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Switchable Lanthanide Luminescence for Detection of Biomolecules

by

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Misteepä sen tietää, mihin pystyy, ennen kun kokkeeloo.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by their Roman numerals (**I**-**IV**):

I **Ulla Karhunen**, Lumi Jaakkola, Qi Wang, Urpo Lamminmäki & Tero Soukka (2010). Luminescence switching by hybridization-directed mixed lanthanide complex formation. *Anal Chem* **82**:751–754.

II **Ulla Karhunen**, Jaana Rosenberg, Urpo Lamminmäki & Tero Soukka (2011). Homogeneous detection of avidin based on switchable lanthanide luminescence. *Anal Chem* **83**:9011–9016.

III **Ulla Karhunen**, Minna Soikkeli, Susanne Lahdenperä & Tero Soukka (2013). Quantitative detection of well-based DNA array using switchable lanthanide luminescence. *Anal Chim Acta* **772**:87–92.

IV **Ulla Karhunen**, Eeva Malmi, Ernesto Brunet, Juan Carlos Rodríquez-Ubis & Tero Soukka. Switchable lanthanide luminescent binary probes in efficient single nucleotide mismatch discrimination. *Submitted manuscript*.

ABBREVIATIONS

$ABTS^{2-}$	2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
AF680	Alexa Fluor 680
BiFC	bimolecular fluorescence complementation
bZip	basic-region leucine zipper
cAMP	cyclic adenosine monophosphate
CC2F/AM	β-lactame conjugate of coumarin and fluorescein
CFP	cyan fluorescent protein
Cy3/5	cyanine dye 3/5
DHF	7,8-dihydrofolate
DTPA	diethylenetriaminepentaacetic acid
(E)YFP	(enhanced) yellow fluorescent protein
Fam	carboxyfluorescein
fMTX	fluorescein conjugated methotrexate
FRET	Förster resonance energy transfer
GFP	green fluorescent protein
HPLC	high-performance liquid chromatography
LOD	limit of detection
LRET	lanthanide resonance energy transfer
LucER	inactivated luciferase
MB	molecular beacon
mDHFR	murine dihydrofolate reductase
MGA	malachite green aptamer
NFκB	nuclear factor kappa B
PCR	polymerase chain reaction
PKA	protein kinase A
PLA	proximity ligation assay
PNA	peptide nucleic acid
QD	quantum dot
Q-STAR	quenched Staudinger-triggered probes
QUAL	quenched autoligation
RCA	rolling circle amplification
*R*Luc	*Renilla* lusiferase
ROX	carboxy-X-rhodamine
RT	room temperature
SNP	singe nucleotide polymorphism
Tamra	tetramethylrhodamine
THF	5,6,7,8-tetrahydrofolate
TEV	tobacco etch virus
TPP	triphenylphosphine
UCP	upconverting phosphor
UV	ultraviolet

ABSTRACT

Binary probes are oligonucleotide probe pairs that hybridize adjacently to a complementary target nucleic acid. In order to detect this hybridization, the two probes can be modified with, for example, fluorescent molecules, chemically reactive groups or nucleic acid enzymes. The benefit of this kind of binary probe based approach is that the hybridization elicits a detectable signal which is distinguishable from background noise even though unbound probes are not removed by washing before measurement. In addition, the requirement of two simultaneous binding events increases specificity. Similarly to binary oligonucleotide probes, also certain enzymes and fluorescent proteins can be divided into two parts and used in separation-free assays. Split enzyme and fluorescent protein reporters have practical applications among others as tools to investigate protein-protein interactions within living cells.

In this study, a novel label technology, switchable lanthanide luminescence, was introduced and used successfully in model assays for nucleic acid and protein detection. This label technology is based on a luminescent lanthanide chelate divided into two inherently non-luminescent moieties, an ion carrier chelate and a light harvesting antenna ligand. These form a highly luminescent complex when brought into close proximity; i.e., the label moieties switch from a dark state to a luminescent state. This kind of mixed lanthanide complex has the same beneficial photophysical properties as the more typical lanthanide chelates and cryptates - sharp emission peaks, long emission lifetime enabling time-resolved measurement, and large Stokes' shift, which minimize the background signal. Furthermore, the switchable lanthanide luminescence technique enables a homogeneous assay set-up.

Here, switchable lanthanide luminescence label technology was first applied to sensitive, homogeneous, single-target nucleic acid and protein assays with picomolar detection limits and high signal to background ratios. Thereafter, a homogeneous four-plex nucleic acid array-based assay was developed. Finally, the label technology was shown to be effective in discrimination of single nucleotide mismatched targets from fully matched targets and the luminescent complex formation was analyzed more thoroughly. In conclusion, this study demonstrates that the switchable lanthanide luminescence-based label technology can be used in various homogeneous bioanalytical assays.

TIIVISTELMÄ

Kahta nukleiinihappokoetinta, jotka hybridisoituvat vierekkäin komplementaariseen kohdenukleiinihappoon, kutsutaan kaksoiskoettimiksi. Kohteen tunnistamiseksi kaksoiskoettimet voidaan leimata fluoresoivilla väreillä, kemiallisilla ryhmillä tai entsyymeillä. Kaksoiskoettimien etu verrattuna yhden nukleiinihappokoettimen käyttöön on, että kaksoiskoettimien hybridisaatio kohteeseen aikaansaa mitattavan signaalin muodostumisen, vaikka hybridisoitumattomia koettimia ei erotettaisi pois reaktioseoksesta pesuvaiheella ennen mittausta. Kaksoiskoettimilla on hyvä spesifisyys, sillä mitattava signaali muodostuu vain, kun koettimet hybridisoituvat kohdenukleiinihappoon yhtä aikaa. Myös entsyymejä ja fluoresoivia proteiineja voidaan jakaa kahteen osaan. Niitä voidaan käyttää reporttereina erotusvapaissa määrityksissä. Jaetut entsyymit ja fluoresoivat proteiinireportterit ovat käyttökelpoisia erityisesti soluissa tapahtuvien proteiinien välisten vuorovaikutusten tutkimisessa.

Tässä tutkimuksessa esiteltiin uusi leimateknologia, kytkeytyvä lantanidiluminesenssi, jota käytettiin kaksoiskoettimien kanssa. Tässä leimateknologiassa luminoiva lantanidikelaatti on jaettu kahteen ei-luminoivaan osaan, ioninkantajakelaattiin ja valoa keräävään antenniin, jotka kytkeytyvät luminoivaksi kompleksiksi kun osat tuodaan lähelle toisiaan. Tällä kahdesta osasta muodostuvalla luminoivalla lantanidikompleksilla on samat fotofysikaaliset edut kuin tavallisemmin käytetyillä luminoivilla lantanidikelaateilla ja -kryptaateilla: terävät emissiopiikit, pitkä emission elinikä, joka mahdollistaa aikaerotteisen mittaamisen, sekä suuri Stokesin siirtymä. Nämä ominaisuudet pienentävät määrityksen herkkyyttä rajoittavaa taustasignaalia. Kytkeytyvän lantanidiluminesenssin lisäetu on, että niitä hyödyntäen voidaan toteuttaa erotusvapaita määrityksiä.

Tässä väitöstutkimuksessa uuden leimateknologian käyttökelpoisuus osoitettiin ensin herkissä erotusvapaissa nukleiinihappo- ja proteiinimäärityksissä, joissa saavutettiin pikomolaarinen herkkyys ja korkea signaalin ja taustan suhde. Seuraavaksi kehitettiin erotusvapaa monianalyyttimääritys neljän kohdenukleiinihapon yhtäaikaiseen havaitsemiseen. Lopuksi uuden leimateknologian osoitettiin soveltuvan myös yhden nukleotidin mutaatioiden erotteluun ja luminoivan kompleksin muodostumista tutkittiin tarkemmin. Loppupäätelmänä voidaan todeta, että tämä tutkimus osoitti kytkeytyvän lantanidiluminesenssin mahdollisuudet erilaisissa erotusvapaissa määrityksissä.

1 INTRODUCTION

The analysis and detection of small molecules, nucleic acids, and proteins and their interaction with each other is based on biomolecular recognition. Oligonucleotide probes and antibodies have a high capability to hybridize to complementary target sequences and to bind their antigens, respectively. Researchers strive to develop simple, sensitive and rapid techniques to detect antigens and nucleic acids in test tubes and to visualize cellular target molecules. Since the first label-based immuno- and nucleic acid assays using radioactive labels by Yalow and Berson (1960), Ekins (1960), and Bolton and McCarthy (1962), numerous assay formats with various detection techniques have been developed. The first immuno- and nucleic acid assays were heterogeneous - requiring separation of the unbound labeled antibody or oligonucleotide probe from the bound molecules. Heterogeneous assays are still widely used due to their sensitivity. However, homogeneous assay formats, wherein the signal of the label is modulated due to the binding reaction, are preferred because they are more efficient - rapid, cost-efficient, and user-friendly. These types of assays are beneficial in point-of-care applications. For example, sensitive and rapid bacterial identification and cardiac marker detection are needed when a patient is suspected to have a blood stream infection with as few as one micro-organism per milliliter of blood or an acute coronary syndrome with low elevation of cardiac troponins (Reichlin *et al.*, 2009; Tissari *et al.*, 2010).

Homogeneous nucleic acid assays can be accomplished using binary oligonucleotide probes. They consist of two reporter molecules conjugated to two nucleic acid strands. The adjacent and simultaneous binding of the two nucleic acid strands to the target; i.e., bimolecular recognition, brings the reporter molecules into close proximity and enables formation of a measurable signal that reveals the presence of the target (Kolpashchikov, 2010). The signal can be a direct fluorescent signal or precede an enzymatic recognition or a chemical reaction. The requirement of two simultaneous binding events for reporter signal formation increases specificity and decreases background noise. For example, in single nucleotide polymorphism detection, the specificity of a binary probe-based approach is higher due to the reduced affinity of two short oligonucleotide probes to a mismatched target when compared to the affinity of one long probe.

In addition to nucleic acid probes and functional nucleic acids, such as aptamers and nucleic acid enzymes, also protein enzymes and fluorescent proteins can be divided into two fragments, which regain their function after target-directed complementation (Porter *et al.*, 2008; Shekhawat and Ghosh, 2011). Protein fragment complementation enables the studying of protein-protein interactions inside living cells.

2 REVIEW OF THE LITERATURE

To enable a sensitive, separation free detection of biomolecules and their interactions, both binary nucleic acid probes conjugated to reporter molecules and fragmented protein reporters fused to targeted proteins can be used. This literature review concentrates on detection strategies used with binary nucleic acid probes, mainly by homogeneous fluorescence-based detection. The review introduces also enzymes and fluorescent proteins, which have been fragmented into two parts and can reform their function after protein interaction induced complementation.

2.1 Binary nucleic acid probes

DNA and RNA can be recognized through specific base pairing by complementary strands. Perfectly complementary strands hybridize readily to each other while even a single nucleotide mismatch can destabilize hybridization enough to prevent it entirely. In theory, 16 base pairs are needed to identify a unique sequence within approximately the size of a human genome (Liu *et al.*, 1997). Binary probes (also called dual probes) are composed of two oligonucleotide probes that hybridize adjacently to a complementary single stranded nucleic acid target. The probes can be modified with fluorescent labels, reactive groups or functional nucleic acids that, when brought into close proximity, enable the formation of a measurable signal, typically the emission of light (Figure 1).

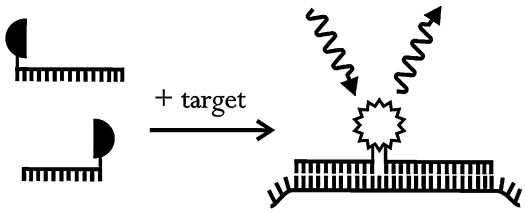

Figure 1. Schematic presentation of binary oligonucleotide probe-based nucleic acid detection. Two probes are conjugated to reporters (black), which are undetectable when the probes are free in solution. After hybridization of the probe pair adjacently to a complementary target oligonucleotide, the close proximity of the reporters elicits a detectable signal that can be, for example, the emission of light at a specific wavelength.

2.1.1 Direct utilization of fluorescence

Nucleic acid detection using the binary probe system is attractive because the requirement of two simultaneous and adjacent hybridization events enables high specificity in target recognition, which is beneficial especially in single nucleotide

polymorphism (SNP) detection. With fluorescent labels, the signal can be generated immediately after the probes have hybridized to the target. This enables real-time detection that can be utilized, for example, in the monitoring of nucleic acid amplification.

Binary probe-based nucleic acid detection using Förster resonance energy transfer (FRET) (Förster, 1948) was pioneered by Heller *et al.* (1983) and Cardullo *et al.* (1988). Binary FRET probes have one of the two probes labeled with a donor fluorophore and the other with an acceptor fluorophore. The prerequisites for resonance energy transfer are close proximity of the two fluorophores, spectral overlapping of the donor emission spectrum with the acceptor excitation spectrum, and proper orientation of the transition dipole moments of the fluorophores. The efficiency of energy transfer diminishes rapidly when the distance between the donor and acceptor increases enabling the detection of closely located donor and acceptor pairs (Juskowiak, 2011). An effective distance between a donor and an acceptor is typically 10–100 Å, which corresponds to 3–30 nucleotides in the double helix structure of DNA (Marras, 2006). The binary FRET probe principle has been commercialized in quantitative real-time polymerase chain reaction (PCR) with LightCycler® (Roche Diagnostics, Indianapolis, IN) wherein the probes are called dual or hybridization probes (Wittwer *et al.*, 1997). The fluorescent dyes used in FRET assays include carboxyfluorescein (Fam), cyanine dyes Cy3 and Cy5, tetramethylrhodamine (Tamra), tetrachlorofluorescein, carboxy-X-rhodamine (Rox) and many others. However, dark quencher molecules such as Dabcyl, or dyes from QSY or the black hole quencher-series can also be used as acceptors, in which case the decrease in donor emission is measured (Marras, 2006). For multiplexing purposes, the use of the aforementioned fluorophores may be problematic due to their broad excitation and emission spectra and short Stokes' shifts. These cause donor cross-talk at the acceptor emission measurement wavelength and direct excitation of the acceptor leading to compromised assay sensitivity. Therefore, various strategies exist to minimize the sensitivity-limiting background signal in fluorescence-based binary probe assays.

A three-dye system can be used to increase the Stokes' shift in FRET-based nucleic acid detection (Marti *et al.*, 2007a). In this system, one probe of a binary probe pair is labeled with Cy5 and the other probe is labeled with Fam and Tamra. Tamra acts as an energy relay that accepts energy from Fam and redirects it to Cy5 when a complementary target is present. The sequential energy transfer increases the Stokes' shift, which reduces direct excitation of the Cy5 acceptor. Binary molecular beacons (MB) labeled with fluorophores and quenchers can also be used to increase the sensitivity of the binary FRET probe-based assay (Tsourkas *et al.*, 2003). In the absence of a complementary target, the fluorescence emissions are quenched by adjacent quenchers, due to the stem-loop structures of the MBs. Hybridization of the loop

sequences with the target separates the fluorophores from the quenchers and the donor can transfer the energy to the acceptor, which then emits light (Figure 2). With binary MB FRET probes, typical problems with single MBs (e.g., increased background signal caused by cellular degradation and nonspecific probe opening) can be avoided. Both only donor quenched (Root *et al.*, 2004), and donor and acceptor quenched (Bratu *et al.*, 2003; Santangelo *et al.* 2004; Tsourkas *et al.*, 2003) binary MB probe-based assays exist. For example, Bratu *et al.* visualized mRNA distribution and transportation in living oocyte cells using an MB pair with Tamra and Texas Red and Santangelo *et al.* used an MB pair with Cy3 and Cy5 for visualization of mRNA in two living cell lines.

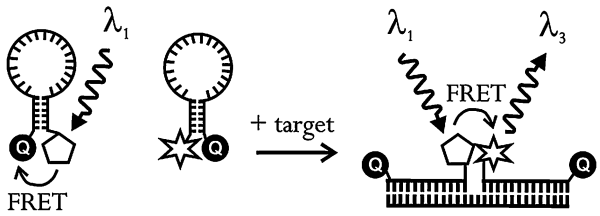

Figure 2. Binary molecular beacons (MBs) in DNA detection. Both the donor (pentagon) and acceptor (star) MB probes are labeled with quenchers (Q). In the absence of a complementary target, both the donor and the acceptor emission is quenched by the quencher due to the hairpin structure of the MB, which brings the fluorophore and the quencher into close proximity. When a complementary target is present, the hairpin structure opens bringing the donor-acceptor pair into close proximity and enables FRET. λ_1, excitation wavelength of the donor; λ_3, emission wavelength of the acceptor.

Lanthanide chelates and cryptates are reporters that have long-lived emission, sharp emission peaks and large Stokes' shifts. These can also be used as donors in binary FRET probe-based assays. The long-lived emission from these donors sensitizes the short-lived organic fluorophore emission, which enables the use of time-resolution in acceptor emission measurement. Sueda *et al.* (2000, 2002) utilized a binary probe pair where the other probe was conjugated to biotin at the 3′ end and the other to Cy5 or Cy3 at the 5′ end. Several terbium(III) (Tb(III)) and europium(III) (Eu(III)) chelate donors were attached to the complexes as streptavidin conjugates that were bound to the biotins. Sensitized, energy transfer enhanced emission from the acceptors in the presence of target DNA resulted in 200 and 30–50 pM limits of detection (LOD). Tsourkas *et al.* (2003) labeled linear oligonucleotide probes directly with Tb(III)- and Eu(III)-chelates and used them with oligonucleotide pairs labeled with Cy3 and Rox acceptors, respectively. Although the spectral properties of the lanthanide chelates would allow omitting the MB structure with a quencher, Root *et al.* (2004) used an MB probe with fluorescein quencher and Tb(III) as a donor for a linear Cy5-labeled probe.

Lanthanide luminescence can be used in binary probe-based nucleic acid detection also without FRET. The principle of splitting a luminescent lanthanide chelate into two label

moieties, an ion carrier chelate and a light harvesting antenna ligand, was originally presented by Oser and Valet (1990). The split label moieties are non-luminescent when they are free in solution, but after adjacent hybridization of a labeled probe pair on a complementary target oligonucleotide, a luminescent lanthanide complex is formed. The signal modulation in the original publication of Oser and Valet was rather low, less than three-fold, and it took over ten years before Wang *et al.* (2001) applied the same principle in their assay. The signal modulation in the assay of Wang *et al.* remained low, although the LOD of the assay was satisfactory, 6 pM. In recent years, several applications for nucleic acid detection using switchable lanthanide luminescent binary probes have been published (Karhunen *et al.*, 2010; Lehmusvuori *et al.*, 2012a; Lehmusvuori *et al.*, 2012b; Lehmusvuori *et al.*, 2013; Karhunen *et al.*, 2013; Alinezhad *et al.*, 2014). In addition to lanthanide-based reporters, transition metal complexes, such as ruthenium(II), also have long-lived emission when compared to the autofluorescence background. Marti *et al.* (2007b) used ruthenium(II) as a donor for Cy5 acceptor in a binary FRET-based nucleic acid assay. Time-gated Cy5-measurement enabled the detection of target DNA in the presence of a colored cell media.

Particulate labels, such as lanthanide-doped upconverting phosphors (UCP) and quantum dots (QD), have also been used as donors in resonance energy transfer-based assays with binary probes for nucleic acid detection. Zhang *et al.* (2006) conjugated a capture probe to UCP and labeled the other probe of the binary probe pair with Tamra. The detection limit of the assay was 1.3 nM. Rantanen *et al.* (2009) utilized UCP with two emission bands in a dual-parameter hybridization assay. The UCP was conjugated to capture probes and the respective probe pairs to AlexaFluor546 and AlexaFluor700 acceptors. The dual-parameter assay detected both targets with a 0.35 nM detection limit. For example, Zhang *et al.* (2005) and Wang *et al.* (2010) attached capture probes on the surface of QD donor particles and used Cy5-labeled probes as acceptors. Zhang *et al.* achieved a 4.8 fM detection limit using a custom-made confocal microscope for the FRET measurement, whereas Wang *et al.* used a commercial Wallac 1420 Multilabel Counter and achieved a 4.0 nM detection limit.

An excimer is a fluorescent dimer of two interacting fluorescent molecules. It is formed when an excited molecule collides with an identical unexcited molecule. The resulting emission from the excimer is located at higher wavelengths than that of the monomer (Birks, 1975). In addition to the increased Stokes' shift, the longer lifetime of the excimer emission compared to the monomer emission makes excimer emission easily distinguishable. Pyrene, with a monomer emission maximum at 390 nm and excimer emission maximum at 480 nm, is the most frequently used fluorophore in excimer-based assays. Pyrene-labeled binary probes have been used for DNA detection from solution (Paris *et al.*, 1998) and for RNA detection from cell extracts (Masuko *et al.*, 1998; Marti *et al.*, 2006). The reported assays vary in the chemistry used to attach

pyrene to oligonucleotides, in the linker length between the pyrene and the oligonucleotide, and in the distance between the excimer-forming hybridization positions. Marti *et al.* (2006) utilized time-resolved measurement in their assay, which resulted in higher signal to background ratio compared to a steady-state measurement. However, in spite of the improvement, the authors concluded that UV excitation at 350 nm of the pyrene excimer may be problematic for *in vivo* applications (Marti *et al.*, 2007b). Exciplexes are also fluorescent dimers but they are formed of two different fluorescent molecules, for example pyrene and naphthalen. Binary probes that utilize exciplex labels are called ExciProbes. They have been used in the detection of specific nucleic acid sequences and SNPs (Bichenkova *et al.*, 2005a; Bichenkova *et al.*, 2005b). The requirement of 80 percent trifluoroethanol for exciplex formation has limited additional applications.

2.1.2 Utilization of enzymatic reactions

In addition to direct utilization of fluorescence in binary probe-based assays, chemical reactions as well as aptamer folding and activity of a nucleic acid enzyme can precede the reporter signal formation. Enzymatic ligation of adjacently hybridized binary probes can also be used for sequence-based nucleic acid and protein detection. For example, HIV-1 RNA has been detected using enzymatic ligation of two oligonucleotide probes (Tyagi *et al.*, 1996). The two probes consisted of recombinant RNA and the probe sequences were embedded at appropriate sites within the sequence of MDV-1 RNA. The MDV-1 RNA is a naturally occurring template for an RNA replicase of bacteriophage Qβ. In the absence of the HIV-1 RNA target, the two probes are separated and the Qβ replicase does not amplify them. However, when the probes are hybridized at adjacent positions on the HIV-1 RNA, the probes can be ligated to a reporter RNA, which is then amplified exponentially with Qβ replicase using radioactive nucleotides. The amplified nucleic acid was visualized by autoradiography and the sensitivity of the presented assay was between 10 and 100 RNA targets.

Ligases can also be used for mismatch discrimination due to the sensitivity of the enzyme to mismatches at the ligation junction. For example, Landegren *et al.* (1988) used T4 DNA ligase and Li *et al.* (2005) used Tth ligase in their single mutation assays. Both assays used two probes of which one was invariant and the other the allele-specific. Only complete hybridization of the allele-specific probe adjacently to the invariant probe induced a ligation reaction. The ligation products were visualized by autoradiography using the radioactively labeled invariant probe (Landegren *et al.*, 1988) or by colorimetry using a gold nanoparticle assembly (Li *et al.*, 2005). Proximity ligation assay (PLA) (Fredriksson *et al.*, 2002), which is also based on enzymatic ligation, enables ultrasensitive protein detection. In PLA a pair (or three, in the case of

3PLA (Schallmeiner *et al.*, 2007)) of oligonucleotide probes are brought into close proximity upon two affinity binding events. The probes hybridize adjacently to a connector oligonucleotide and are ligated enzymatically. The ligation product can then be amplified with, for example, PCR or rolling-circle amplification (RCA), and detected with various techniques (Conze *et al.*, 2009).

2.1.3 Utilization of chemical reactions

Ligase enzymes have limitations such as low activity with modified nucleic acids and with short binary probes that are commonly used for enhancing the specificity in nucleic acid detection. Autoligation using chemical reactions can be used as an alternative to enzymatic ligation (Figure 3). An oligonucleotide probe with 3′ phosphorothioate reacts with an adjacently hybridized 5′ iodide probe resulting in a ligated product and in the release of iodine (Xu and Kool, 1998). The iodide can be replaced with a 5′ Dabsyl quencher that quenches the emission from Fam placed three nucleotides from the 5′ terminus (Figure 3a). The autoligation reaction releases the quencher, which restores the fluorescence of Fam (Sando and Kool, 2002). The high background, due to the inefficient quenching by Dabsyl, can be improved by utilizing FRET with the autoligation: the phosphorothioate and the quencher probes can be labeled with a FRET pair, for example with Fam and Cy5 (Abe and Kool, 2006). The donor fluorophore emission is quenched until the probe pair hybridizes to a complementary target oligonucleotide, which induces an autoligation reaction that releases the quencher (Figure 3b). This kind of autoligation was used for detection of RNAs with flow cytometry and imaging with confocal microscopy in human cells (Abe and Kool, 2006). Another type of chemical ligation reaction utilized with binary probes is condensation (Huang and Coull, 2008). In this assay, the probes were modified with cyanine dye precursors containing an aldehyde and an indol that formed a fluorescent hemicyanine dye after the condensation reaction (Figure 3c). Hemicyanine formation between peptide nucleic acid (PNA) probes has been used for the sensing of hairpin and G-quadruplex DNA (Meguellati *et al.*, 2010; Koripelly *et al.*, 2010). An advantage of the formation of a fluorescent hemicyanine is that the fluorescence signal is emitted at greater than 650 nm, which is usable for *in vivo* applications. Cu(I) catalyzed azide-alkyne cycloaddition (termed the "Click"-reaction) has also been utilized in a ligation-based DNA assay (Figure 3d; Sun and Peng, 2013). In this application, the other probe is labeled with alkylated coumarin and the other with an azide group. The presence of a complementary DNA and Cu(I) induced the ligation reaction, which switched on coumarin fluorescence.

A typical limitation of chemical ligation reactions is that they require several hours and are thus not practical for real-time hybridization monitoring. Moreover, ligated products have higher affinity to the target nucleic acid, which causes product inhibition

and prevents high catalytic activity (Grossmann and Seitz, 2006). High affinity of the ligation product to the target has been decreased using a rearrangement of the originally formed ligation product between a glycine thioester and isocysteine modifications on a PNA probe pair. The rearrangement lengthened the ligation product in the middle of the probe thereby decreasing its stability towards the target DNA (Dose *et al.*, 2006; Dose and Seitz, 2008).

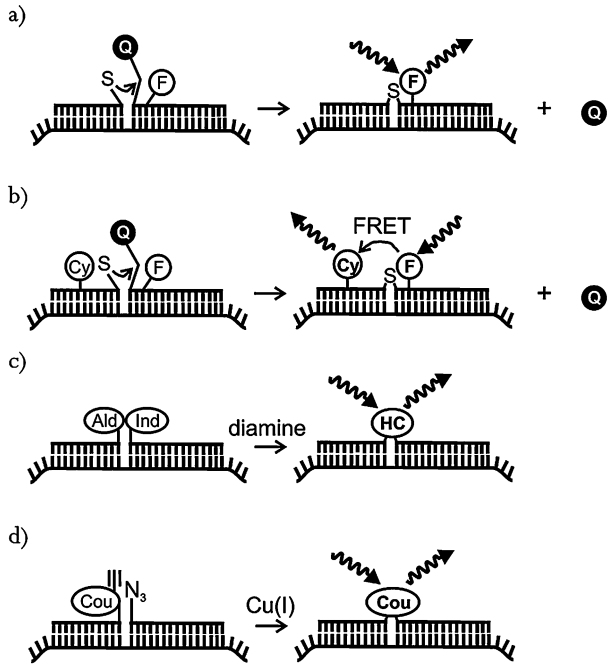

Figure 3. Schematic examples of DNA directed chemical ligation reactions used with binary probes. a) Quenched autoligation (QUAL) probes by Sando and Kool (2002). Phosphorothioate nucleophile (S) attacks at the target carbon thereby releasing the Dabsyl quencher (Q) into the solution. The product is a ligated probe where the Fam (F) emission is unquenched; b) QUAL-FRET probes by Abe and Kool (2006). Fluorescence background of the QUAL probes can be lowered by utilizing Fam (F) as a FRET donor for Cy5 (Cy); c) Hemicyanine condensation by Huang and Coull (2008). An aldehyde (Ald) and an indol (Ind) modified cyanine dye precursors react in the presence of diamine catalyst to produce a ligation product with fluorescent hemicyanine (HC); d) Fluorogenic "Click"-chemistry ligation by Sun and Peng (2013). Nonfluorescent alkylated coumarin (Cou≡) and azide (N_3) react in the presence of a Cu(I) catalyst to produce a fluorescent coumarin (Cou) and a ligated product.

Chemical reaction between two molecules on an adjacently hybridized binary probe pair can switch on fluorescence also without ligation of the two probes to each other (Figure 4). A modified Staudinger reduction between an azide and a reducing agent, such as triphenylphosphine (TPP), is the most typical chemical reaction used in these applications. Rhodamine and coumarin can be modified to nonfluorescent forms with an azide group, but after the reaction between the azide and the TPP, the azide is reduced

to an amino group, and fluorescence is switched on (Figure 4a) (Abe *et al.*, 2008; Franzini and Kool, 2008). Light-triggered ruthenium-promoted azide reduction, which switches on fluorescence, has also been used in nucleic acid detection (Figure 4b). Both azide-coumarin and azide-rhodamine reduction-based DNA detection schemes have been presented (Röthlingshöfer *et al.*, 2012) and applied to microRNA detection in living cells (Sadhu and Winssinger, 2013). The azide-modified rhodamine utilized in both publications was attached to a PNA-probe with immolative linkers that decomposed after photoreduction of the azide, which led to the release of fluorescent rhodamine. The assay presented by Sadhu and Wissinger had a detection limit of 5 pM, although it required a 24 hour reaction time. The difference to the previous examples of Staudinger reduction is that, here, the ruthenium-labeled probes acted as catalysts that could promote the reduction of several profluorophore labeled probes. Moreover, TPP, which is commonly used as the reducing agent, is sensitive to oxidation. This may lead to the excess use of TPP and, as a consequence, to the reduction of the azide also in the absence of the target nucleic acid (Röthlingshöfer *et al.*, 2012).

Staudinger reduction of an azide to an amine in the presence of TPP can be used to release a quencher from a probe to increase the fluorophore emission. In quenched Staudinger-triggered (Q-STAR) probes, the other probe is labeled with a fluorophore and a quencher. The quencher is conjugated to the probe through an α-azidoether linker. The other probe contains the TPP, which reduces the azide moiety and triggers the cleavage of the linker and the release of the quencher, which switches on the fluorescence signal (Figure 4c) (Franzini and Kool, 2009; Franzini and Kool, 2011a). The authors presented the utility of the two probe pairs to discriminate bacteria targets having only a single nucleotide difference. An improved version of the technique, named 2-STAR, includes probes with two releasable quenchers. The release is carried out sequentially with two TPP-probes. This lowers the signal from unspecific reactions and improves the single nucleotide mismatch discrimination specificity (Franzini and Kool, 2011b). A recent publication demonstrates microRNA detection using the original Q-STAR probes combined with initial RNA template-dependent autoligation of a circle precursor and subsequent RCA. The presented assay had a 200 pM detection limit in solution-based detection of microRNA (Harcourt and Kool, 2012).

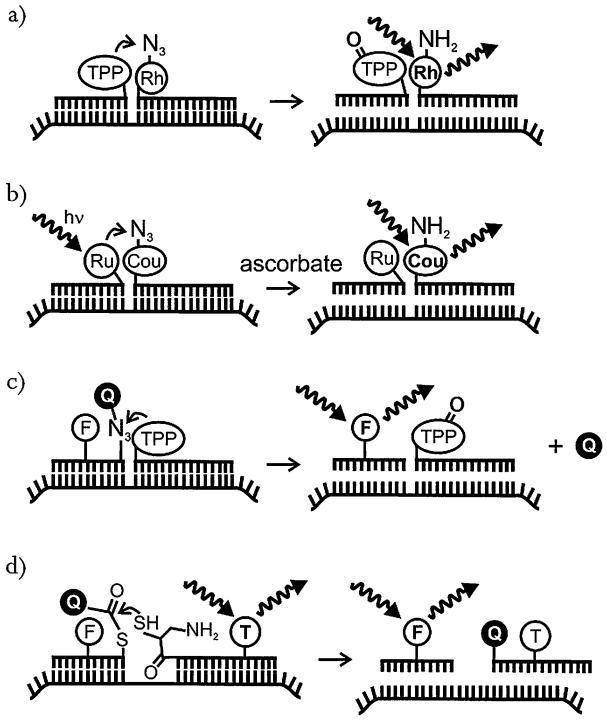

Figure 4. Schematic examples of DNA directed binary probe-based chemical reactions without the use of ligation. a) Staudinger reduction presented by Abe *et al.* (2008). Staudinger reduction between an azide-modified rhodamine (Rh-N_3) probe and a TPP probe results in azide reduction, which turns on rhodamine fluorescence; b) Ru-catalyzed photoreduction by Röthlingshöfer *et al.* (2012). Light triggers ruthenium (Ru) promoted azidocoumarin (Cou-N_3) reduction to fluorescent coumarin (Cou-NH_2) in the presence of an ascorbate catalyst; c) Q-STAR-probes by Franzini and Kool (2009). The other probe is labeled with both Fam (F) and an azido-modified Dabcyl quencher (Q-N_3) and the other with the reducing agent TPP. The reduction triggers the cleavage of the linker between the probe and the Dabcyl, which releases the Dabcyl into the solution; d) DNA-directed transfer of quencher by Grossmann and Seitz (2006). Before the transfer reaction, the fluorescence from Fam (F) is quenched with Dabcyl (Q), whereas Tamra (T) on the other probe is fluorescent. Target DNA catalyzed *iso*-cysteine-mediated transfer of the Dabcyl-quencher from the Fam-probe to the Tamra-probe switches on the Fam emission.

Molecules can also be transferred from one probe to another using a chemical reaction. Complementary DNA and RNA targets have been shown to catalyse *iso*-cysteine (*i*Cys)-mediated transfer of a Dabcyl-quencher between adjacently hybridized PNA probe pairs (Grossmann and Seitz, 2006; Grossmann *et al.*, 2008). The probes were labeled with Fam and Tamra, whose emission is quenched by Dabcyl when in close proximity. The Dabcyl is attached to the probe with a thioester linkage. The presence of target nucleic acid was followed with both the increase in Fam emission and the decrease in Tamra emission due to the transfer of Dabcyl from the Fam probe to the Tamra probe (Figure 4d). The *i*Cys-mediated transfer has also been utilized in transfer of pyrene from one

probe to another that already contains one pyrine. The transfer enables the formation of a fluorescent pyrene excimer (Grossmann and Seitz, 2009). An advantage of the pyrene reporter is that the competing reaction, the hydrolysis of the thioester linkage, does not increase the background noise because the excimer formation requires a correct transfer reaction.

2.1.4 Utilization of split aptamers

Modification of binary oligonucleotide probes with reactive groups and fluorescent molecules is time-consuming and the extra groups may affect the hybridization affinity. Therefore, labeling-free detection strategies are favored. Aptamers are composed of single-stranded RNA or DNA that is folded to a structure that specifically recognizes a certain target (Smuc *et al.*, 2013). Kolpashchikov (2005) separated a malachite green aptamer (MGA) into two strands and added target nucleic acid binding arms to the separated strands to create a binary MGA. The original MGA was composed of RNA, and upon binding to the malachite green dye, the fluorescence of the dye was increased. The binary MGA was used for the detection of a fully complementary target and also for the discrimination of single nucleotide mismatches with maximal discrimination factor of greater than 20. Similar split aptamers have been developed for Hoechst-dye (Sando *et al.*, 2007) and for Cy3-dye (Endo and Nakamura, 2010). These were used similarly to the binary MGA aptamer for DNA and RNA detection. Split aptamers can also be used in the detection of small molecule targets other than nucleic acids. Examples of such assays include detection of cocaine and adenosine-5′-triphosphate with electrochemical (Zuo *et al.*, 2009), visual (Zhang *et al.*, 2008) and FRET-based detection (He *et al.*, 2013). The detection limits of the reported assays are in the micromolar range.

2.1.5 Utilization of nucleic acid enzymes

Ribozymes are ribonucleic acid enzymes that can perform enzymatic reactions. Analogously, deoxyribozymes (or DNAzymes) are catalytically active deoxyribonucleic acids. Collectively, ribozymes and deoxyribozymes are called nucleic acid enzymes. Natural ribozymes catalyze RNA cleavage, splicing and peptide bond formation in ribosomes. Artificial ribozymes made by *in vitro* selection have been shown to catalyze a variety of reactions such as polymerization, alcohol dehydrogenation, ligation, and phosphorylation (Liu *et al.*, 2009). Cleaving and peroxidase-like nucleic acid enzymes have also been utilized as split reporters.

Split cleaving nucleic acid enzymes

Kolpashchikov (2007) demonstrated the proof-of-concept for a binary cleaving deoxyribozyme-based assay for DNA that used fluorescence detection (Figure 5). A

Mg^{2+}-dependent deoxyribozyme E6 (originally presented by Breaker and Joyce (1995)) was divided into two fragments (A and B in Figure 5) and nucleic acid target-binding arms were added to the fragments. In the presence of complementary target, the split fragments, A and B, hybridized to it, and to a reporter substrate labeled with a fluorophore-quencher-pair. This enabled the formation of the enzyme catalytic core. The reformed enzyme then cleaved the substrate embedded with one ribonucleotide, which led to increased fluorescence. The assay had 1 nM detection limit, which was 20 times more sensitive than a reference assay based on an MB detection of the same target. However, the assay required a 30 hour incubation.

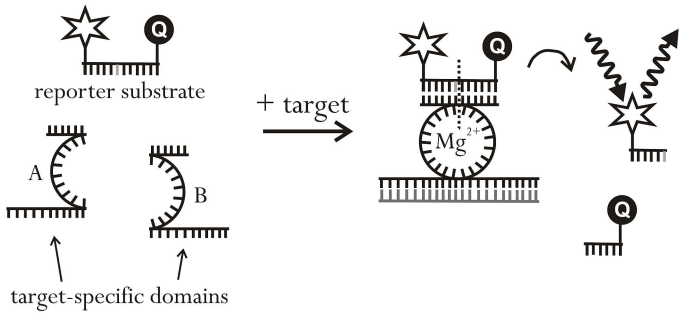

Figure 5. Schematic presentation of a binary cleaving DNAzyme-based assay. A DNAzyme is separated in two parts, A and B, which contain also target-specific domains. Hybridization of the target nucleic acid (grey) to the target specific domains brings the enzyme parts together and enables the formation of an active catalytic core, which cleaves the reporter DNA substrate at the embedded ribonucleotide (grey). The cleaving separates the reporter fluorophore (star) and the quencher (Q), which increases the emission signal of the fluorophore.

Several proof-of-principle reports with improved detection limits (10 fM–160 pM) have been published using binary DNAzymes derived from different original cleaving nucleic acid enzymes (Gerasimova *et al.*, 2010; Mokany *et al.*, 2010; Wang *et al.*, 2011a; Wang *et al.*, 2011b). However, none of these have applied the technique for *in vivo* nucleic acid detection. The low catalytic activity of the used DNAzymes still required assay times of several hours with the high sensitivity assays (Wang *et al.*, 2011a; Wang *et al.*, 2011b). Recently, colorimetric detection of DNA sequences (Zagorovsky and Chan, 2013), a fiveplex nucleic acid analysis in real-time quantitative PCR (Mokany *et al.*, 2013) and a library of programmable DNAzymes used for RNA detection in cell lysates (Kahan-Hanum *et al.*, 2013) have also been reported.

Unlike with the split DNAzyme-based assays, there are only a few reports of using split ribozymes. One example is a *cis*-splicing ribozyme from *Tetrahymena*, which was split in two fragments and used as a reporter for mRNA detection in monkey kidney cell line cells (Hasegawa *et al.*, 2006). The ribozyme fragments were conjugated to split halves of TEM-1 β-lactamase (Bla) mRNA and to two antisense sequences for target mRNA

detection. The presence of target mRNA generated a complete ribozyme, which spliced the mRNA of the Bla reporter to complete mRNA, which was then translated to an active β-lactamase enzyme that was assayed using a fluorogenic substrate. The cleavage efficiency of split ribozymes has been improved by incorporating modified nucleotides to the original ribozyme RNA sequences (Vorobjeva *et al.*, 2006; Christiansen *et al.*, 2007), but they are rarely used as reporters.

Split peroxidase-like nucleic acid enzymes

Peroxidases, like horseradish peroxidase, are enzymes that can oxidize for example 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) ($ABTS^{2-}$) in the presence of H_2O_2 to a colored radical anion ($ABTS^{\bullet-}$) or can produce chemiluminescence from a luminol substrate (Kosman and Juskowiak, 2011). Xiao *et al.* (2004) showed that two single-stranded guanine-rich nucleic acid segments self-assembled with hemin and formed an enzyme complex that generated chemiluminescence from luminol in the presence of H_2O_2. The enzyme complex formation was inhibited by hybridization of the two segments to a complementary nucleic acid sequence. In later studies 3-3′-diaminobenzidine tetrahydrochloride has also been used as an oxidizable substrate. The principle of binary DNAzyme-based target detection is presented in Figure 6. In the presence of target DNA, the hybridization of the target binding arms of the split DNAzymes to the target brings also the DNAzyme-forming strands into close proximity. This enables folding with hemin to form an active enzyme complex. The color change of the oxidizable substrate is visible by eye and can also be measured quantitatively using an absorbance at 500 nm. The first reports of binary DNAzymes (Kolpashchikov, 2008; Deng *et al.*, 2008) showed the identification of SNPs in the target sequences.

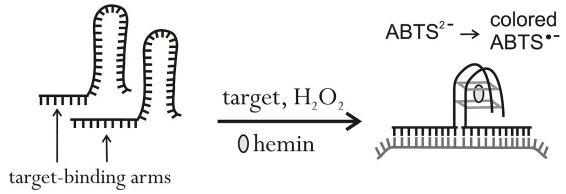

Figure 6. Schematic presentation of the binary peroxidase-like DNAzyme-based nucleic acid assay with colorimetric read-out. The split guanine-rich DNAzyme sequences (black) form an active peroxidase with hemin in the presence of target nucleic acid (grey). The active enzyme catalyzes H_2O_2-mediated oxidation of a colorless substrate ($ABTS^{2-}$) to a colored product ($ABTS^{\bullet-}$), which can be detected visually.

After the proof-of-principle reports, split peroxidase-like DNAzymes have been used for the sensing of bacterial DNA (Darius *et al.*, 2010; Deng *et al.*, 2012), small molecule coralyne (Hou *et al.*, 2013a; Hou *et al.*, 2013b), Hg^{2+}-ion (Kong *et al.*, 2010) and thrombin (Zhu *et al.*, 2012) with sensitivities from 0.5 nM to 31 nM. A higher signal

amplification and three orders of magnitude lower detection limit (0.1 pM) can be achieved with target-induced formation of conjugated DNAzyme nanowires (Shimron *et al.*, 2012). Both cleaving and peroxidase-like DNAzymes include a signal amplification step in the assay: once reformed cleaving DNAzymes and G-quadruplexes can cleave and catalyze the oxidization of multiple substrates. Another benefit is that the denaturation problems encountered with protein enzymes are absent. However, the spontaneous formation of an active nucleic acid enzyme in the absence of the target often limits the sensitivity (Deng *et al.*, 2008).

An interesting application combined the activity of a peroxidase-like DNAzyme and a cleaving DNAzyme in assays for Cu^{2+}, Pb^{2+}, and Hg^{2+} (Zhang *et al.*, 2012). The cleaving DNAzyme, a substrate, hybridized into a hairpin form with the G-quadruplex-containing enzyme strand thereby blocking the formation of active enzyme core. After a metal-ion triggered cleavage of the enzyme strand by the substrate strand, an active G-quadruplex was formed and thereafter the presence of a metal ion was detected through the peroxidase activity of the G-quadruplex. The sensitivities of the assays for Cu^{2+}, Pb^{2+}, and Hg^{2+}, all with respective enzyme and substrate oligonucleotide sequences, were 4–14 nM.

2.2 Protein fragment complementation

Protein fragment complementation, also called split-protein assembly, is one of the methods that can be used to study protein-protein interactions, protein locations, and protein modifications in living cells. It is a technique where proteins with known or suspected interaction are fused to protein fragments that reconstitute their activity after dimerization (Figure 7). The activity can be a fluorescent, a bioluminescent, or an enzymatic signal. The first paper describing the reassembly of an enzyme, ribonuclease A, from its polypeptide parts is from the year 1958 (Richards). Johnsson and Varshavsky (1994) were the first to actually use a split protein (ubiquitin) as a sensor for polypeptide interactions *in vivo*.

The yeast two-hybrid system, pioneered by Fields and Song (1989), is a genetic method that is also based on protein fragment complementation. The idea is to fuse the two proteins of interest into two domains of a fragmented transcription factor, a DNA-binding domain and an activator domain, such that the interaction between the proteins of interest reconstitutes a functional transcription factor, which then activates the expression of a reporter gene located downstream in the DNA sequence (Brückner *et al.*, 2009). The classic yeast two-hybrid system is, however, limited to the studying of proteins that are capable of entering the cell nucleus, and the technique is not applicable to kinetic studies.

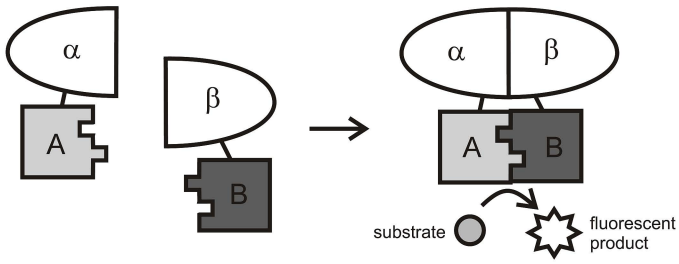

Figure 7. Principle of the protein fragment complementation-based assay. Proteins of suspected interaction, α and β, are fused to reporter protein fragments A and B. The protein fragments are inactive as separate components, but through the interaction of α and β, a functional protein is formed. The functional protein is, for example, an enzyme that converts a non-fluorescent substrate into a fluorescent product.

Protein candidates for protein fragment complementation-based assays have certain characteristics: the protein fragments should not exhibit activity on their own, the fragments should have minimal affinity towards each other in the absence of the attached interacting proteins, and the reassembled protein should provide an easily measureable read-out. Both enzyme and fluorescent protein reporters have been used in protein fragment complementation assays (Shekhawat and Ghosh, 2011).

2.2.1 Enzyme fragment complementation

In vivo assays

Several studies using enzyme fragment complementation for the characterization of protein-protein interactions *in vivo* have been published since the first report by Johnsson and Varshavsky in 1994 (Table 1). β-galactosidase is a tetrameric enzyme that hydrolyses D-galactosyl residues from polymers, oligosaccharides or secondary metabolites (Husain, 2010). Rossi *et al.* (1997) utilized β-galactosidase deletion mutant fragments that were fused to two proteins, which interact in the presence of the low molecular weight compound, rapamycin. The reassembled enzyme in myoblast cells was visualized using a histochemical Fluor-X-Gal stain that contains a dye, which is converted to a fluorescent precipitate by an active β-galactosidase. The precipitate can be detected with fluorescence microscopy. DiscoveRx Corporation (Fremont, CA) has commercialized the β-galactosidase complementation as trade name PathHunter® for cell-based assays (Olson and Eglen, 2007).

Dihydrofolate reductase (DHFR) is a ubiquitous enzyme found in all organisms. The enzyme comprises three structural fragments that form two domains, which catalyze the reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF) using

nicotinamide adenine dinucleotide phosphate as a cofactor (Schnell *et al.*, 2004). The complementation of an active murine DHFR (mDHFR) from two inactive fragments was first demonstrated in *E. coli* (Pelletier *et al.*, 1998) and soon thereafter in mammalian cells (Remy and Michnick, 1999). In *E. coli* cells, the complementation was detected when the enzyme converted DHF to fluorescent THF, whereas in mammalian cells the quantitation was made using a fluorescein conjugated methotrexate (fMTX) as a substrate for the enzyme. The fluorescence of fMTX is quenched when the dye is free in the cell, but when it is bound to the reconstituted mDHFR, the fluorescence intensity is increased and can be measured with, for example, fluorescence microscopy.

Several pathogenic bacteria strains produce β-lactamase that is responsible for the resistance to β-lactam antibiotics (Philippon *et al.*, 1998). In 2002, three research groups published protein-protein interaction assays based on *E. coli* TEM-1 β-lactamase enzyme fragment complementation (Galarneau *et al.*, 2002; Spotts *et al.*, 2002; Wehrman *et al.*, 2002). The TEM-1 β-lactamase is usable for a protein fragment complementation assay as it is a relatively small, monomeric, non-toxic, and an easily expressed protein. The enzyme can be split into two 19 kDa fragments. In the three publications from 2002, CC2F/AM was used as a fluorescent substrate for β-lactamase activity. CC2F/AM is a cell-permeable dye that consists of coumarin and fluorescein dyes that are conjugated to each other through a β-lactame ring. An intact CC2F/AM exhibits FRET from coumarin to fluorescein, whereas an active β-lactamase catalyzes the opening of the β-lactame ring. This results in increased donor fluorescence from coumarin as fluorescein is released from the molecule and FRET no longer occurs. Galarneau *et al.* (2002) presented the assaying of several known protein-protein interactions *in vitro* and also *in vivo* in HEK 293 cells. Wehrman *et al.* (2002) presented β-lactamase complementation in myoblasts through rapamycin-inducible interaction of two proteins. Furthermore, Spotts *et al.* (2002) reported a protein phosphorylation-dependent protein-protein interaction between a cyclic adenosine monophosphate response element binding protein (CREB) and a CREB binding domain. They also extended the measurement to time-lapse imaging of dynamic protein association in single primary cortical neurons. A drawback of β-lactamase fragment complementation is that the hydrolysis products of CC2F/AM are freely diffusible, which limits the monitoring of the location of protein interaction within the cells.

Tobacco etch virus (TEV) protease complementation can also be used for detection of protein-protein interactions (Wehr *et al.*, 2006). The technique is based on the proteolytic activation of reporters. The reporter in the split TEV technique can be either a transcription factor that activates the reporter genes or an inactivated protein that activates upon proteolysis. The detection of protein-protein interaction using split TEV was presented both at the membrane and in the cytosol of Chinese hamster ovarian

(CHO) cells (Wehr *et al.*, 2006). The reporter was a firefly luciferase conjugated to two protein domains that strongly reduce the activity of the luciferase moiety (LucER). The complemented protease activity of the TEV releases the luciferase, which catalyzes the light production from luciferin. A more recent study presented a modified version of the split TEV-based assay: the enzyme was engineered to decrease the affinity of the split fragments to each other (Gray *et al.*, 2010). In a cellular context, the engineered fragments were used to identify and characterize caspase activation.

Luciferases are enzymes that oxidate luciferins to produce oxyluciferin with concurrent emission of light. Luciferases from many organisms have been used in bioluminescent assays (Thorne *et al.*, 2010) and luciferases from *Renilla*, *Gaussia* and firefly have also been used as split reporters (Stains *et al.*, 2010). The first reported split luciferase was from the firefly (Ozawa *et al.*, 2001). The fragments of luciferase were fused to fragmented intein and to two proteins of interest. The interaction between the protein pair of interest brought the fragmented inteins into close proximity and induced the formation of a splicing intein protein that linked the luciferase fragments to each other by a peptide bond. The linked mature luciferase produced luminescence from a chemiluminescent substrate. Ozawa *et al.* used insulin-induced interaction between two proteins, insulin receptor substrate and its target, to demonstrate the assay principle in CHO cells. The luminescence intensity showed insulin concentration dependence. Split luciferase has also been shown to be useful in the imaging of protein-protein interactions with cell implants in living mice (Paulmurugan *et al.*, 2002). Although most enzymes used in protein fragment complementation assemble irreversibly, a reversible form of split *Renilla* luciferase (*R*Luc) exists (Stefan *et al.*, 2007). The two fragments of *R*Luc were fused to regulatory (R) and catalytic (C) subunits of a protein kinase A (PKA). The interaction of R and C subunits was regulated by cyclic adenosine monophosphate (cAMP). The fusion proteins were expressed first without cAMP, which enabled the formation of PKA and thus the reconstitution of an active *R*Luc that oxidized benzyl-coelenterazine in a reaction that produced blue light. However, after the cells were treated with a cAMP-elevating agent forskolin, the PKA complex dissociated, which led to the disassembly of active *R*Luc and to the decreased intensity of the blue light.

Table 1. Examples of enzymes used in *in vivo* protein fragment complementation assays.

Enzyme	Host cell	Substrate	Reference
β-galactosidase	Myoblast	Fluor-X-Gal stain	Rossi *et al.*, 1997
mDHFR	*E. coli* CHO	DHF fMTX	Pelletier *et al.*, 1998 Remy and Michnick, 1999
β-lactamase	Myoblast HEK 293 Cortical neuron	CCF2/AM	Wehrman *et al.*, 2002 Galarneau *et al.*, 2002 Spotts *et al.*, 2002
TEV protease	CHO	Luciferin with LucER	Wehr *et al.*, 2006
Luciferase	CHO	Chemiluminescent substrate	Ozawa *et al.*, 2001

Abbreviations: mDHFR, murine dihydrofolate reductase; CHO, Chinese hamster ovarian cell line; DHF, 7,8-dihydrofolate; fMTX, fluorescein conjugated methotrexate; HEK293, human embryonic kidney cell line; CCF2/AM; β-lactame conjugate of coumarin and fluorescein; TEV protease, tobacco etch virus protease; LucER, inactivated luciferase.

In vitro assays

Split enzymes have also been used as reporters in cell-free assay formats. For example, a cloned enzyme donor immunoassay (CEDIA) utilizes fragmented β-galactosidase (Henderson *et al.*, 1986). In CEDIA, the enzyme is split into a large polypeptide called the enzyme acceptor (EA) and to a smaller peptide called the enzyme donor (ED). These combine spontaneously in solution to form an enzymatically active β-galactosidase. For example, a hapten can be conjugated to the ED without disturbing the enzyme complementation. However, if a hapten antibody binds to the ED conjugated hapten, the enzyme complementation is inhibited. The free hapten from the sample to be analyzed competes with the ED-hapten conjugate for binding to the antibody, and thus the amount of enzyme activity is directly proportional to the amount of the free hapten. CEDIA kits for drug of abuse testing are currently available from Thermo Fisher Scientific (Waltham, MA). A fragmented β-galactosidase is also used in the HitHunter® assay (from DiscoveRx), which has applications in high through-put screening (Golla and Seethala, 2002). Like CEDIA, HitHunter® is a homogeneous competitive assay where the analyte of interest competes for antibody binding with the enzyme donor conjugated analyte. In addition to split β-galactosidase, split luciferase has gained interest as a cell-free reporter for detection of native proteins (Stains *et al.*, 2010) and protein interactions (Porter *et al.*, 2008).

2.2.2 Bimolecular fluorescence complementation

In studying protein-protein interactions, the advantage of enzyme fragment complementation compared to, for example, FRET, is signal amplification: the enzymatic activity of the reporter enzyme determines the catalytic rate at which substrates are converted to measurable products. However, for signal production, most enzymes require external substrates, which are not needed when fluorescent proteins exhibiting intrinsic fluorescence are used as reporters. Protein fragment complementation is called bimolecular fluorescence complementation (BiFC) when fluorescent proteins are reassembled. The first BiFC report demonstrated *Aequorea Victoria* green fluorescent protein (GFP) complementation (Ghosh *et al.*, 2000). The non-fluorescent fragments were fused to leucine zippers, whose interaction resulted in a reformed fluorescent protein. Examples of the various fluorescent proteins used for BiFC are presented in Table 2.

BiFC in the detection of protein-protein interactions in living cells was pioneered by Hu *et al.* (2002). Fragmented enhanced yellow fluorescent protein (EYFB), a variant of GFP, was fused to a basic-region leucine zipper (bZIP)-domain and to nuclear factor kappa B (NF-κB) -family proteins. The fusion proteins were used for studying the bZIP and NF-κB dimerization and localization patterns in three mammalian cell lines. Hu *et al.* also studied in detail the formation of the fluorescent protein from its non-fluorescent parts. They observed that the rate-limiting step after EYFP refolding is the maturation of the endogenous chromophore. This autocatalytic reaction involves oxidation and requires several minutes to take place and, therefore, BiFC is not applicable to the real-time visualization of protein-protein interactions.

Soon after the discovery of BiFC, it was realized that GFP and its variants were sensitive to the physiological environment. Moreover, the EYFP chromophore maturation is temperature-sensitive and thus requires preincubation at lower temperature before measurement of the protein emission (Hu and Kerppola, 2003). Therefore, fluorescent proteins that would be brighter under physiological conditions were searched. For this purpose, three engineered fluorescent proteins (Venus, Citrine and Cerulean) were fragmented in several alternative ways (Shyu *et al.*, 2006). Venus and Citrine are yellow fluorescent protein (YFP) derivatives (Griesbeck *et al.*, 2001; Nagai *et al.*, 2002) and Cerulean originates from the cyan fluorescent protein (CFP) (Rizzo *et al.*, 2004). The process resulted in several fragments that were effective for BiFC analysis in cellular environment. The protein fragments were attached to bZIP proteins Fos and Jun and their subnuclear heterodimer localization was examined. Moreover, the fluorescent protein maturation of these proteins was fast and, unlike with EYFP, did not require pre-incubation at 30°C.

In addition to the cyan, green, and yellow fluorescent proteins, also red-emitting fluorescent proteins derived from red fluorescent protein from coral *Discosoma* sp. (Matz *et al.*, 1999) have been used in BiFC. The advantage of a BiFC protein, which emits at longer wavelengths, is that it enables the visualization of protein-protein interactions in deep inside tissues. The red fluorescent protein from *Discosoma* sp. (DsRED) is, however, a tetrameric protein and cannot be used for protein complementation. Jach *et al.* (2006) presented an improved monomeric DsRED variant (mRFP1-Q66T) that was fragmented and the fragments were fused to humanized *Renilla* GFP that forms stable homodimers. The fusion proteins expressed in tobacco protoplasts were incubated at 26–28°C and they produced strong red fluorescence indicating formation of a functional BiFC. A second generation red emitting mRFP1 variant was named mCherry (Fan *et al.*, 2008). The applicability of the mCherry-based BiFC was demonstrated using a previously demonstrated interaction between the large T antigen and the human p53 protein in kidney epithelial cells after overnight incubation at 25°C. Eventually, the site-directed mutagenesis of a far-red monomeric fluorescent protein mKate (Shcherbo *et al.*, 2007) resulted in an improved far-red emitting protein named mLumin that was functional at 37°C and under physiological conditions. (Chu *et al.*, 2009) The applicability of BiFC using this new protein was demonstrated with the typical example of protein interaction, bFos - bJun, in COS-7 cells. In addition to the elimination of the lower temperature preincubation, the mLumin was twice as bright as the mCherry or the mRFP1-Q66T.

Table 2. Examples of fluorescent proteins used in *in vivo* BiFC assays.

Fluorescent protein	Excitation/emission maximum (nm)	Host cell	Reference
EYFP	500/535	Mammalian cell lines	Hu *et al.*, 2002
Venus; Citrine; Cerulean	515/528; 516/529; 439/479	COS-1	Shyu *et al.*, 2006
mRFP1-Q66T	549/570	Tobacco protoplasts	Jach *et al.*, 2006
mCherry	587/610	Vero	Fan *et al.*, 2008
mLumin	587/621	COS-7	Chu *et al.*, 2009

Abbreviations: EYFP: enhanced yellow fluorescent protein; COS-1 and COS-7: monkey kidney tissue cell lines; mRFP1-Q66T: monomeric red fluorescent protein variant; Vero: African green monkey kidney epithelial cell line.

BiFC has also been applied in bioluminescene RET (BRET) and FRET analyses of protein interactions. BRET has been demonstrated from *R*Luc to YFP-based BiFC (Rebois *et al.*, 2006). The *R*Luc and fragments from the YFP were fused to G-protein

subunits and their effectors. After coelenterazine substrate was added, YFP emission was detected indicating the close proximity of the studied G-protein subunits and their effectors. On the other hand, FRET has been shown from Cerulean, the variant of CFP, to a Venus-based BiFC (Shyu *et al.*, 2008). Here, the Cerulean was fused to a Rel homology region of p65, and bFos and bJun were fused to C- and N-terminal fragments of Venus, respectively. The coexpression of these three fusion proteins in cells generated FRET signals that demonstrated a ternary complex among the three interacting proteins. Moreover, multiple protein interactions can be visualized in the same cell using multicolor BiFC analysis, where two or more fluorescent proteins with distinct excitation and emission spectra are reconstituted. Multicolor BiFC has been shown in the simultaneous detection of the bFos - bJun heterodimer and the bJun homodimer. This was achieved using N-terminal fragments of YFP and CFP with a common partner, the C-terminal fragment of CFP (Hu and Kerppola, 2003). Yet another example of multicolor BiFC is the analysis of G-protein subunit interactions (Hynes *et al.*, 2008).

An advantage of BiFC compared to enzyme fragment complementation is that the complete protein itself is fluorescent without adding any exogenous substrate molecules. It allows the visualization of protein interactions *in vivo* with minimal perturbation of the normal cellular environment (Kerppola, 2006). A limitation of BiFC is that the formation of most, if not all, fluorescent proteins are irreversible and thus the analysis of transient and weak protein-protein interactions may be overestimated and dynamic interactions cannot be analyzed. The development of reversible forms of BiFC complexes is underway in several laboratories by means of splitting fluorescent proteins at different locations and by means of mutagenesis (Hynes *et al.*, 2008; Kodama and Hu, 2012). The irreversibility of the BiFC complex can also be seen as an advantage: weak and transient protein-protein interactions are difficult to observe using for example FRET and BRET analysis, whereas the irreversible BiFC has the potential to detect these as well (Ohashi *et al.*, 2012).

2.3 Recent trends of binary probes and protein fragment complementation

One recent trend in binary probes is utilization of padlock probes. In padlock probes, two target-specific nucleic acid strands are connected to each other with a flexible linker (Nilsson *et al.*, 1994). The conventional binary probes, reviewed in section 2.1.1, are composed of two separate oligonucleotide probes that hybridize independently to the target nucleic acid. A challenge with such probes is hook effect, which leads to signal decrease at high target nucleic acid concentrations. The hook effect can be alleviated using the padlock probes. With padlock probes, when the 5′ and the 3′ ends of the padlock probe are labeled with a FRET pair, the hybridization of the padlock probe to

the target nucleic acid can be measured directly (Rosmarin *et al.*, 2006; Yang *et al.*, 2006). A more recent trend is to combine padlock probes with proximity ligation. Although PLA is a slightly complicated assay format including many assay components, it enables highly sensitive target detection (Li *et al.*, 2010; Barisic *et al.*, 2013; Yaroslavsky and Smolina, 2013; Xiang *et al.*, 2013). Moreover, in PLA, the target-specific nucleic acid strands are not labeled or modified in any other way, either. From this point of view, PLA is an easily adaptable assay format.

Recent trends in avoiding labeling of the target-specific nucleic acid strands include the split cleaving and peroxidase-like DNAzymes, reviewed in section 2.1.5 (Mokany *et al.*, 2013; Zagorovsky and Chan, 2013). Yet another novel technique to avoid direct labeling of the target-specific nucleic acid strands is to use four-way junctions (Kouguchi *et al.*, 2011; Nguyen *et al.*, 2011; Gerasimova and Kolpashchikov, 2013). In four way junction-based nucleic acid detection, two nucleic acid strands are composed of a target-specific region and of a universal region. In the presence of a target, the hybridization of the target-specific regions brings the universal regions available for universal MB hybridization. The MB hybridization opens the MB, which increases fluorescence signal. The MB does not bind to the target directly, which enables detection of several nucleic acid targets using one labeled MB.

Enzyme and fluorescent protein fragment complementation assays are valuable tools for detection of various protein-protein interactions in many cell types. A requirement for efficient reporter enzyme and fluorescent protein formation is the correct folding and maturation of the protein complex. A common drawback of all fluorescent proteins is their phototoxicity and photobleaching concerns, which can both be alleviated by enhancing the brightness of the fluorescent protein (Lowder *et al.*, 2011). Reversible forms of protein fragment complementation, apart from the *R*Luc (Stefan *et al.*, 2007), are unknown. Thus the detection of dynamic interactions using protein fragment complementation remains a challenge.

Typical applications, advantages, and disadvantages of binary probe and protein fragment complementation-based assays are summarized in Table 3. In conclusion, both binary probes with various reporters and protein fragment complementation enable sensitive, separation free nucleic acid and protein interaction detection. Of these two techniques, binary probes are more versatile and have wider applications.

Table 3. Applications, advantages, and disadvantages of binary nucleic acid and protein fragment complementation-based biomolecule detection techniques.

Binary technique	Applications	Advantages	Disadvantages
Direct utilization of fluorescence	Real-time quantitative PCR Detection of DNA and RNA *in vitro* and *in vivo*	Fast signal formation Enables multiplexed assays High sensitivity	Requires labeling of the probe pair
Enzymatic reactions	Detection of DNA, RNA and proteins SNP discrimination	No labeling High sensitivity	Requires many assay components
Chemical reactions	Detection of DNA and RNA *in vitro* and *in vivo*		Requires chemical modification of the probe pair Slow reactions
Split aptamers	Detection of DNA, RNA and small molecules	No labeling	Limited sensitivity
Split nucleic acid enzymes	Detection of DNA, RNA, small molecules and metal ions SNP discrimination	No labeling of the target-specific probes Signal amplification	Slow enzymatic activity Limited sensitivity
Enzyme fragment complementation	Monitoring of protein-protein interactions *in vivo* Immunoassays and high through-put screening	Signal amplification	Requires substrate Irreversible protein complementation
Bimolecular fluorescence complementation	Monitoring of protein-protein interactions *in vivo*	Enables localized detection Substrate-free	Slow maturation of the chromofore Irreversible protein complementation Phototoxicity

3 AIMS OF THE STUDY

The main objective of this thesis was to develop a novel switchable lanthanide luminescence reporter technology and to demonstrate that this technology can be used in the detection of nucleic acid and protein targets in homogeneous assay formats.

More specifically, the aims of the original publications were:

I To establish the proof-of-principle of using switchable lanthanide luminescent binary probes in a sensitive homogeneous nucleic acid assay.

II To demonstrate that switchable lanthanide luminescence, with an improved ion carrier chelate structure, can be used in homogeneous protein detection.

III To show that switchable lanthanide luminescence enables wash-free multiplexed array-based nucleic acid detection where the luminescent complexes are formed on spatially separated spots on a solid surface.

IV To characterize the formation of the mixed chelate complex with a binary oligonucleotide probe approach and to investigate the utility of the switchable lanthanide luminescent binary probes in single nucleotide mismatch discrimination.

4 SUMMARY OF MATERIALS AND METHODS

The summary of the materials and methods used in the study are presented here. More detailed information can be found in the original publications (**I–IV**).

4.1 Labels and biomolecules

4.1.1 Lanthanide chelates

The ion carrier chelate (N1-Eu^{III}) [*N*1-(4-isothiocyanatobenzyl)diethylenetriamine-*N*1,*N*2,*N*3,*N*3-tetrakis(acetato)europium(III)] used in publication **I** was synthesized as described earlier (Mukkala *et al.*, 1989). The ion carrier chelate used in publications **II**, **III** and **IV** (DOTA-Eu^{III}) [2,2,2''-(10-(3-isothiocyanatobenzyl)-1,4,7,10-tetraaza-cyclododecane-1,4,7-triyl)tri(acetato)-europium(III)] was synthesized as described in publication **II**. The intrinsically luminescent heptadentate chelate (7d-Eu^{III}) [2,2',2'',2'''-[[4-[(4-isothiocyanatophenyl)ethynyl]pyridine-2,6-diyl]bis(methylene-nitrilo)]tetrakis(acetato)-europium(III)] used as a donor in publication **II** was synthesized as described earlier (Takalo *et al.*, 1994). The structures of the lanthanide chelates are presented in Figure 8.

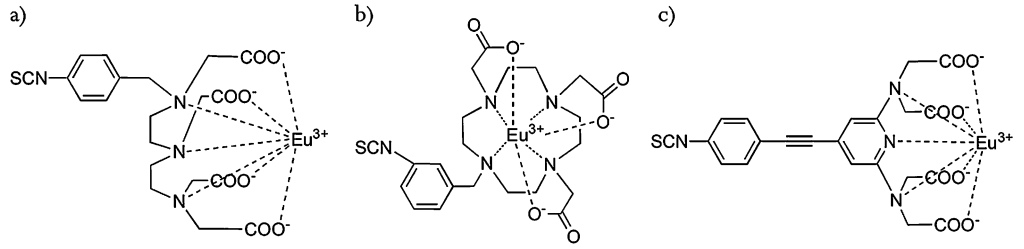

Figure 8. Structures of the non-luminescent isothiocyanate-activated ion carrier chelates a) N1-Eu^{III} (**I**, Mukkala *et al.*, 1989) and b) DOTA-Eu^{III} (**II**, **III**, **IV**, synthesis described in **II**). c) Structure of the isothiocyanate-activated intrinsically luminescent 7d-Eu^{III} (**II**, Takalo *et al.*, 1994).

4.1.2 Light harvesting antenna ligand and acceptor fluorophore

The light harvesting antenna ligand (4-((4-isothiocyanatophenyl)ethynyl)pyridine-2,6-dicarboxylic acid) (Figure 9) used in the switchable lanthanide luminescence assays in all four publications was synthesized as described in **I**. The near-infrared fluorescent label Alexa Fluor 680 (AF680) was used as an acceptor fluorophore in the reference assay based on lanthanide resonance energy transfer (LRET) (**II**) and was purchased from Molecular Probes (Eugene, OR).

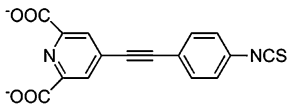

Figure 9. Structure of the isothiocyanate-activated light harvesting antenna ligand (**I**-**IV**, synthesis described in **I**).

4.1.3 Biomolecules

The oligonucleotide probes, primers, and synthetic oligonucleotide targets used in publications **I**, **III**, and **IV** were purchased from Sigma-Aldrich (St. Louis, MO), Biomers.net GmbH (Ulm, Germany) and Thermo Fisher Scientific (Waltham, MA). In publication **II**, the amine-PEG-biotin conjugated to DOTA-Eu^{III} and AF680 was purchased from Pierce Biotechnology (Rockford, IL), aminohexylbiotin labeled with antenna ligand was synthesized as described earlier (Collot *et al.*, 2008) and avidin was purchased from Sigma-Aldrich.

4.1.4 Labeling of biomolecules

Oligonucleotide probes used in publications **I**, **III** and **IV**, and biotins used in **II** were labeled with an ion carrier chelate (N1-Eu^{III} or DOTA-Eu^{III}) or with a light harvesting antenna ligand. The labelings were made using a reaction between a primary amino on aliphatic carbon linkers at the oligonucleotide probes or biotins and isothiocyanate at the ligand or chelate. The reactions with 3–100-fold molar excess of the label molecule in 50–150 mM carbonate buffer, pH 9.8, were incubated overnight at 50°C or 37°C and purified with reverse-phase high-performance liquid chromatography (HPLC) using C18 columns from Thermo Scientific and Phenomenex (Torrance, CA) with a linear acetonitrile gradient in 50 mM triethyl ammonium acetate buffer (Sigma-Aldrich). The aminoderivative of D-biotin coupled to the intrinsically luminescent 7d-Eu^{III} (bio-7d-Eu^{III}, Välimaa *et al.*, 2004) was kindly provided by Lasse Välimaa (Department of Biotechnology, University of Turku).

In publication **II**, the amine-PEG-biotin was labeled with AF680 using a reaction between the succinimidyl ester on the AF680 and the primary amino group on the biotin. The reaction was carried out in 50 mM carbonate buffer, pH 9.0 with 10-fold molar excess of the label molecule for 2 hours at 35°C and was purified with HPLC similarly to the previous reactions.

4.2 Instrumentation and measurement settings

4.2.1 Plate readers

The luminescence of the Eu(III)-chelate complexes were measured in time-resolved mode with a 1420 Victor Multilabel Counter (**I**, **II**) or with an EnVision Multilabel Plate Reader (**III**, **IV**) (Perkin Elmer Life and Analytical Sciences, Boston, MA) using a flash lamp and 340 nm excitation filter or 337 nm laser for excitation, respectively. In all original publications, the emission filter was 615 nm and measurements had a delay time of 400 μs and a measurement window of 400 μs. The sensitized emission from AF680 generated by LRET (**II**) was measured with a Victor 1420 Multilabel Counter that was modified with a 730 nm bandpass emission filter with a bandwidth of 10 nm and 70% transmission maximum (Nabburg, Interferenzoptik Elektronik GmbH, Germany) and a red-sensitive Model R4632 photomultiplier tube (Hamamatsu Photonics, Hamamatsu, Japan). The excitation wavelength was 340 nm, the measuring wavelength 730 nm, the delay time was 75 μs and the measurement window was 50 μs. In **III**, Eu^{III} luminescence from the bottom of the microtiter wells was measured by scanning a 10×10 raster (with 0.5 mm between the measurement points) using the EnVision Multilabel Plate Reader.

4.2.2 Fluorescence spectrophotometer and UV-VIS spectrophotometer

Emission and excitation spectra and emission lifetimes of the lanthanide complexes were measured with a Cary Eclipse fluorescence spectrophotometer (Varian Scientific Instruments, Mulgrave, Australia) (**I**, **II**). In luminescence emission spectrum measurement, the complex was excited at 325 nm (**I**) or at 340 nm (**II**) with a 20 nm slit and the emission at 550–750 nm was measured with 5 nm slit using 0.1 ms delay and 0.4 ms measurement time. The excitation spectrum was measured using the 5 nm excitation slit and emission wavelength of 615 nm with a 10 nm slit, 0.1 ms delay and 0.7 ms measurement time. In the emission lifetime measurement, the complex was excited at 325 nm (**I**) or at 340 nm (**II**) and emission at 615 nm using 20 nm slit was measured with 0.05 ms delay and 0.05 ms gate.

Absorbance measurement in the characterization of labeled oligonucleotide probes and absorption spectrum of the light harvesting antenna ligand for determination of the molar absorptivity were made with NanoDrop 1000 spectrophotometer from Thermo Scientific.

4.3 Homogeneous assays based on switchable lanthanide luminescence

4.3.1 Homogeneous nucleic acid assay (I)

In the original publication **I**, a sensitive homogeneous nucleic acid assay using switchable lanthanide luminescence was constructed. The principle of the assay is presented in Figure 10.

The assay was performed at room temperature (RT) in a buffer containing 50 mM Tris (pH 7.75), 600 mM NaCl, 0.1% Tween 20, 0.05% NaN_3 and 30 μM diethylenetriaminepentaacetic acid (DTPA). The hybridization assay was performed in low fluorescence 96-well Maxisorp microtitration wells from Nunc (Roskilde, Denmark). The probe labeled with the light harvesting antenna and the probe labeled with the N1-Eu^{III} (10 or 50 nM) and the complementary target oligonucleotide (0–50 nM) were combined to a total volume of 60 μL. The hybridization reactions were incubated for 15 min before the measurement of the europium(III) emission at 615 nm with a Victor 1420 Multilabel Counter.

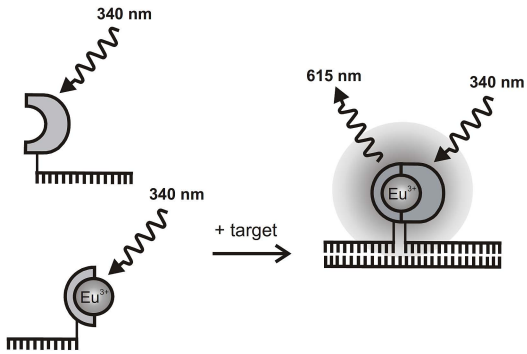

Figure 10. Principle of the switchable lanthanide luminescence-based nucleic acid assay. Labeled oligonucleotide probes are non-luminescent (in the dark state) in the absence of the complementary target oligonucleotide. After the hybridization of a labeled probe pair adjacently on the target oligonucleotide, the label moieties form a luminescent complex (switch to a bright state), which can be excited at 340 nm and the long lifetime emission can be measured in time-resolved mode at 615 nm.

4.3.2 Homogeneous avidin assay (II)

In the original publication **II**, the switchable lanthanide luminescence assay principle was applied for protein detection (Figure 11) and the assay performance was compared to a reference assay based on LRET. In the LRET assay, the bio-7d-Eu^{III} and the bio-AF680 formed an LRET pair. In the presence of avidin, the biotinylated labels bound to

the protein. Upon excitation at 340 nm, the intrinsically luminescent 7d-Eu^{III} donor transferred energy to the AF680 acceptor in close proximity, which enabled the measurement of the sensitized emission at 730 nm in time-resolved mode.

The assays were performed at RT in a buffer containing 10 mM Tris (pH 7.5) and 50 mM NaCl in low fluorescence 96-well microtitration wells from Nunc coated with bovine serum albumin to prevent nonspecific binding of avidin to the well surface. In the switchable lanthanide luminescence based assay, biotin labeled with DOTA-Eu^{III} (20 nM) and biotin labeled with a light harvesting antenna ligand (15 nM) were combined with 0–100 nM avidin to a total volume of 60 μL. The reactions were incubated for 15 min and thereafter the emission from europium(III) at 615 nm was measured in time-resolved mode with a Victor 1420 Multilabel Counter. In the LRET-based assay, biotin labeled with a 7d-Eu^{III} (20 nM) and biotin labeled with an acceptor fluorophore AF680 (1 nM) were combined with 0–100 nM avidin in a total volume of 60 μL. The reactions were incubated for 15 min and thereafter the sensitized emission from AF680 at 730 nm was measured with a Victor 1420 Multilabel Counter.

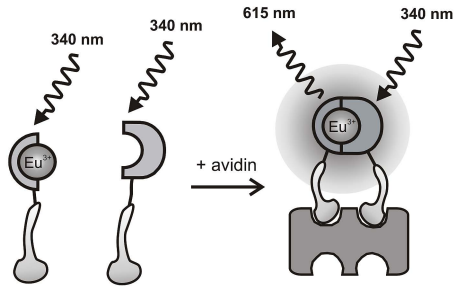

Figure 11. Principle of the switchable lanthanide luminescence-based avidin assay. Without avidin, the biotinylated DOTA-Eu^{III} and the biotinylated light harvesting antenna ligand are non-luminescent. The presence of avidin induces the formation of the luminescent complex.

4.3.3 Multiplexed array-based nucleic acid assay (III)

In the publication **III**, the switchable lanthanide luminescence was applied in a homogeneous multiplexed array-based nucleic acid detection. The principle of the assay is presented in Figure 12.

A primer and a probe pair were designed for *Escherichia coli gyrB* gene amplification and detection and three other probe pairs and three oligonucleotide targets were designed as control sequences. The probe pairs were labeled with the antenna ligand and the DOTA-Eu^{III}. For the array-based assay, four 3′ DOTA-Eu^{III} labeled capture probes containing also biotin at 5′ were spotted on streptavidin wells (Kaivogen Oy, Turku, Finland) at VTT Medical Biotechnology Centre (Turku, Finland). Four 5′ light

harvesting antenna ligand labeled detection probes (50 nM) and up to four synthetic target oligonucleotides or amplified DNA from asymmetric PCR were added to the spotted wells in a buffer containing 50 mM Tris (pH 7.7), 600 mM NaCl, 0.1% Tween 20, 0.05% NaN_3, and 100 μM DTPA. The hybridization reactions in a total volume of 60 μL were incubated first for 20 min at 50°C and then cooled to RT for 15 min in slow shaking before the scanning measurement of the europium(III) luminescence from the bottom of the wells at 615 nm with an EnVision Multilabel Plate Reader.

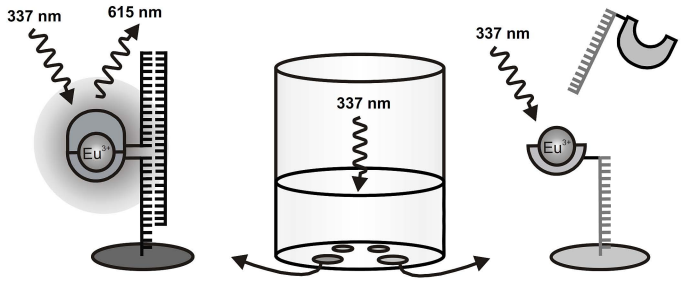

Figure 12. Principle of the switchable lanthanide luminescence-based multiplexed nucleic acid assay. Four capture probes conjugated to both biotin (at 5′) and to DOTA-Eu^{III} (at 3′) are spotted onto streptavidin coated 96-wells. The detection probes containing the light harvesting antenna ligand at 5′ and up to four target oligonucleotides are added. In the presence of the complementary target oligonucleotide, the spotted capture probe and the corresponding detection probe hybridize adjacently to the target. Consequently, a luminescent Eu^{III} complex is formed on the spot and the luminescence signal can be measured in time-resolved mode by scanning the bottom of the wells without a wash step. In the absence of the complementary target oligonucleotide, the detection probe is free in the liquid phase and thus the spot remains non-luminescent.

4.4 Optimal probe hybridization distance and single nucleotide mismatch discrimination (IV)

In publication **IV**, the characteristics of the switchable luminescent binary probes were more thoroughly investigated. For studying the optimal labeled probe hybridization distance, the oligonucleotide probe pairs with aminolinkers of different length and with different position for the label moiety conjugation were used (Figure 13). In addition, complementary target oligonucleotides with increasing amount of thymines between the specific hybridization positions of the two labeled probes were acquired. For studying SNP discrimination efficiency, target oligonucleotides with single mutations placed at five positions on two target oligonucleotides were used.

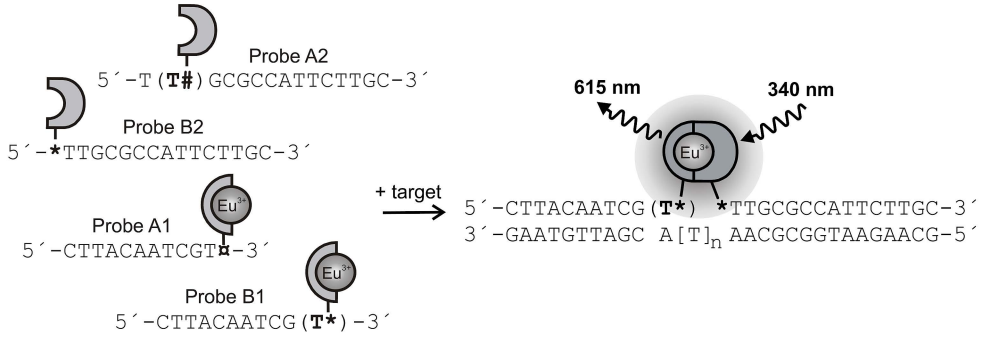

Figure 13. Schematic presentation of the oligonucleotide probe sequences and label moiety positions used for studying the optimal probe hybridization distance. Amino modifications for the label moiety coupling: ¤: Amino C3; (T#): Amino C2dT; (T*): Amino C6dT; *: Amino C12. n: 0–30.

Both the probe hybridization distance and the SNP discrimination experiments were performed in low fluorescence 96-well microtitration wells from Nunc. The labeled probe pairs (20 nM) and the fully matched targets of different length or the single nucleotide mismatched targets (10 nM) were combined in a buffer containing 50 mM Tris (pH 7.6), 0.05% Tween 20, 0.05% NaN_3, and 50 μM DTPA. In the hybridization distance measurement, the assay buffer contained 300 mM NaCl, and in the SNP assays the NaCl concentration was 50 mM. The reactions were incubated first at 50°C for 30 min and then at slow shaking at RT for 1 hour before the europium(III) luminescence measurement at 615 nm in time-resolved mode with an EnVision Multilabel Plate Reader.

5 SUMMARY OF RESULTS AND DISCUSSION

5.1 Photophysical properties of the luminescent complexes

In this study, the ion carrier chelates for the switchable luminescent complex were non-luminescent N1-Eu^{III} (**I**) and DOTA-Eu^{III} (**II**, **III**, **IV**) (see Figure 8). Soon after publication **I**, it was found out that the originally used linear heptadentate N1-Eu^{III} was not stable during PCR conditions (Lehmusvuori *et al.*, 2012a) and a more stable DOTA-Eu^{III} was developed and used. The light harvesting antenna ligand introduced in publication **I** (Figure 9) was used in all four publications. The emission spectra and emission decay spectra of a target oligonucleotide and protein induced antenna-N1-Eu^{III} and antenna-DOTA-Eu^{III} complexes, respectively, are presented in Figure 14. The label moieties switch from a dark state (narrow lines in the figure) to a bright state (thick lines in the figure) and form a luminescent complex upon addition of the analyte. The luminescent complexes of the both ion carrier chelates with the antenna ligand have their main emission peaks at 615 nm and the spectra are similar to that of heptadentate (Karhunen *et al.*, 2010) and nonadentate (von Lode *et al.*, 2003) intrinsically luminescent europium(III) chelates. The luminescence decay times of the antenna-N1-Eu^{III} and the antenna-DOTA-Eu^{III} complexes were 618 μs and 950 μs, respectively. The long emission lifetime of the antenna-DOTA-Eu^{III} complex implies that the mixed chelate complex excludes most of the quenching water molecules and that the quantum yield is close to 0.38 determined for a similar ligand (but lacking the isothiocyanate activation) in 3:1 ratio with Eu^{III} by Latva *et al.* (1997).

The excitation spectrum of a target oligonucleotide induced antenna-DOTA-Eu^{III} complex and the absorption spectrum of the antenna ligand are presented in Figure 15. The antenna ligand has its excitation maxima around 260 and 325 nm. The excitation efficiency at the excitation wavelength used in the homogeneous assays, around 340 nm, remained 80 percent of the maximum. The molar absorptivity of the light harvesting antenna ligand at 330 nm was determined to be 16 400 M^{-1} cm^{-1}.

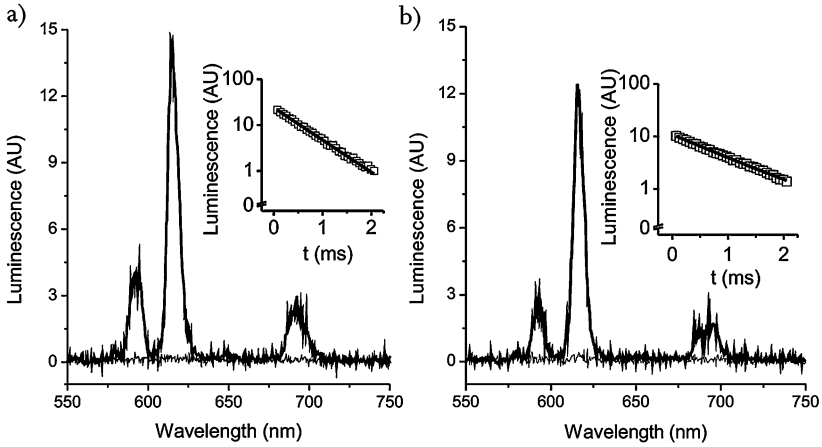

Figure 14. Luminescence emission spectrum of a) complex formed of oligonucleotide probe pair labeled with N1-Eu^{III} and antenna together with 0 nM (narrow line) and 10 nM (thick line) target oligonucleotide and b) protein induced complex formed of biotinylated DOTA-Eu^{III} and antenna with 0 nM (narrow line) and 25 nM (thick line) avidin. The fitted single exponential emission decay spectra are presented in the insets.

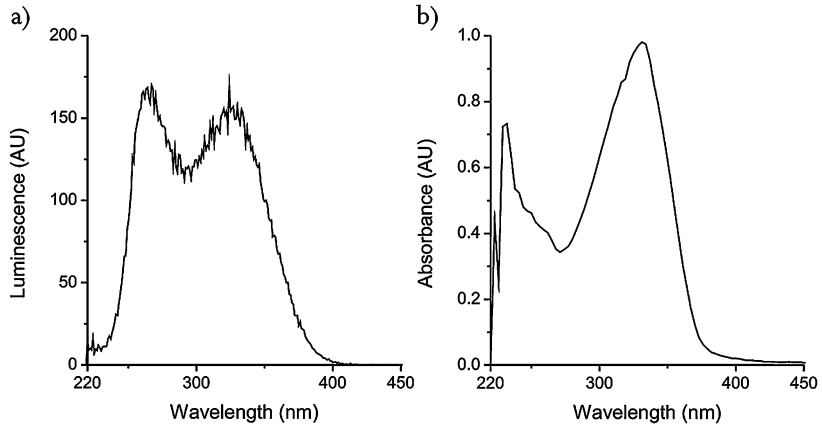

Figure 15. a) Excitation spectrum of a complementary target oligonucleotide induced complex formed between a probe pair labeled with a DOTA-Eu^{III} and an antenna ligand. The emission was recorded at 615 nm. b) Absorption spectrum of the light harvesting antenna ligand.

5.2 Performance of the homogeneous assays

5.2.1 Nucleic acid assay (I)

Binary probes consist of two oligonucleotide probes that are complementary to adjoining positions on a target nucleic acid. To detect the formation of the ternary

complex, the two probes have functional groups, which produce a detectable signal only when the probes hybridize simultaneously to the target nucleic acid, an essential property that enables homogeneous assays (Kolpashchikov, 2010). In the original publication **I**, a homogeneous nucleic acid assay, based on the switchable lanthanide luminescent binary probes, was constructed. The idea of splitting a luminescent lanthanide chelate into two non-luminescent label moieties and using them in homogeneous nucleic acid detection was originally presented by Oser and Valet in 1990 as reviewed in section 2.1.1.

In publication **I**, a novel light harvesting antenna ligand and an intrinsically non-luminescent europium(III) chelate, N1-Eu^{III}, were conjugated to a binary probe pair and used successfully in homogeneous nucleic acid detection. The LOD in the developed assay, determined as target concentration corresponding to 1.2× background signal, was 22 pM (Figure 16) corresponding to 1.3 fmol of nucleic acid per assay well. The detection limit was nearly 1000-fold better than what Oser and Valet (1990) reported. Furthermore, the obtained signal to background ratio of 1400 and the dynamic range of four orders of magnitude are exceptional among homogeneous fluorescence-based assays. For example, Wang *et al.* (2001) achieved a signal to background ratio of 3 in their assay using similar switchable lanthanide luminescent binary probes and Sueda *et al.* (2002) reported a 30-fold ratio in their LRET-based nucleic acid assay.

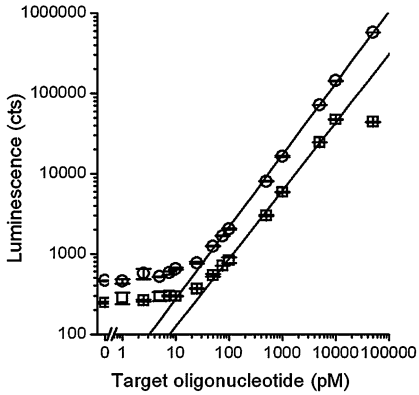

Figure 16. Target oligonucleotide titration using two probes of which the other is labeled with the N1-Eu^{III} and the other with the antenna. Square, 10 nM binary probe pair; circle, 50 nM binary probe pair. The error bars represent the standard deviation of two replicate wells.

5.2.2 Protein assay (II)

After the proof-of-principle nucleic acid assay, the switchable lanthanide luminescence was applied to protein detection in the original publication **II**. Avidin, although it is not for example a clinically relevant protein, was chosen as the model analyte because it is a

tetrameric protein with high affinity to biotin enabling a simple assay set-up (Livnah *et al.*, 1993). In this publication, also the thermally more stable DOTA-Eu^{III} was presented. An intrinsically luminescent heptadentate chelate, 7d-Eu^{III}, was used as a donor for an AF680 acceptor fluorophore in the LRET-based reference assay. The donor and the acceptor fluorophores and the two label moieties of the switchable lanthanide luminescence system were conjugated to biotin. Both assays were optimized for the ratios of the two biotin conjugates.

The biotin conjugates forming the luminescent lanthanide complex were optimal at a nearly 1:1 ratio (Figure 17a), as would be expected because the luminescent complex forms in 1:1 ratio of the two label moieties. Optimization of the LRET assay was demanding. The optimal ratio between the donor and the acceptor was 20:1 (Figure 17b). Higher biotinylated AF680 concentrations resulted in dramatically decreased sensitized emission signal. This may be explained by the saturation of the avidin by both energy donor and acceptor. This, however, results in a sensitized emission that is too rapid to be effectively measured (Vogel and Vedvik, 2006). Another possible explanation is acceptor self-quenching occurring at high acceptor concentrations (Didenko, 2001).

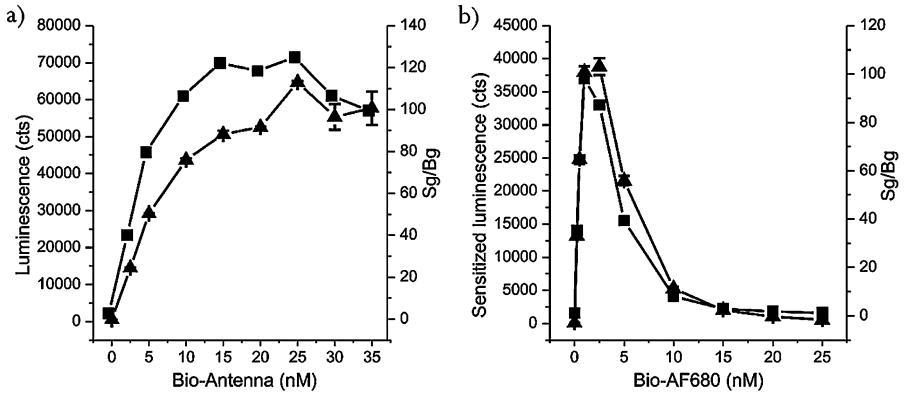

Figure 17. Optimization of the biotinylated light harvesting antenna ligand and AF680 concentrations in the avidin assays. a) Bio-antenna titration using 20 nM biotinylated DOTA-Eu^{III}; b) Bio-AF680 titration using 20 nM biotinylated 7d-Eu^{III}. Triangles, specific signals; squares, signal to background ratios. Error bars indicate the standard deviation of three replicate reactions, and cts denotes counts.

Avidin titration with optimized switchable luminescence and LRET assays are presented in Figure 18. With switchable lanthanide luminescence, a LOD of 4 pM, defined as the concentration of avidin corresponding to 3×SD of the background luminescence, was achieved. The dynamic range of the assay was four orders of magnitude. With the LRET-based reference assay, a LOD of 15 pM and a dynamic range close to three orders of magnitude were achieved. Both assays have sensitivity that is outstanding when

compared to other reported homogeneous avidin assays (Oh *et al.*, 2005; Wang *et al.*, 2005; Hernandez *et al.*, 2009; Liu *et al.*, 2012) with LODs at best in the low nanomolar concentration. The presented assay shows that switchable lanthanide luminescence enables homogeneous detection of a protein analyte, which has also been shown by Päkkilä *et al.* (2013) in a thrombin assay using two aptamer binders. Further exploitation could be in point-of-care applications where there is a need for rapid and sensitive assays for, for example, cardiac markers (Aldous, 2013).

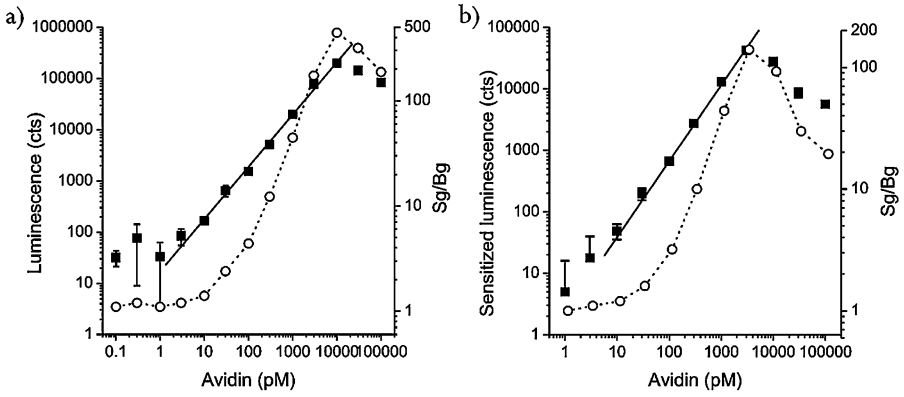

Figure 18. Avidin titration using a) switchable lanthanide luminescence and b) LRET. Squares, specific signals; circles, signal to background ratios. Error bars indicate the standard deviation of three replicate reactions, and cts denotes counts.

5.2.3 Multiplexed nucleic acid assay (III)

Microarrays using spatially separated spots of binders, such as antibodies or oligonucleotide probes, have greatly improved the throughput of biomedical research like analysis of gene expression and genetic variations, and diagnostics. A variety of technologies including fluorescence and colorimetry, and label-free methods such as surface plasmon resonance are used for microarray detection. Out of these, fluorescence is the most widely used (Nagl *et al.*, 2005; Wu *et al.*, 2008). A wash step to separate the signal of the bound assay components from the unbound components is, however, usually needed in current microarrays formats.

In publication **III**, a homogeneous multiplexed array using switchable lanthanide luminescence was constructed. One of the four probe pairs, *gyrB*, was designed for *gyrB* gene-based species identification of the *E. coli*. *gyrB* is a commonly used gene for bacterial identification in PCR-based assays (Järvinen *et al.*, 2009; Huang *et al.*, 2012; Lee *et al.*, 2014). Four 5′ biotinylated and 3′ DOTA-Eu^{III}-labeled capture probes were spotted on streptavidin-coated microtiter wells. After this, four 5′ antenna-labeled detection probes and up to four synthetic target oligonucleotides were added. When all

four target oligonucleotides were titrated simultaneously, four luminescent spots were formed at the bottom of the wells (Figure 19 I–IV). When the synthetic *gyrB* target was titrated in the absence of the other targets, only one luminescent spot was formed. To determine the limit of detection of the single target assay, an average signal from nine measurement points at the *gyrB* spot area was calculated. The respective titration curve is presented in Figure 20. A LOD of 0.32 nM, corresponding to 3×SD of the background luminescence, was achieved. In a multiplexed assay, where the three control target concentrations were kept at 100 nM and only the *gyrB* target was titrated, the LOD for was 0.60 nM due to higher luminescence background at the *gyrB* spot area (Figure 19 V-VIII).

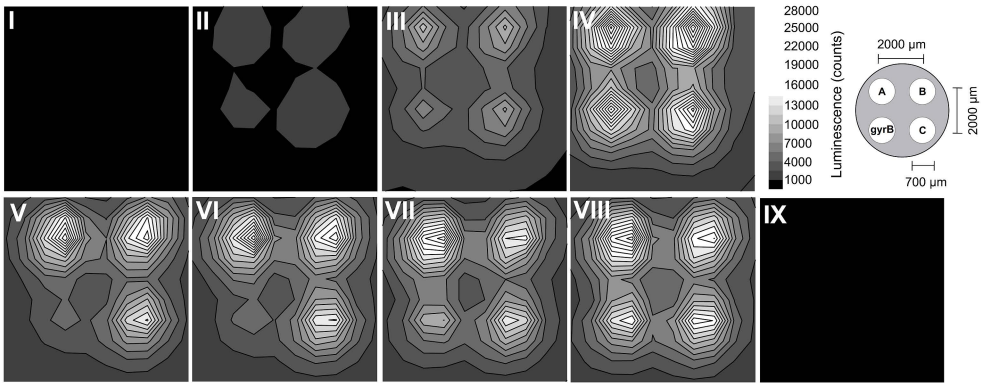

Figure 19. Luminescence images of spotted wells and a schematic microarray pattern. The images are from wells containing four spotted capture probes with 3′ DOTA-Eu^{III} and four detection probes with 5′ antenna ligand (50 nM) with different concentrations of synthetic target oligonucleotides. (I-IV) Simultaneous titration of four different target oligonucleotides: (I) 0 nM; (II) 1 nM; (III) 10 nM; (IV) 100 nM synthetic targets. (V-VIII) Titration of *gyrB* synthetic target oligonucleotide when concentration of each control (A-C) is 100 nM: (V) 0 nM; (VI) 1 nM; (VII) 10 nM; (VIII) 100 nM synthetic *gyrB* target oligonucleotide. (IX) Luminescence image from a background well with only spotted capture probes and assay buffer.

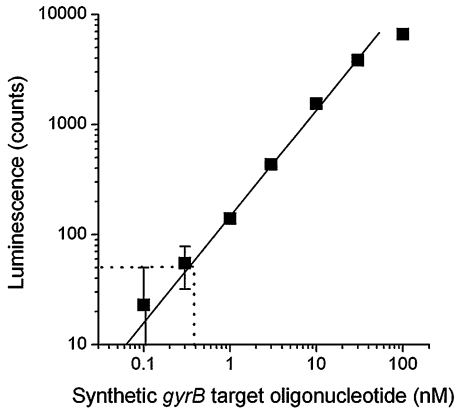

Figure 20. Titration of the synthetic *gyrB* target oligonucleotide using switchable lanthanide luminescence as a label in a homogeneous microarray. Luminescence signal was measured in time-resolved mode by scanning. Limit of detection is presented with a dotted line and error bars indicate the standard deviation of three replicate wells.

To demonstrate the detection of a PCR product with the array, primers for specific amplification of *gyrB* gene from *E. coli* were designed and used for asymmetric PCR amplification. After thermocycling, the reaction mixtures were diluted 1:5 in the assay buffer and transferred to the spotted wells containing also the four antenna labeled detection probes. Luminescent spots were observed at the *gyrB* spot area when samples were from amplification reactions containing 1×10^4 to 1×10^6 initial copies of the genomic DNA of *E. coli* (Figure 21 II-IV). The sample without an initial DNA template did not form a luminescent spot (Figure 21 I).

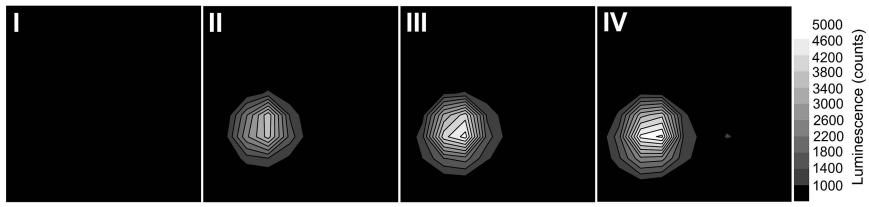

Figure 21. Luminescence images of wells containing four spotted capture probes and four detection probes in the liquid phase with asymmetric amplification product from I) 0; II) 10 000; III) 100 000 and IV) 1 000 000 initial copies of the *E. coli* genomic DNA.

The multiplexing degree of the constructed four-plex array is modest when compared to the commercially available nucleic acid arrays wherein hundreds or thousands of genes can be determined simultaneously (Matsuzaki *et al.*, 2004). Nevertheless, we were able to show for the first time that switchable lanthanide luminescence enables separation-free array-based nucleic acid detection. In the future, a combination of nucleic acid amplification and array-based detection in the same reaction would enable

closed-tube multiplexed assays needed, for example, in blood stream infection diagnostics.

5.3 Optimal probe pair distance and single nucleotide mismatch discrimination

5.3.1 Optimal probe pair distance (IV)

In publications **I** and **III**, the labeled probes were positioned adjacently or at a single nucleotide distance from each other, and the label moieties were conjugated to the oligonucleotide probes with two- or six-carbon (C2 and C6) aliphatic linkers. In publication **IV**, the optimal hybridization distance between the labeled probes and carbon linker lengths and positions for label moiety conjugation were more thoroughly studied. Based on preliminary experiments with eighteen probe pair combinations, two probe pairs, A and B, were selected. The probe pair A contained a C3 linker at the 3′ phosphate for DOTA-Eu^{III} conjugation and an internal C2 on penultimate thymine at 5′ for antenna ligand conjugation (see Figure 13). The linker positions were reversed on the probe pair B - the DOTA-Eu^{III} was conjugated to an internal C6 linker on the ultimate thymine at 3' and antenna ligand to the C12 linker at 5' phosphate. The distance variation between the labeled probes was accomplished with a set of targets containing 0–30 thymines between the probe pairs' hybridization positions.

When the labeled probe pairs hybridized at a short distance of 0–3 thymines to each other, the pair B resulted in low luminescence intensity, whereas the probe pair A resulted in relatively high luminescence intensities already at a distance of one thymine (Figure 22). When the distance between the labeled probes increased, the luminescence intensity also increased. Ten thymines was the limit and then the signal intensity started to decrease. This indicates that the luminescent complex requires certain space for formation and that the single stranded, non-hybridized part of the target is quite flexible allowing the label moieties to reach each other even though the probes are hybridized at a relatively long distance to each other.

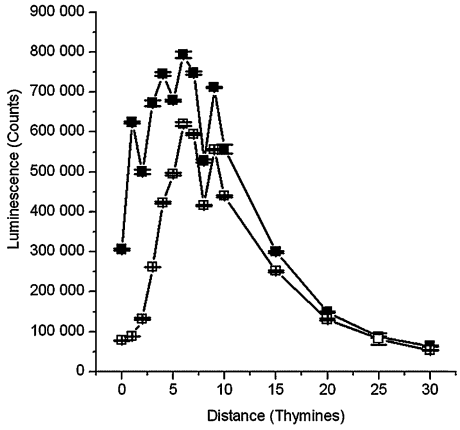

Figure 22. Luminescence intensity of the lanthanide complexes formed from labeled probe pairs hybridized at increasing distance. Black square, pair A with C2 and C3 alkyl linkers; white square, pair B with C6 and C12 alkyl linkers. The error bars indicate the standard deviation of three replicate wells.

5.3.2 Single nucleotide mismatch discrimination (IV)

Single nucleotide polymorphisms represent the most common class of variation in the human genome (Collins *et al.*, 1998). For example, differential hybridization and DNA sequencing can be used to genotype single mutations (Soler *et al.*, 2011). The longer the probe used for differential hybridization, the higher its binding affinity and lower its specificity. In addition to locked nucleic acids, chaotropic agents and an elevated measurement temperature, binary probes can be used to improve mismatch discrimination (Kolpashchikov, 2010). In publication **IV**, the mismatch discrimination efficiency of the switchable lanthanide luminescent binary probes was also investigated. For this, mutations were placed at five positions on two target oligonucleotides. The other target oligonucleotide had one thymine and the other had six thymines separating the probe pair hybridization. The mutations were placed complementary to 11-nucleotide DOTA-Eu^{III}-labeled probes starting from the labeled 3′ end. Six thymines separating the probe pair resulted in slightly higher mismatch discrimination efficiency than only one separating thymine (Figure 23). For example, a cytosine mismatch with an adenosine at position 5 resulted in 1400-fold signal difference with six separating thymines and in 900-fold signal difference with one separating thymine. With both probe pairs, mismatches at positions 1 and 2, that is, complementary or next to the DOTA-Eu^{III}-labeled nucleotide, were weakly or not discriminated from the fully matched targets. This is in accordance with earlier studies of single nucleotide mutation discrimination accuracy (Urakawa *et al.*, 2002; Urakawa *et al.*, 2003; Yan *et al.*, 2012). The signal differences between fully matched targets and single nucleotide mismatch targets achieved in this study are outstanding. To illustrate this, Liu *et al.* (2011)

reported a 30-fold, Ihara *et al.* (2011) a 7-fold, and Kolpashchikov *et al.* (2011) a 4-fold difference.

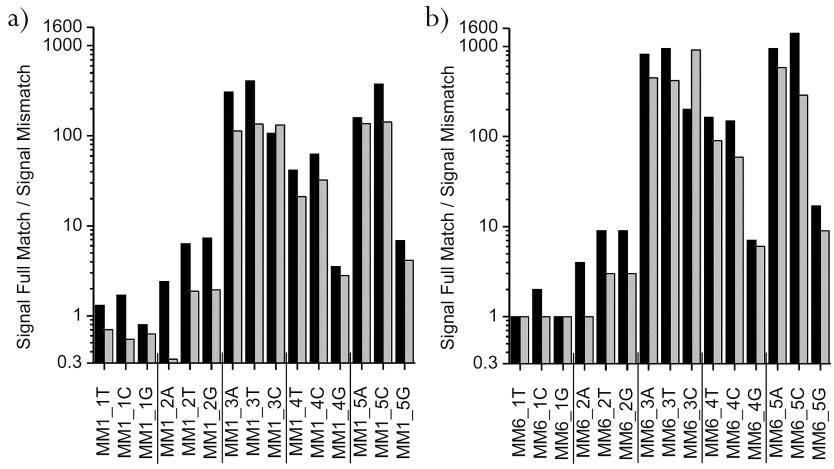

Figure 23. Luminescence signal of the labeled probe pairs with C2 and C3 (black columns) and C6 and C12 (grey columns) alkyl linkers hybridized with the fully matched target compared to the signal when hybridized with the mutated targets. a) One thymine separates the labeled probes; b) Six thymines separate the labeled probes.

6 CONCLUSIONS

Lanthanide luminescence has been utilized for over three decades in various bioanalytical assay formats. Thus far homogeneous assays using lanthanide chelate reporters have however been limited to FRET and to luminescent oxygen channeling (LOCI^{TM}) chemistry. In assays using FRET, the lanthanide emission energy is redirected to a fluorescent acceptor or to a quencher in close proximity, whereas in assays using LOCI, the lanthanide chelate is excited in a reaction involving singlet oxygen. The switchable lanthanide luminescence reporter technology, used in this study, is a novel application for lanthanide chelate-based labels. The close proximity of two, as such non-luminescent, molecules enables the formation of a highly luminescent mixed chelate complex.

The conclusions, based on the original publications are:

I A novel three-dentate light harvesting antenna ligand and a non-luminescent seven-dentate Eu^{III} chelate were conjugated to a binary oligonucleotide probe pair. The label moieties formed a highly luminescent complex in the presence of a complementary target oligonucleotide. The low background signal, in the absence of the complementary target, indicated that the label moieties had low affinity to each other, but when brought together, they were able to effectively exclude the quenching water molecules from the vicinity of the Eu^{III}. The long emission lifetime of the mixed chelate complex enabled time-resolved measurement, which minimized the background signal and enabled high sensitivity to the assay. The limit of detection (22 pM), signal to background ratio (maximally 1400), and dynamic range covering four orders of magnitude achieved in this study are exceptional among homogeneous fluorescence-based methods.

II For the first time, the switchable lanthanide luminescence reporter technology was used in protein detection. Comparison to a reference assay based on LRET revealed that switchable lanthanide luminescence is at least as sensitive as the reference assay. The optimization of the LRET assay was difficult because the optimal donor to acceptor ratio was high. A possible reason for this is the short distance between the donor and the acceptor when they are bound to avidin in equal amount. This creates too efficient energy transfer to be measured. The dynamic range of the switchable lanthanide luminescence-based assay was larger by one order of magnitude. The applicability of switchable lanthanide luminescence could be extended to generic protein detection, for example, by conjugating the label moieties to oligonucleotide linkers coupled to an antibody pair.

III There are only few reports of array-based assays using lanthanide chelate reporters and even fewer reports of separation-free array-based assays. We constructed a homogeneous four-plex nucleic acid array using switchable luminescent binary probes for quantitative target detection. Higher multiplexing was limited by the spatial resolution of the measurement device. An asymmetrically amplified DNA from *E. coli* was also qualitatively detected with the array. Immobilization of the light harvesting antenna ligand instead of the ion carrier chelate might improve the limit of detection of the assay. Moreover, covalent attachment of the immobilized capture probe instead of the biotin-streptavidin linkage, used in this study, could be beneficial in applications requiring tolerance to elevated temperatures.

IV In the original publications **I** and **III**, the switchable lanthanide luminescent binary probes were positioned at a short distance to each other when hybridized to a complementary target. By increasing the labeled probe pair hybridization distance in publication **IV**, the luminescence intensity of the mixed complex was increased. The effect was more dramatic when long aliphatic carbon chains were used as linkers between the label moiety and the oligonucleotide probe. Therefore, in practical cases when the probes have to be positioned next to each other or at a distance of only few nucleotides, it may be advantageous to select rather short linkers for label moiety conjugation. The switchable chelate complex showed also potential to discriminate single mismatches from fully matched targets. The high discrimination originates from the high luminescence intensity of the mixed chelate complex when the probe pair is hybridized to a fully matched target. This brings the label moieties in molecular contact, whereas weak or non-existent hybridization to mismatched target results in low background signal.

In conclusion, the beneficial photophysical properties of luminescent lanthanide chelates are preserved in the mixed chelate complex. The switchable lanthanide luminescence technology has the additional advantage in being applicable to separation-free assay formats. The requirement of two simultaneous binding events and close molecular contact of the label moieties strictly controls the signal formation and produces high specificity. This technology showed high sensitivity in homogeneous nucleic acid and protein detection, and enabled the construction of a separation-free array-based four-plex nucleic acid assay. Furthermore, single nucleotide mismatch discrimination using this technology proved outstanding. In the future, the high sensitivity and high signal to background ratio of the switchable lanthanide luminescence technology, presented in this thesis, could be utilized for developing novel nucleic acid assays.

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Turku, February 2014

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