imaging of GPCR, as is shown in Figure 9. After labeling vasoactive intestinal peptide receptor 1 (VPAC1)-ST receptors with SNAP−Lumi4-Tb, the labeled receptors are mainly present at the cell surface. After stimulation with the natural agonist vasoactive intestinal peptide (VIP), internalization of the labeled receptors is evident as vesicles are formed within the intercellular compartments.

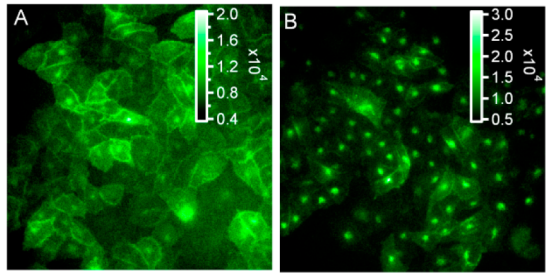

Figure 9. VPAC1-ST internalization monitoring by time-resolved imaging.[100] (a) CHO cells expressing VPAC1-ST are labeled with **9b** and imaged in time-resolved mode. (b) Stimulation with 1 μM of the natural agonist VIP leading to internalization of the VPAC1-ST−Lumi4-Tb complex. Color bars represent time-resolved intensity counts. Note: these data were previously published at a conference.[100]

Time-resolved spectral imaging of the very bright europium complex **13**, which can be localized in mitochondria,[41] was recently also achieved. The development and time-resolved imaging of pH[101] or anion sensors[102] derived from **13** prove the versatility of these types of europium complexes, and its possible future implication in time-resolved and TR-FRET imaging in drug-discovery and clinical applications.

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CONCLUDING REMARKS

On the bench side, biochemical assays are dealing mostly with isolated biological elements taken out of their cellular environment and are indeed far from being representative of the reality of true molecular interactions in biology. Taking advantage of the unique properties of rare-earth macrocycles and particularly cryptates and HTRF to probe molecular interactions in the nanometer range, we have opened up new tools and paths to explore such interactions in living cells and on the cell surface in collaboration with many academic partners, and particularly with the IGF "Institut de Genomique Fonctionnelle" in Montpellier in the field of GPCR.

From the early experiments using rare-earth cryptates and fluorescent cyanine-labeled antibodies against tags fused to the external parts of $GABA_B$ receptors to study the obligatory heterodimer to more sophisticated experimental setups using a suicide enzyme toolbox, we have investigated many different biological processes including binding, oligomerization, internalization, modulation of receptor activity, and deciphered their pharmacological behavior.

The next challenges lie on the bedside, dealing with analysis of new biological events and new paradigms, on native tissues in the frame of molecular medicine. Although we showed that the new arsenal of rare-earth macrocycles and HTRF technology allows quantification of receptor expression and oligomerization on human biopsy, still more challenges rely on optimization of the current compounds and technology to increased sensitivity and selectivity of the measurement, leading to more precise quantification and imaging conditions.

To achieve this, an ensemble of new characteristics that are hardly predictable on the basis of simple physicochemical properties must be fulfilled, among which are the following:

(1) The brightness of the donor needs to be increased. Care should be taken at the intensity levels of $^5D_0$ to $^7F_4$ and $^7F_3$ transitions between 650 and 700 nm, which are not predictable and contribute dramatically to increase the background of HTRF detection.

(2) Nonspecific binding of rare-earth complexes on tissues and the cell surface is also detrimental to sensitivity.

(3) Although not discussed here, the photophysical properties of the acceptor (quantum yield and emission wavelength) as well as solubility and nonspecific interactions with biomolecules are critical.

This is the reason why designing and synthesizing new macrocyclic complexes and cryptates remains of crucial interest and is still part of our research programs of collaboration with academia.

ASSOCIATED CONTENT

Supporting Information

Synthesis of compound **9b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): The authors are employees of the company Cisbio Bioassays.

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