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LUMINESCENCE-BASED ASSAY METHODS IN DRUG DISCOVERY

by

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To my home town Turku

Luminescence-based assay methods in drug discovery

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ABSTRACT

The drug discovery process is facing new challenges in the evaluation process of the lead compounds as the number of new compounds synthesized is increasing. The potentiality of test compounds is most frequently assayed through the binding of the test compound to the target molecule or receptor, or measuring functional secondary effects caused by the test compound in the target model cells, tissues or organism.

Modern homogeneous high-throughput-screening (HTS) assays for purified estrogen receptors (ER) utilize various luminescence based detection methods. Fluorescence polarization (FP) is a standard method for ER ligand binding assay. It was used to demonstrate the performance of two-photon excitation of fluorescence (TPFE) vs. the conventional one-photon excitation method. As result, the TPFE method showed improved dynamics and was found to be comparable with the conventional method. It also held potential for efficient miniaturization. Other luminescence based ER assays utilize energy transfer from a long-lifetime luminescent label e.g. lanthanide chelates (Eu, Tb) to a prompt luminescent label, the signal being read in a time-resolved mode. As an alternative to this method, a new single-label (Eu) time-resolved detection method was developed, based on the quenching of the label by a soluble quencher molecule when displaced from the receptor to the solution phase by an unlabeled competing ligand. The new method was paralleled with the standard FP method. It was shown to yield comparable results with the FP method and found to hold a significantly higher signal-to-background ratio than FP.

Cell-based functional assays for determining the extent of cell surface adhesion molecule (CAM) expression combined with microscopy analysis of the target molecules would provide improved information content, compared to an expression level assay alone. In this work, immune response was simulated by exposing endothelial cells to cytokine stimulation and the resulting increase in the level of adhesion molecule expression was analyzed on fixed cells by means of immunocytochemistry utilizing specific long-lifetime luminophore labeled antibodies against chosen adhesion molecules. Results showed that the method was capable of use in a multi-parametric assay for protein expression levels of several CAMs simultaneously, combined with analysis of the cellular localization of the chosen adhesion molecules through time-resolved luminescence microscopy inspection.

Keywords: drug discovery, high-throughput-screening, estrogen receptor, fluorescence polarization, two-photon excitation, time-resolved luminescence, adhesion molecule expression, immune response

Luminesenssiin perustuvista menetelmistä lääkekehityksessä

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YHTEENVETO

Uusien johtoyhdisteiden etsintä lääkekehitysprosessissa on jatkuvasti uusien haasteiden edessä, koska tähän tarkoitukseen syntetisoitujen yhdisteiden määrä kasvaa jatkuvasti. Uusien yhdisteiden soveltuvuutta testataan yleensä määrittämällä yhdisteen sitoutumisvoimakkuus kohdemolekyyliin tai -reseptoriin tai toiminnallisilla testeillä mittaamalla yhdisteen aiheuttamia vaikutuksia kohdesoluissa tai -kudoksessa tai kohteeksi valitussa eliössä.

Estrogeenireseptoriin (ER) sitoutuvia yhdisteitä eristetyillä reseptoreilla testattaessa käytetään usein homogeenisia luminesenssiin perustuvia tehoseulontamenetelmiä. Fluoresenssipolarisaatio (FP) on standardimetodeja mitattaessa ER:iin sitoutumista. Kaksifotoniviritteisen fluoresenssi -tekniikan soveltuvuutta perinteiseen yksifotoniviritteiseen verrattuna testattiin ER:n ligandin sitoutumisen FP-sovelluksella. Tuloksena todettiin kaksifotoniviritystekniikan olevan yhtäläinen yksifotonivirityksen verrattuna sekä lisäksi havaittiin mahdollisuudet määritysdynamiikan tehostamiseen ja tehokkaaseen määritystilavuuden pienentämiseen. Jotkut luminesenssiin perustuvat ER:n ligandinsitoutumismenetelmät hyödyntävät energiansiirtoa pitkäikäisen luminoivan leiman kuten lantanidikelaatin (Eu, Tb) ja lyhytikäisen luminoivan leiman välillä. Vaihtoehtona näille menetelmille kehitettiin uusi yksileimamenetelmä (Eu), jossa leiman luminesenssi sammuu liukoisen sammutinmolekyylin johdosta silloin, kun sitoutumisesta kilpaileva leimaamaton ligandi syrjäyttää leimamolekyylin reseptorista liuokseen. Uutta menetelmää testattiin FP:tä verrokkimenetelmänä käyttäen ja tulokset osoittivat uudella menetelmällä saavutettavan huomattava signaali/tausta -suhteen parannus sekä menetelmän olevan vertailukelpoinen FP:n kanssa.

Solupohjainen pintaproteiiniekspression määritys yhdistettynä mikroskooppianalyysiin antaisi arvokasta lisätietoa kohdeproteiinien olemuksesta verrattuna pelkkään ekspressiotason määritykseen. Tässä työssä immuunivastetta jäljiteltiin endoteelisolujen sytokiinistimulaatiolla, ja siitä seurannutta adheesiomolekyylien ekspressiotason nousua mitattiin fiksoiduista soluista immunosytokemian menetelmin käyttämällä pitkäikäisillä luminoivilla leimoilla varustettuja vasta-aineita, jotka spesifisesti tunnistivat valitut kohdeproteiinit. Tuloksena saavutettiin solun pintaproteiinien kvantitatiivinen määritysmenetelmä, jolla useita proteiineja voidaan määrittää samassa kokeessa ja lisäksi tarkastella näiden lokalisaatiota soluissa pitkäikäiseen luminesenssiin soveltuvalla mikroskoopilla.

Avainsanat: lääkekehitys, tehoseulonta, estrogeenireseptori, fluoresenssipolarisaatio, kaksifotoniviritys, aikaerotteinen luminesenssi, pintaproteiinimolekyylieksпressio, immuunivaste

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ABBREVIATIONS

AF	activation function
AP	alkaline phosphatase
AP-1	activator protein 1
AR	androgen receptor
β-gal	β-galactosidase
BSA	bovine serum albumin
CAM	cell adhesion molecule
DBD	DNA-binding domain
DELFIA	dissociation-enhanced lanthanide fluorescence immunoassay
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
E_2	estradiol
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
ERE	estrogen responsive element
ERK	extracellular signal regulated kinase
FAMA	fluorescence anisotropy microplate assay
FITC	fluorescein isothiocyanate
FMAT	fluorometric microvolume assay technology
FP	fluorescence polarization
FPIA	fluorescence polarization immunoassay
FRET	Förster resonance energy transfer
GPCR	G-protein coupled receptor
GPR30	G-protein coupled receptor 30
HCS	high-content screening
Her	herceptin
HRP	horseradish peroxidase
HTS	high-throughput screening
HUVE	human umbilical vein endothelial
IC_{50}	half-maximal inhibitory concentration
ICAM	intercellular adhesion molecule
IL	interleukin
K_d	dissociation constant

K_i	inhibition constant
LBD	ligand-binding domain
LBS	ligand-binding site
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
mRNA	messenger RNA
NFκB	nuclear factor κB
PKA	protein kinase A
QRET	quenching resonance energy transfer
RBA	relative binding affinity
RNA	ribonucleic acid
SERM	selective estrogen receptor modulator
SPA	scintillation proximity assay
SRC	steroid receptor coactivator
TGF	transforming growth factor
TNF	tumour necrosis factor
TPEF	two-photon excitation of fluorescence
TRF	time-resolved fluorescence
TR-FRET	time-resolved FRET
VCAM	vascular cell adhesion molecule
uv	ultra-violet
vis	visible

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by Roman numerals I-IV:

I Marko E. Tirri, Roope J.Huttunen, Juha Toivonen, Pirkko L. Härkönen, Juhani T. soini and Pekka E. Hänninen: Two-photon excitation in fluorescence polarization receptor-ligand binding assay. *Journal of Biomolecular Screening* 2005 10(4):314-9.

II Roope J. Huttunen, Tomas C. O'Riordan, Pirkko L. Härkönen, Juhani T. Soini, Niko J. Meltola and Aleksi E. Soini: Quantitative detection of cell surface protein expression by time-resolved fluorimetry. *Luminescence* 2007 22(3):163-70.

III Roope Huttunen, Juhani Soini, Pirkko Härkönen, Pekka Hänninen and Harri Härmä: Multiparametric luminescence method for quantitative cell surface protein expression analysis and imaging. *Journal of Immunological Methods* 2011 367(1-2):40-6.

IV Roope Huttunen, Shweta, Eija Martikkala, Merja Lahdenranta, Pasi Virta, Pekka Hänninen and Harri Härmä: Single-label time-resolved luminescence assay for estrogen receptor-ligand binding. *Analytical Biochemistry* 2011 415(1):27-31.

1 INTRODUCTION

During the development process of a successful drug molecule, many important steps are involved. From the target discovery and evaluation, through computer-aided molecule design and synthesis of combinatorial small molecule libraries (Dolle et al. 2009) together with libraries of natural products to lead compound screening and lead optimization, and finally, after animal tests to clinical examinations with humans. Despite the large number of human genes (> 20 000), gene transcripts and proteins (> 100 000), the number of molecular targets with drugs approved by the year 2006 was still limited to 324, of which 266 were human-genome-derived proteins, and the rest were pathogenic organism targets (Overington et al. 2006). Some of the potential targets might be intractable for modulation via low molecular weight compounds or simply be inaccessible using current technologies (Mayr and Bojanic 2009).

As new targets for drug discovery are emerging, efforts to develop new technology for lead compound screening and validation are becoming increasingly important. New analysis methods for screening assays should be robust, highly specific, simple, informative and low in material cost. Modern screening campaigns are based on high-throughput-screening (HTS) methods, assaying from 100 000 to millions of compounds a day. As such, the development of automation and detection technology is a key factor. HTS approaches can be carried out using small animals like fish or frogs, but more frequently, cell cultures or purified receptors are chosen for the first stage screening efforts. For validation of the assay methods used for screening purposes, a measure of assay quality and readiness called the Z'-factor (Zhang et al. 1999) has emerged as a commonly used standard in drug discovery.

Detection methods based on different forms of luminescence (e.g. fluorescence, phosphorescence and (bio)chemiluminescence) are by far the most applied drug screening applications. Fluorescence-based methods especially, are multifaceted and ever evolving in an attempt to create new and innovative screening strategies for HTS platforms. Fluorescence based detection methods are relatively simple, highly sensitive and amenable for miniaturization. They have a high dynamic range and especially, they lack the drawbacks of the radioactivity based detection methods, such as the short shelf-time of labeled probes and the laborious handling of both hazardous material and waste. Fluorescence-based methods, however, may suffer from certain disturbances caused by library compounds or assay matrix. These include sample autofluorescence, quenching of the fluorescence emission signal or damping of the excitability of the fluorescent probe. These limitations can be overcome using e.g. multiphoton excitation (Lakowicz et al. 1999) or red-shifted fluorescent dyes. Also, ratiometric readout methods like fluorescence polarization (FP) and Förster resonance energy transfer (FRET) may be used, or alternatively measurement of the fluorescence lifetime or methods based on long-lifetime fluorophores. As a whole, new robust and economical screening methods are being developed for the validation of an ever-growing number of "druggable" targets. The methods used are often specifically

designed to reject ineffective or potentially adverse effects causing compounds in the very early phases of drug molecule development. It would be especially advantageous to avoid the appearance of unexpected adverse phenomena in the first clinical trials, as was the case in a widely-known clinical trial accident, in the form of a "cytokine storm" (Stebbings et al. 2007), although this was partly caused by the misinterpretation of inappropriate tests with primates, preceding the first clinical trials with humans. This kind of fatal action during the clinical trials or at a later stage when a drug is already on the market, could be economically disastrous.

2 REVIEW OF THE LITERATURE

Two different realms in the drug discovery related screening field are introduced. The first part deals with purified estrogen receptor screening applications, their possibilities for miniaturization and also the goal to reduce any unspecific signal originating from the sample or the analysis instrument. The second part deals with cell surface expression analysis of endothelial cell adhesion molecules on fixed endothelial cells, describing different screening approaches in detail and touching on the objective of reducing unspecific signal.

2.1 Purified estrogen receptors as targets for lead compound screening

Estrogenic steroids estradiol, estriol and estrone mainly act as female sex hormones but play an important role also in the male biology. Estrogens are essential for the development and maintenance of female sex characteristics (Taylor 2009, Archer 2010) important for the development and growth of bones and maintenance of the bone mass and the connective tissue (Khosla 2008) and they have a positive effect in lipid metabolism and on the vascular tone as well (Zirilli et al. 2008). In males, estrogens also act as specific regulators of the prostatic function and have the capacity to exert both beneficial and adverse effects within the prostate (Ellem and Risbridger 2009).

Estrogen effects are mediated through genomic and non-genomic pathways. Genomic pathways are divided in two main classes, classical and non-classical. A simplified illustration of the main pathways of estrogen receptor (ER) mediated signalling is shown in Figure 1. In the classical pathway, the estrogenic signal is mediated through dimerized ERs (ERα or ERβ), complexed together with coactivator or corepressor proteins acting as transcription factors in the nucleus on a palindromic or pseudo-palindromic consensus sequence AGGTCAnnnTGACCT (with n standing for any nucleotide) called the estrogen responsive element (ERE). This finally leads to transcription of the target gene mRNA and the synthesis of target proteins like oxytocin, prolactin, cathepsin D, progesterone receptor, vascular endothelial growth factor, insulin-like growth factor or c-fos (Klinge 2001). In the non-classical pathway, the signal is mediated in the nucleus through ERs bound to other transcription factors such as Fos/Jun (AP-1 responsive elements), c-Jun/NFκB and specificity protein 1 (Sp1). These recruit co-regulators to form initiation complexes that regulate the transcription of genes whose promoters do not harbour EREs (Cheskis et al. 2007, Safe and Kim 2008). The coregulator proteins include coactivators (e.g. SRC-1, GRIP-1, ACTR and CBP) and corepressors (e.g. SMRT and NCoR) (Klinge et al. 2004). The coregulator proteins of nuclear receptors contain an amino acid sequence called LXXLL (with X standing for any amino acid), for recognition between the nuclear receptor and the coregulator protein. These coregulators bind to the ER complexed with the target site in DNA, generating a fully functional transcription complex. In

addition to the ligand, also the sequence of a given ERE-site influences the physical and functional interaction of ERα and ERβ with their coregulators. This allows gene-specific recruitment of coregulators to ERα and ERβ, which along with the cell-specific ratios of coregulator expression leads to E_2-induced tissue-specific transcription of genes (Klinge et al. 2004).

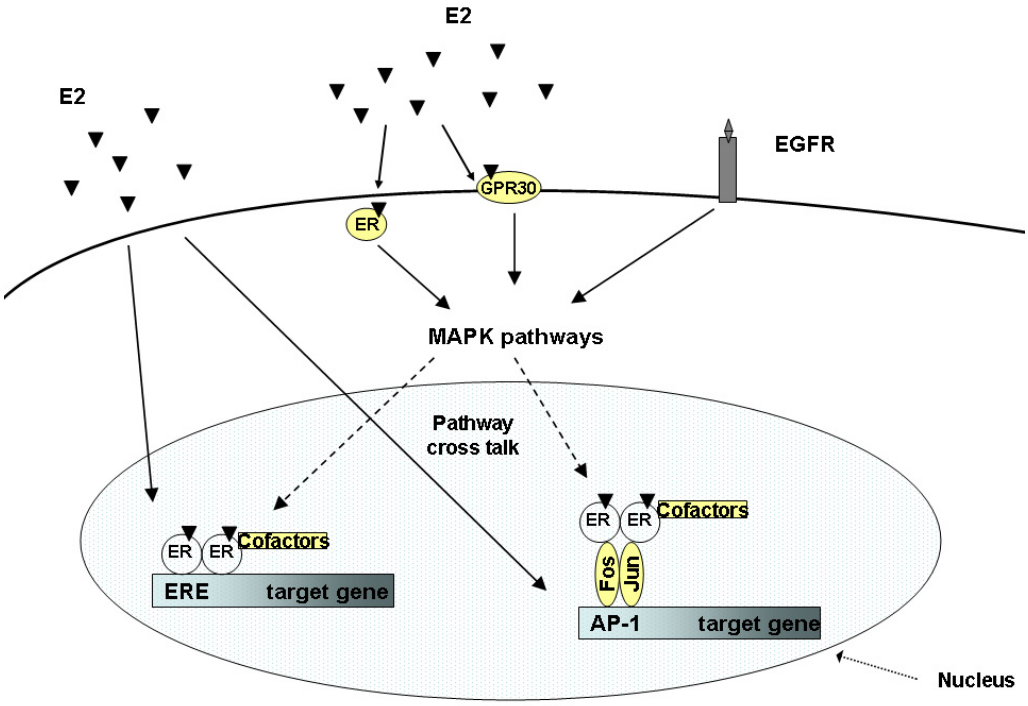

Figure 1. Simplified overview of ER function as a transcription factor. Classical (ERE-site) and non-classical (AP-1 site) transcription pathways work in concert with membrane-initiated estrogen signalling pathways and growth factor signalling pathways (EGFR).

Non-genomic ER signalling pathways refer to responses that estrogens exert within seconds or minutes of stimulus. These include the activation of the phosphatidylinositol-3 (PI3) kinase / Akt pathways and mitogen-activated protein (MAP) kinases, especially the extracellular signal regulated kinase 1/2 (ERK 1/2) and p38 MAPK (Cheskis et al. 2007). The detailed mechanisms behind these rapid membrane-initiated steroid signalling events (Nemere et al. 2003) are still to be revealed. These responses may be elicited through a small ER population and/or a certain estrogen-binding G-protein coupled receptor termed GPR30, localized at or close to the cell membrane (Levin 2002, Revankar et al. 2005). The cell membrane localization of ERα has been suggested to be in close association with caveolin-1 or Shc proteins (Evinger and Levin 2005).

Ligand-independent signalling pathways bring additional complexity to estrogen signalling. In these pathways, ERs or co-regulators associated with them can be activated via kinase phosphorylation in the cross-talking pathways of growth factor signalling, through highly active interactions in both the nuclear and cytoplasmic compartments (Kato 2001, Massarweh and Schiff 2006). This cross-talk signalling is also a very important aspect in the development of endocrine resistance in the progression of breast cancer.

Antiestrogens and selective estrogen receptor modulators (SERMs) targeted to bind ERα or ERβ or both are designed as therapeutic compounds for the prevention or acute treatment of various age- or sex-specific diseases. Examples of the therapeutic scope cover cancer, bone and metabolic homeostasis, depression, vasomotor symptoms and neurodegenerative diseases. Traditionally, synthetic therapeutic compounds have been designed to target the ligand-binding domain to trigger various cellular events such as the conformational changes of ERs, receptor dimerization and binding to specific response elements on DNA, and the interaction with coregulators (the essential effectors of the biological activities of ligand-activated ERs) (Nilsson and Gustafsson 2011). In the early phase of development of new SERM lead compounds, after the preliminary target validation and molecule modelling steps, followed by various organic synthesis chemistry efforts, the most common way to probe the binding affinity and efficacy of new leads is through assays using purified estrogen receptors (ER). The purified ERs usually are either recombinant full-length human receptors produced in Sf9 insect cells or ligand-binding domains (LBD) of the human ERs produced in yeast.

The binding characteristics of new lead SERMs on purified ERs can be assessed via straight-forward ligand binding site (LBS) affinity assays, using a labeled LBS-binding probe, a labeled co-regulator protein (or part of one), a labeled probe peptide bearing the co-regulator binding motif (e.g. LXXLL) or a labeled target gene nucleotide sequence bearing e.g. ERE-site. In addition, combinations of these forms of assays can potentially be used for screening purposes. Traditionally the compound libraries used for screening have included various types of natural products derived from plants (e.g. flavonoids and other natural polyphenols) and chemically synthesized substances.

2.1.1 Methods for screening

2.1.1.1 Fluorescence polarization

Fluorescence polarization or anisotropy, originally described by F. Weigert (Weigert 1920) and theorized by F. Perrin (Perrin 1926), can be utilized in binding assays by comparing the rotational state of a fluorophore bound to a faster rotating small molecule or a slowly rotating large molecule. More precisely; when excited with plane-polarized (parallel orientation) light, the large fluorescing molecules rotating slowly retain the polarization level of emitting light, while the radiative emission from small fast-rotating fluorescing molecules is depolarized. Thus, the ratio of fluorophores bound to small and large molecular entities (e.g. free and receptor-bound fluorescent probes) can be determined as polarization (P) by observing emission intensities through polarizers oriented both in parallel and perpendicular direction to the polarization plane of the excitation light (Jameson and Croney 2003). Polarization $P = [(I_{par}-I_{perp}) / (I_{par}+I_{perp})]$ is dimensionless but often expressed in mP units. mP is simply the polarization multiplied by 1000 (e.g. a P of 0.250 equates to 250 mPs). As the polarization for a spherical molecule in a liquid solvent is relative to the rotational relaxation time and its volume, the polarization contrast between the free and receptor-bound fluorescent probe is in direct relation to the difference in the molecular sizes of the labeled probe and the receptor.

Fluorescence polarization (FP) is a beneficial detection technique because it is ratiometric and thus less prone to interferences from inner-filter effects when compared with those methods measuring absolute intensity values. Supposedly, FP based assay methods are still by far the most established techniques to assess the binding of a ligand or a ligand-associated co-regulator to purified ERs, in a competitive manner. FP based detection techniques could be described as the gold standard concerning homogeneous assay methods for receptor-ligand binding in general.

The pioneering work of Bolger and co-workers (Bolger et al. 1998) to assess ligand binding to ERs for screening purposes, used purified full length ERα and an inherently fluorescent diethylstilbestrol derivative (tetrahydrochrysene (ES1)) as a fluorescent LBS probe (Hwang et al. 1992). This method had some drawbacks. For the excitation of the probe, a uv light source of around 380 nm was required and in addition, the fluorescence intensity of ES1 slightly increased upon binding to the receptor. Additionally, the reaction required dimethylformamide (DMF) to inhibit the enhancement of ES1 fluorescence polarization caused by dimethylsulfoxide (DMSO), a commonly used solvent for screening library compounds. This may be problematic as DMF is a hazardous compound. Further, in the ES1 based ER ligand binding assays the assay mixture included three different organic solvents: DMSO, DMF and ethanol (solvent for ES1), which made the assay more complex.

Later, Parker and co-workers (Parker et al. 2000) reported on an improved form of FP ER-ligand binding assay utilizing a fluorescein-labeled estradiol conjugate ES2 (Invitrogen) as a probe. The method is considered to be improved since the probe is based on the major natural binder 17β-estradiol, instead of the synthetic diethylstilbestrol derivative and no DMF is required. This assay was later developed further by introducing a probe with red-shifted emission (EL Red, Invitrogen) with an excitation maximum of ~ 535 nm and an emission maximum of ~ 590 nm. The benefit of the red-shifted emission probe is the reduced interference derived from the incidental autofluorescence of library molecules, which typically decreases towards the longer wavelengths of the visible light spectrum. A number of studies have reported the use of prompt fluorophore labeled 17α-conjugates as probes for FP-based ER-ligand binding assays (Ohno et al. 2002, Ohno et al. 2003) and in one study a fluorescein-labeled derivative of diethylstilbestrol (Adamczyk et al. 2002) was used. These assays have not been reported as being applied in a microplate assay format.

FP measurements have also been useful in the determination of ligand-associated coactivator binding to ERs. These methods have been utilized to measure the ligand efficacy in either the binding of a labeled coactivator e.g. SRC1-4 (Margeat et al. 2001) or a labeled LXXLL-motif-containing peptide (Rodriguez et al. 2004, Ozers et al. 2005) to ERs. In these methods, the less polarized fluorescence from the unbound labeled coactivator or LXXLL-motif-peptide turns more polarized when complexed with larger ER. Generally, in these studies, the antagonistic compounds were observed to inhibit and the agonistic compounds to enhance the binding of the labeled probe peptides on the ER dimers, as expected. The dual-mode assay method (Ozers et al. 2005) could discriminate between agonistic and antagonistic test compounds, as they performed with either increased or decreased efficacy when compared with the “basal” efficacy, in the presence of non-binder molecules like testosterone, or a solvent vehicle only.

The potential benefits of FP-based coactivator binding affinity measurements in screening approaches to discover new LBS binding SERMs, may become fully attainable when combined with assaying the ER binding to target DNA sequence. FP based DNA binding analysis methods apply a fluorescein-labeled oligonucleotide bearing an ERE-site, while the receptor or the ligand remains unlabeled. The fluorescence from the unbound labeled oligonucleotide is less polarized than the fluorescence from ER-bound labeled oligonucleotide. The potency of ER binding to a plain oligonucleotide bearing ERE-site is ligand-independent as such, as indicated in FP studies (Ozers et al. 1997, Boyer et al. 2000). These confirm earlier reports (Reese and Katzenellenbogen 1991, Reese and Katzenellenbogen 1992, Furlow et al. 1993). No difference in ER-ERE association between agonist or antagonist exposure has been shown in the absence of a coactivator protein in general, and only subtle differences between various ligands in ERE-ER interactions were detected in a single study (Margeat et al. 2003). Taking into account that binding of ER to the ERE is thought to precede coactivator binding, and also that the ERE influences ER conformation and its the ability to recruit coactivators, it is important that the ER-coactivator interaction is examined in a context where the ER is pre-bound to its DNA binding site (Wang et al.

2007). Wang and co-workers developed a fluorescence anisotropy microplate assay (FAMA) for HTS purposes, in order to identify inhibitors of ER-ERE interaction beyond the ligand binding pocket (Wang et al. 2004). The assay principle was similar to the FP assays described above and it was applicable for the 384-well format and amenable to automation. The FAMA assay platform has been used successfully in screening campaigns and potent lead compounds for the treatment of breast cancer have been identified (Mao et al. 2008). Interestingly, during a cell-based secondary screening campaign, another potent lead compound for breast cancer therapy was found (Kretzer et al. 2010). This compound however does not disrupt ER-ERE interaction or bind in the ligand-binding pocket, but greatly enhances proteasome-mediated degradation of ERα. It is noteworthy that this compound would not have been identified with the conventional screening strategies used in the steroid receptor related drug discovery discipline.

2.1.1.2 TR-FRET

In a proximity-based time-resolved-Förster resonance energy transfer (TR-FRET) assay method, the beneficial low background signal derived from long-lifetime labels as lanthanide chelates or cryptates is combined with a FRET-based ratiometric homogeneous assay format. The unique luminescence properties of these lanthanide complexes, such as long Stokes' shift and decay times on a microsecond to millisecond scale, provide possibilities to overcome disadvantages related to the use of conventional prompt fluorescence labels (decay times of nanoseconds). These may include background noise derived from the scattered excitation light and the inherent short-lifetime fluorescence from the sample and other assay materials (Hemmilä and Laitala 2005, Degorce et al. 2009). In a similar manner to the conventional FRET, TR-FRET applies two different fluorophores, a donor and an acceptor. According to the Förster theory (Förster 1948), the donor molecule is excited by an energy source (e.g. flash lamp or laser) and the excitation energy is non-radiatively transferred to the acceptor molecule. This is dependant upon the donor and the acceptor being situated at a proper angle with respect to each other and in close proximity (in practice, 5 – 105 Å, depending on the dimensions of the interacting molecules) and the absorption spectra of the acceptor overlapping efficiently with the emission spectra of the donor. This reflects in the quantum yield of the FRET phenomenon. Essentially, the detection of acceptor emission has to be performed in a spectral region separate from the emission of the donor. In TR-FRET assays typically, luminescent chelates or cryptates of lanthanide ions such as terbium (Tb) or europium (Eu), act as donor species and a prompt fluorescence label as an acceptor (Selvin 2002).

TR-FRET based methods, as well as AlphaScreen and FP methods can be used to study plain ligand-receptor interaction or more functional ligand-dependent coregulator binding. Indeed, the TR-FRET technology has made it possible to study these binding events simultaneously in multiplexed assays (Hilal et al. 2010, Kim et al. 2010). At present, Tb is usually used as the donor, because its emission spectrum is characterized by four sharp peaks centred at 490, 546, 584 and 620 nm with distinct

silent regions of ≥ 15 nm between the peaks which can be used to measure the specific acceptor emission with suitable filters. In contrast, Eu emits primarily beyond 600 nm, limiting the range of acceptor species that may be used (Vogel et al. 2008). In practice this means that the acceptor emission intensity can be measured using filters for regions between (Blomberg et al. 1999, Riddle et al. 2006) or beyond (Kupcho et al. 2007) the Tb emission peaks, whereas if Eu is being utilized as the donor, the emission signal should be measured beyond the ~ 610 nm emission peak.

TR-FRET methods probing simply ER-ligand association, include a lanthanide-labeled antibody against tagged or intact receptor (or tagged LBD), in addition to a labeled tracer. This makes TR-FRET slightly more complex in comparison to the sole receptor-tracer-pair used in FP based ligand-receptor binding assays. The extent of competition on the binding of the ligand to the receptor is detected as a ratio of lanthanide (donor) and acceptor emissions, which greatly reduces the variability of the data (Shukla et al. 2009). When a competitor ligand binds to the ER and displaces the bound prompt fluorescent acceptor fluorophore, the FRET is disrupted. These assays often use a glutathione-S-transferase (GST)-tagged LBD of ERs instead of an intact full-length receptor. The GST-tag originally used for receptor purification purposes is also applicable to binding the lanthanide-labeled antibody (the donor). The easy to perform purification process presents a major advantage in addition to the donor "taggability". Although one could theoretically "mark" the intact receptor by a labeled antibody that recognizes another domain of the receptor (outside LBS), this approach has rarely been reported.

Coactivator binding assays for ERs based on TR-FRET thus far developed, have been largely based on already established FP assays. A TR-FRET based assay for measuring the binding of a coactivator SRC3 derived peptide to ER (Gunther et al. 2009) was desirable because the FP based assay of Rodriguez et al.. had a low dynamic range and required excessively high ER concentrations (Rodriguez et al. 2004). The new assay utilized a biotinylated ER-LBD and streptavidin conjugated europium chelate as the donor system while the SRC-peptide was labeled with a Cy5 acceptor. The method is able to identify ligands that act as conventional ER antagonists that bind to the LBS and indirectly block the binding of the SRC-peptide. Nevertheless, the explicit aim of Gunther et al.. was to set up an assay for identifying compounds that directly block SRC-peptide binding in a competitive manner in the presence of a high concentration of agonist estradiol (1 μM). The positive "hits" were then re-assayed with a simpler method to exclude false-positives. In this confirmatory assay, the LBS of the receptor was probed with a Cy5-labeled ligand as an acceptor and the setup was otherwise similar to the primary assay excluding the labeled SRC-peptide acceptor. If the test compounds in the secondary assay reduced the FRET signal significantly, they were categorized as false positives due to a predominant conventional antagonist character. The assay was found to be highly robust and reproducible and the excellent Z'-factors indicated its amenability for HTS purposes. Consequently, the need for a receptor in this assay was reduced over 100-fold, when compared to the former FP assay. The method of Gunther et al.. was also essentially simpler and more economical than the first TR-FRET based ER-coactivator binding assay (Liu et al. 2003), which was

composed of a FLAG-tagged ER-LBD, a costly Eu-labeled anti-FLAG antibody, a biotinylated LXXLL-motif peptide and a costly and cumbersome streptavidin-conjugated allophycocyanin (APC) protein as acceptor.

Another screening approach combined the fluorescein-labeled estradiol ES2 from the FP assay (Parker et al. 2000) as donor, with two separate acceptor molecules associated with the ER in a dual TR-FRET assay for simultaneous ligand and coactivator binding (Hilal et al. 2010). This assay used GST-tagged ER-LBD and terbium chelate-labeled anti-GST antibody as the donor system and an LXXLL-motif peptide (PGC1a-peptide) labeled with Dy (a red fluorescing molecule) and used the fluorescein-labeled ES2 probe as acceptor. If the test compound did not displace ES2; only the FRET emission derived from ES2 was detected when excited with uv light (Tb exc.) and the compound was categorized as non-binder. Agonist compounds were able to displace ES2, the emission of which was decreased. The red-shifted emission from Dy-labeled PGC1a-peptide was simultaneously increased. Antagonist compounds for their part displaced ES2, decreasing its emission but without affecting emission from Dy-labeled PGC1a-peptide. This was due to the fact that antagonists do not enhance but rather inhibit the coactivator binding to ER. The method was also able to identify unconventional agonists; in those cases ES2 was probably only partly displaced from ER as its emission decreased only moderately, whilst the emission from the labeled PGC1a peptide increased prominently. Z'-factors of between 0.6 and 0.9 and a signal-to-background (S/B) ratio of 7 were obtained for the binding readout, whereas the recruitment readout showed a S/B of 2.3 and Z' factors of 0.5. The method was amenable for automation and miniaturization and in principle was also found to be applicable for screening purposes with other nuclear receptors.

2.1.1.3 Scintillation proximity technology

Unstable isotopes (radioisotopes) are commonly used as tracers for biomolecule detection in various biological applications and in heterogeneous ligand binding or diagnostic assays and can also be utilized as tracers for a homogeneous method called Scintillation Proximity Assay (SPA). The SPA technology was first introduced in the late 1970's (Hart and Greenwald 1979). In SPA measurements, the signal detected is a consequence of a radiolabeled tracer molecule bound in close proximity to the scintillation material incorporated into the fluomicrosphere beads. These beads can be functionalized with affinity tags (e.g. streptavidin, protein A or wheat germ agglutinin), to bind receptors or other biological target molecules (Glickman et al. 2008)

Once a radiolabeled tracer binds in close proximity to the SPA bead-bound receptor, the energy derived from the radioactive decay, instead of being adsorbed by the medium, is transferred to the scintillation material resulting in the emission of photons. The proximity for an effective energy transfer from the bound radiotracer varies between 1 and 125 μm depending on the isotope used. The emitted photons are usually quantified with photomultiplier tube-based scintillation readers; also charge-

coupled device (CCD) camera based imagers can be applied for detection (Zheng et al. 2001, Bryant et al. 2004, Merk et al. 2004). Using the SPA technology, any nonspecific signal derived from the assay reagents or sample sources can be quite efficiently avoided. Nevertheless, some background signal from unbound radiolabeled tracers can be of significance in cases where an unbound isotope (β-particle emitter) is close enough to stimulate a bead to emit, or when there is a requirement for a high concentration of additional radiolabeled tracers (Glickman et al. 2008). These non-proximity effects (NPE) can often be diminished with the proper choice of the radiolabel tracer and the scintillating material used and also by setting optimal concentrations for them.

For some targets, a solid-phase approach is a more relevant way to perform scintillation proximity based assays. In these assays, the scintillators are incorporated within the walls of specific scintillant multi-well plates as solid support. The receptor, ligand or mediating molecule is bound to the plastic well surface by adsorptive mechanisms or via attached affinity tags as used in the bead-based applications previously described.

Both direct ligand binding to ERs (Carlsson and Häggblad 1995, Allan et al. 1999) and ligand-controlled DNA-ER interactions (Carlsson and Häggblad 1995, Häggblad et al. 1996), have been assayed using scintillant-containing multi-well plates. In the method used by Allan and co-workers (Allan et al. 1999), the binding of the radiolabeled estradiol was assayed using ER adhered on the well bottom via an ER-specific antibody that was bound to a secondary antibody precoated on the bottom of the well. Antibody-assisted adhering of ER in this method was one example of the methods for immobilizing the protein target on the well surface via an intermediate affinity capture motif, in order to yield a better signal-to-background ratio when compared to methods using target bound adhering on the wells by means of simple adsorption, as used in the method of Häggblad and co-workers (Häggblad et al. 1996). As a whole, both approaches for assaying estradiol-ER binding were comparable, though Allan's method claimed to save receptor material by using a small amount of receptor per well. In the scintillation proximity approach, a tritium-labeled estradiol was used as a tracer while the biotinylated oligonucleotide was attached to the bottom of the well using streptavidin coated plates. Additionally, the effects of the ligand on the binding properties of ER were assessed using a palindromic consensus sequence-containing oligonucleotide. The most remarkable finding in those studies was that the binding of ER to the ERE-oligonucleotide immobilized on the bottom of the well was clearly dependent on the concentration of estradiol, in contradiction to the results from studies applying FP where the labeled ERE-oligonucleotide is diffusing freely in the solution (Ozers et al. 1997, Boyer et al. 2000). This phenomenon was explained by hypothesizing that the system composed of immobilized ERE-oligonucleotide and freely moving ligand and receptor is more similar to the events in the nucleus of a cell when ER binds to its target sequence e.g. ERE (Carlsson and Häggblad 1995). However, other ER-ligands than estradiol were not tested and similar studies using SPA technology have not been reported so far.

2.1.1.4 AlphaScreen technology

A bead-based assay platform called AlphaScreen utilizes special donor and acceptor beads to report biological interactions e.g. between ligand and receptor, or receptor and coactivator. AlphaScreen technology was originally reported as a luminescent oxygen channelling immunoassay (LOCI) technique (Ullman et al. 1994). The initial signal is derived from the donor beads containing a photosensitizer, phthalocyanine, which converts ambient oxygen to an excited and reactive form of O_2 (singlet oxygen) upon laser illumination at 680 nm. Taking into account that the lifetime of the excited singlet oxygen is only ~ 4 μs, during this time a molecule can diffuse only for a distance of approximately 200 nm in solution. When the donor and acceptor beads are withine this close proximity, a highly amplified signal in the 520-620 nm range is detected due to a luminescent cascade initiated by energy transfer from the singlet oxygen to the fluorophores in the acceptor beads. In the absence of an acceptor bead, the reactive singlet oxygen falls to ground state and only a low background signal is detected.

In the AlphaScreen ER assay for agonist and antagonist screening (Rouleau et al. 2003) the acceptor beads were coated with protein A in order to capture anti-ERα antibodies which recognize and bind to the full length ERα. The donor beads were coated with streptavidin in order to capture the biotinylated LXXLL-sequence containing motif for SRC-1 coactivator binding. The agonist-induced change in the conformation of the ER is responsible of the increased affinity of the receptor towards the coactivator-motif peptide triggering the beads into close proximity and leading to the generation of the specific AlphaScreen signal. The determined binding characteristics for two well-known ER agonists and two antagonists were in fair agreement with the literature values determined by other methods. The determined Z'-factor of 0.8 showed that the method could be adapted for HTS purposes.

The most striking benefit of AlphaScreen technology is the negligible background signal due to the fact that the excitation is performed using light of an explicitly higher wavelength than that used for detection. This leads to a large S/B ratio and high sensitivity, subsequently allowing for efficient miniaturization and reduced reagent consumption. Although the method is robust and amenable for HTS studies ($Z' > 0.5$) and allows assessing both agonist and antagonist potencies and the usage of the intact full-length receptor, it still is a highly complex assay method composed of several costly components. The AlphaScreen method also requires use of a specialized instrument, and based on the time needed to measure a plate using a multilabel reader, one could classify it as a medium throughput method.

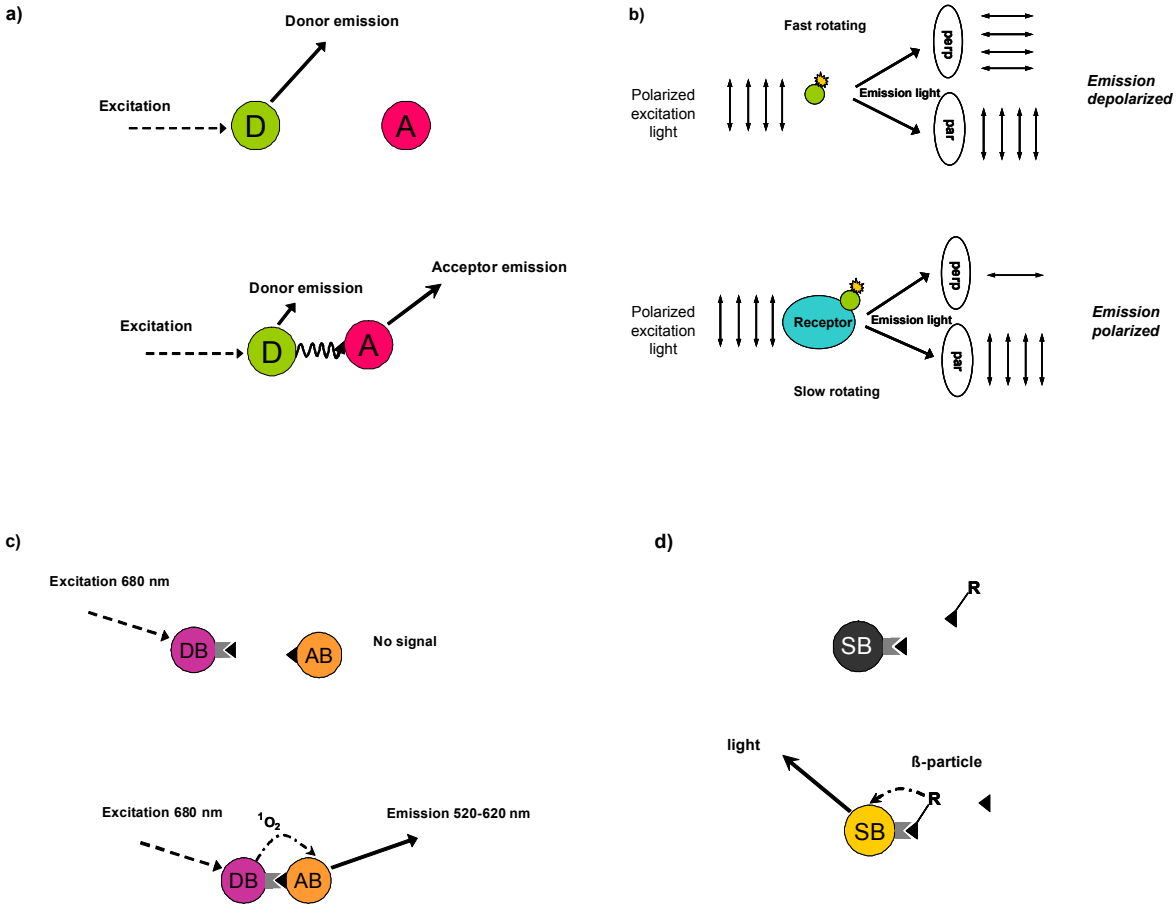

Figure 2. Mechanism principles of luminescence based detection methods. a) Förster resonance energy transfer (A = acceptor, D = donor). b) Fluorescence polarization (par = parallel, perp = perpendicular). c) AlphaScreen (AB = acceptor bead, DB = donor bead). d) Scintillation proximity (SB = scintillating bead, R = radiolabel).

2.2 Cell-surface expression modulation of endothelial cell adhesion molecules as subject for drug discovery

Glycoproteins at the cell surface mediating the contact between two cells or between cells and the extracellular matrix are called adhesion molecules (Carlos and Harlan 1994, Gumbiner 1996). They regulate intercellular interactions, mediate the composition of the intercellular network and have an important role in the regulation of inflammatory and immune responses. Adhesion molecules have also been recognized as mediator molecules of intercellular signal transduction processes (Crockett-Torabi and Fantone 1995, Hubbard and Rothlein 2000). The number and average affinity of the cell adhesion molecules determine the strength of cellular adhesion. Cell surface expression levels of the adhesion molecules can be enhanced by induced release from intracellular stores or synthesis of new adhesion molecules. The cell surface levels are regulated by a number of cytokines and other inflammatory mediator molecules (Meager 1999). A simplified illustration of the temporal expression pattern of adhesion molecules of the endothelium during cytokine-mediated immune response is shown in Figure 3.

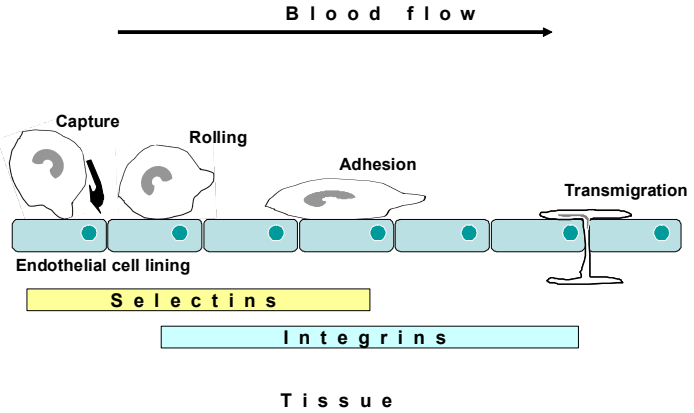

Figure 3. Simplified overview of cell adhesion molecule (CAM) expression and progression of leukocyte infiltration to the inflamed tissues due to enhancement of CAM expression on endothelium as a response to cytokines released from the tissue.

Most cell adhesion molecules are classified in certain families by their functional and structural characteristics. The main families are cadherins, integrins, selectins and the immunoglobulin super-family. Cadherins are Ca^{2+}- dependent hemophilic adhesion receptors for other cells, essential in the regulation of morphogenesis and the organization of tissues. Their intracellular part interacts with the cytoskeleton via catenin proteins to ensure that cells within a tissue are bound together (Behrens 1993). Integrins are composed of a heterodimer of subunits named α and β, associated with each other through non-covalent interactions. Cells can have multiple types of integrins on their surface. The heterodimers are composed of x different α- and y different β-subunits ($\alpha_x\beta_y$) and the integrin families are defined by the identity of the β-subunit (Kavanaugh 1996). Integrins mediate cell-cell and cell-matrix interactions through activation by binding with their cognate receptors (Yamada and Even-Ram

2002). Many integrins function as receptors for other cell adhesion molecules, like the other integrins as e.g. $\alpha_4\beta_7$ for $\alpha_{2b}\beta_3$ and $\alpha_v\beta_3$ or members of the immunoglobulin super-family e.g. $\alpha_4\beta_1$ for VCAM-1, β_2-integrins for ICAM-1,-2 or -3 and $\alpha_4\beta_7$ for MadCAM-1 or VCAM-1 (Kavanaugh 1996). The adhesion molecule family of selectins includes E-, P- and L-selectin, where; E stands for endothelial, P for platelet and L for leukocytes. Selectins share many structural properties with lectins that are carbohydrate-recognizing proteins. In principle, since the sugar moieties of the other cell surface molecules act as the ligands for selectins, they could be classified as a specific class of lectins (Ishida 2010). L-selectin is constitutively expressed on leukocytes, functioning as a "homing receptor" for them to enter the secondary lymphoid tissues via high endothelial venules (Kawashima 2006). E-selectin and P-selectin mediate early and reversible inflammatory events involving "leukocyte rolling" and margination along the luminal surface of microvascular cells (Foster et al. 1994). This "rolling" phenomenon is thought to be due to the weak affinity of E-selectin to leukocyte receptor molecules causing the temporary molecular interactions to be made and broken at a high pace. During the inflammatory response, P-selectin is released from storage granules within minutes of stimulation by thrombin or histamine, while the cytokine-induced expression of E-selectin and P-selectin takes place within 2-6 hours. Ligands for E-selectin differ from those for P-selectin suggesting the separate roles of these selectins in early inflammatory events. Members of the adhesion molecules of the immunoglobulin super-family are called cellular adhesion molecules (CAMs) and they play roles in antigen recognition, complement-binding or cellular adhesion. Characteristic of these proteins is a variable number of extracellular Ig-like domains (Williams and Barclay 1988).

ICAM-1 and CD2 play an important role in the interactions between immune cells and MadCAM-1 in the homing of lymphocytes in high endothelial venules. ICAM-1, ICAM-2, VCAM-1 and PECAM-1 are essential for leukocyte adhesion on the surface of endothelial cells, followed by migration of leukocytes into tissues. The expression of ICAM-1 and VCAM-1 in endothelial cells is up-regulated from minimum basal levels upon induction by several various cytokines, while ICAM-2 and PECAM-1 are expressed constitutively. Upon stimulation with cytokines, the expression levels of ICAM-1 and VCAM-1 on endothelial cells can be up-regulated for several days (Bevilacqua et al. 1994).

The inhibition of leukocyte emigration to the inflammation site due to cytokine stimulus followed by increased cell surface adhesion molecule expression levels, is an important target for drug therapy (Stewart et al. 2001, Blankenberg et al. 2003, Ulbrich et al. 2003, Mrowietz and Boehncke 2006). Notably, TNF-α and IL-1β and a number of other cytokines act through various receptors and signalling pathways (e.g. MAPK). This leads to the activation of NFκB and AP-1 transcription factors (Niessen et al. 2002), followed by many cellular events e.g. induced adhesion molecule expression.

Adhesion molecules are potential target molecules for therapy also in a variety of other pathological conditions beyond the endothelium, including allergy and asthma,

Crohn's disease, diabetes, inflammatory bowel disease, multiple sclerosis, psoriasis and rheumatoid arthritis (Simmons 2005). Drug therapy in cancer treatment is also a possible implication for adhesion molecule control as they have been reported to play a significant role in the progression of various cancers (Rosette et al. 2005, Francavilla et al. 2009, Makrilia et al. 2009). In addition to the attenuation of adhesion molecule expression, their function can be inhibited through small-molecules or antibodies which block their binding to cognate receptor molecules (Hopkins et al. 2004, Simmons 2005). Drug molecules already established and emerging for therapy, are specifically targeted to control the expression levels or the binding capacity of endothelial adhesion molecules and often have their origin in the molecular libraries of specifically designed synthetic substances, antisense oligonucleotides and monoclonal antibodies, targeted against either the adhesion molecules or their receptors (Stewart et al. 2001, Ulbrich et al. 2003). Other potential compounds are currently under intense research for the future development of inhibitors of cell adhesion molecule expression. These include natural compounds, flavonoids, steroids and phenolic compounds (Wheller and Perretti 1997, Norata et al. 2006, Wang et al. 2006, Wu et al. 2006, Lee et al. 2007). Thus far, potential drug molecules have completed clinical trials quite poorly (Yonekawa and Harlan 2007). In addition, statins originally designed for treatment of high cholesterol levels, have been reported to affect the cell adhesion molecule expression levels directly or post-translationally via cleavage shedding (Greenwood and Mason 2007), which are clearly off-target effects.

2.2.1 Methods for screening

2.2.1.1 High-content screening method (image-based)

Image-based screening methods arose in the mid-1990s, by which time imaging tools had established their role in other aspects of the life sciences (Ramm 2005). Especially, high-throughput microscopy (HTM) technologies - an important element of high content screening (HCS) methods, are considered to provide extra value in cell-based assays compared to the whole-well cell population measurements on conventional plate readers. HCS methods also enable pattern change measurements such as the translocation of a molecule from one cellular compartment to another, as covered in the first reported automated HTM assay for cytokine induced nuclear translocation of NFκB (Ding et al. 1998). HCS methods also provide special gating algorithms for the precise selection of the actual targeted cell subpopulation and for the elimination of assay disturbing artifacts originating from e.g. debris, deteriorated or dead cells, and precipitates of fluorescent molecular species (Bushway et al. 2008). Rapid progress in algorithm and software development has enabled the extraction of a broad array of image-based cellular information in the form of numerical and computable data along with subsequent processing and analysis of the collected data.

Bushway and co-workers (Bushway et al. 2008) evaluated the performance of a plate imager (IN Cell 1000 Analyzer) and two commonly used plate readers (Beckman

Coulter DTX and PerkinElmer EnVision) in a primary fluorescent cellular screen of 10 000 library compounds for up- and down-regulation of vascular cell adhesion molecule 1 (VCAM-1). In brief, all three instruments were initially evaluated for titration of fluorescently labeled MIN6 cells. The cells were made fluorescent by addition of the fluorescent DNA stain DAPI or transfecting the cells with the fluorescent proteins eGFP and DsRed. The cell titer varied from 280 to 9000 cells seeded on the wells of a 384-well plate. After fixation with paraformaldehyde, the specific fluorescent signals were measured using proper filter sets. Results pointed out that the imager showed best performance in this preliminary test: the imager was able to reliably detect cells in the lowest concentration of 280 cells / well whilst the DTX and EnVision readers could detect 2250 and 560 cells per well, respectively.

Agonism or antagonism on the TNF-α induced VCAM-1 expression on the surface of HUVE cells was then studied using a compound library, to perform the evaluation of the screening capacity of the instruments. The assay was designed for screening both agonists and inhibitors of VCAM-1 expression by setting the used TNF-α concentration to half of the value inducing maximum detected expression of VCAM-1 in the assay development step. The HUVE cells were treated with TNF-α and fixed with paraformaldehyde. Following blocking with serum and washing, a primary mouse monoclonal anti-VCAM-1 antibody was added. After washing, a secondary anti-mouse antibody labeled with Alexa-488 fluorescence stain was introduced. After a further washing step, the cells were finally mounted in glycerol and analyzed with the instruments.

For the inhibitor assay, Z' -factors of 0.41 and 0.16 were measured for the IN Cell 1000 and EnVision respectively. In the assay for agonists, Z' -factors of 0.57 and 0.65 were measured for the IN Cell 1000 and EnVision respectively. In terms of Z' values, the imaging platform was more capable than the plate reader platforms in identifying inhibitors, while all three platforms performed more evenly in the field of agonist identification. A reasonable explanation for this could be that plate readers have a lower dynamic range and sensitivity than the imager in the range of limited signal, as one could predict already based on the results of the cell number assay evaluation. On the other hand, agonists enhance VCAM-1 expression, thereby elevating the signal into a range where plate readers may detect the signal more reliably. In-turn, this leads to a comparable detection performance with the plate imagers. The authors, however, did not report any detection limits or deviation of the detected signals for VCAM-1 expression (as TNF-α conc.). Neither was any comparison between the throughput levels of the different assay platforms shown. The authors expressed a notion that the InCell 1000 takes about a 5- and 2-fold measurement time per plate compared to the respective EnVision and DTX platforms. Additionally, the imager platform assay requires additional analysis of data, involving a rigorous and labour intensive process.

One major advantage of the imaging platform is the capability of filtering out wells with an artifact, e.g. wells containing cells with misshaped nuclei or fluorescent debris, by using certain discriminating computational criteria. Other advantages are the possibility for re-analysis of the existing datasets with additional algorithms and

the option to carry out a more detailed image analysis of defined wells, which could be especially valuable as a means of troubleshooting.

2.2.1.2 Assay using cell-based DELFIA technology

An approach for analyzing the expression levels of multiple adhesion molecules on the surface of endothelial cells in a single assay was reported by Zerwes and co-workers (Zerwes et al. 2002). HUVE cells were seeded on the bottom of gelatin-coated microplates and let to grow near confluence. The enhancement in expression levels of E-selectin, ICAM-1 and VCAM-1 was stimulated with IL-1β or TNF-α. The incubation time with cytokine was set to 6 h as the peak point of E-selectin was determined to be 8 h by the authors. After incubation, the cells were fixed with paraformaldehyde and briefly washed with a buffer. In the next step, a mixture of lanthanide chelate-labeled primary mouse monoclonal antibodies against each target was added. The antibody against E-selectin was labeled with Eu(III)-chelate, the anti-ICAM-1 antibody with Sm(III)-chelate and the anti-VCAM-1 antibody with Tb(III)-chelate. After antibody incubation, the cells were briefly washed with a DELFIA wash buffer containing DTPA. Then the DELFIA enhancement solution for Eu and Sm was added, followed by measurement of europium and samarium fluorescence with a microplate reader. Next, the DELFIA enhancement solution for Tb was added without removing the first enhancement solution and finally the terbium fluorescence was read with a microplate reader. The method was proven to be highly capable of separating distinguished effects on the expression levels of the target proteins, caused by various substances in a genuine high-throughput screening campaign with a throughput of 12 000 samples per week. The Z'-factors determined were 0.72, 0.68 and 0.69 for the expression of E-selectin, ICAM-1 and VCAM-1, respectively. The authors also demonstrated that the labeled antibodies still recognized the target antigens on paraformaldehyde-fixed cells and did not interfere with each other in the assay mixture via cross-reactions. The authors erroneously described the method as the first description of a multiple readout based on a cell-based ELISA system, as no enzymatic reaction or enzyme is involved. In fact, the method resembles an ELISA-type assay platform but the signal is not amplified before measurement (typically characteristic of the ELISA platform). A notably beneficial feature of the method however, in addition to the negligible interference from fixation-induced autofluorescence, was that the routine protocol included only two wash steps after fixation.

2.2.1.3 Assays using cell-based ELISA

A widely used method for the quantification of cell surface protein expression is cell-based enzyme-linked immunosorbent assay (ELISA), a modification of the conventional ELISA used for analyte quantification in liquids. In the classical ELISA method, the soluble analyte is captured on a solid surface (usually a multi-well plate).

The bound analyte is recognized by a primary antibody and then quantified with an enzyme-linked secondary antibody: common enzymes used include alkaline phosphatase (AP), horseradish peroxidase (HRP) and β-galactosidase (β-gal), and detection is performed by absorbance, fluorescence or chemiluminescence. In the cell-based ELISA method, the adhered cells form the solid support matrix as the surface expressed protein functions as the analyte. In principle, the cells can be fixed or non-fixed for the assay, but a fixation process preceding the assay is usually recommended to avoid any disturbing effects resulting from exposure to antibodies, buffers or detection reagents. As in the traditional ELISA methodology, the unbound detection antibody is washed off at the end of the antibody incubation step and the detection step may be initiated through substrate addition to the cells. For the most commonly applied enzymes used in ELISA assays (AP, HRP, β-gal), there are a plethora of different chromogenic, fluorogenic or luminogenic substrates to choose from. The detection capabilities of the microplate reader used and naturally the costs of the assay per well, are limiting the choice of detection mode.

In a typical assay scheme, the confluent monolayer of cells is treated with cytokines, with and without the compounds studied. After the incubation time is complete, the medium is removed and cells are washed with PBS, 1-3 times. Next, the cells are fixed according to a chosen method, after which the monolayer is washed 3 times with PBS. The primary antibody is added and after the incubation period, the antibody solution is removed and the cells washed 3 times with PBS. The secondary antibody is then added. When incubation is complete, the antibody solution is again removed and the cells washed 3 times with PBS. Finally, the detection substrate can be added to the cells and measured after a proper reaction time has elapsed. A typical cell-based ELISA then includes 10-12 laborious and time-consuming washing steps, which significantly hamper the automation of the assay method and limit throughput extensively. In spite of these limitations Rice and co-workers (Rice et al. 1996) developed such an automated microplate-based cell ELISA assay, to identify inhibitors of endothelial cell activation by monitoring the cell surface expression of E-selectin. During the development of this cell-based ELISA method, the two major obstacles were to assess the reproducible fixation method for the treated monolayers and to identify an automated washing technique, which would not detach the cells as a result of the multiple wash steps required. In the final assay developed, the HUVE cells were fixed for 2-3 min with 100% methanol and the washing steps were run with an automated plate washer with controlled flow rates of liquid addition. The authors also multi-plexed the assay through the determination of cytotoxicity and by assaying cellular attachment. In this way, they looked to reveal real “hits” instead of compounds generating non-specific effects on the integrity of the cellular monolayer, e.g. by interfering with the general metabolism of the cells or detaching the cells from the surface of the plate. Cytotoxicity was assayed by measuring the calcein fluorescence prior to the fixation step and the ELISA process. The fluorescent dye calcein AM (cleaved by intracellular esterases in viable cells generating impermeable calcein) was added to the cells 30 min before the end of the incubation period with drug compounds and the fluorescence was determined (ex 485 nm, em 532 nm). Cellular

attachment was determined using a crystal violet stain following the aspiration of the colour development solution after running the ELISA assay. Crystal violet dye dissolved in methanol remained inside the cells. After a washing step, the dye was extracted with extraction solution in the wells and an absorbance of 562 nm was measured at the end of the assay.

2.2.1.4 Assay based on FMAT assay format

Fluorescence microvolume assay technology (FMAT) was designed as a platform for homogeneous ligand binding and immunological assays (Mellentin-Michelotti et al. 1999, Swartzman et al. 1999). In brief, the optical format of FMAT uses for excitation, collimated laser light that is focused on the bottom of the microtiter plate well to achieve a Gaussian beam focus of approximately 8 μm with a depth of focus of approximately 100 μm (Miraglia et al. 1999). The emitted fluorescence is also collimated by the focusing objective (NA 0.45) epi-fluorescence configuration) and separated from the excitation light by a dichroic beam splitter and a long-pass spectral filter. This fluorescence is further split into two channels using a dichroic filter and collected by two photomultiplier tubes. The microtiter plate is mounted on an XYZ stage and an image of the well can be reconstituted by moving the detector head in the Y direction, while the stage is moved in the X direction at a rate of 1 mm/sec to achieve a 1 mm^2 area scan per well. The homogeneous nature of the FMAT assay is achieved by image processing using a software algorithm that ignores the background fluorescence derived from unbound fluorescent molecules in the wells. The unbound fluorophores provide a level of baseline fluorescence within the entire scanned area. Through image processing, the specific signal is detected as localized and concentrated fluorescence within the wells, while the remaining background fluorescence is ignored in the final processing of the image data. This method is capable of analyzing the binding processes on adherent cells as well as on suspension cells or bead bound target molecules.

Miraglia and co-workers (Miraglia et al. 1999) demonstrated the usage of the FMAT assay format for the measurement of TNF-α- and IL-1β-induced enhancement of ICAM-1 expression on adherent HUVE and HepG2 (liver cell line) cells. Adhered cells at subconfluent density on 96-well plates were exposed to the medium with or without the addition of cytokines. After an overnight incubation, the medium was removed and the cells were stained with an anti-ICAM-1 antibody labeled with Cy5.5 fluorochrome and analyzed. Treatment with both cytokines resulted in significant up-regulation of ICAM-1 in both cell types used. Up-regulation of ICAM-1 was clearly more intense in HUVECs than in HepG2 cells and the experiment with HUVECs plated on a 384-well format gave similar results. The expression level of ICAM-1 was assayed as the mean fluorescence of HepG2 cells or as in the number of HUVE cells stained. However, the authors did not mention how the imaging software identified the individual cells. Overall, the FMAT assay format was considered to have a homogeneous character and to be capable of measurement of adhesion protein expression levels on the surface of adherent cells in an immunocytochemical assay.

However, the method requires a costly and highly specified instrument with specifically designed image analysis software. Multi-plexing of the assay is restricted to the use of two labels in the same assay, but a positive property is that with the method it is possible to measure two separate binding events simultaneously; one on the micro well surface and the other in the liquid phase.

2.2.2 Other assays

2.2.2.1 Luminescence assay based on ICAM-1 gene transcription

Transcriptional reporter-based assays have been used for screening purposes since the early stages in the HTS scheme, especially those bioluminescence based assays where light is produced by the interaction luciferase and its substrate luciferin (Roelant et al. 1996). A luciferase reporter assay for adhesion molecule expression in the cells of an immortal endothelial cell line is achievable by transfecting the cells with a region of the target protein promoter ligated to a luciferase reporter vector. If an outer stimulus activates the target protein promoter, the luciferase product is continuously synthesized and accumulates in the cytoplasm. After the assay is complete, the cells are lysed and luciferin is added. The resulting light is measured with a conventional plate reader or a scintillation counter. The luminescence reaction can be either glow-type (low throughput) or flash-type (high throughput): the flash-type measurements require an injector connected to the detection instrument (rarely available for scintillation counters).

Davis and co-workers (Davis et al. 2002) developed an automated luciferase reporter glow type assay for cytokine-induced ICAM-1 gene transcription using an immortal cell line ECV304. For the assay, ECV304 cells stably transfected with the human ICAM-1 promoter-luciferase/pGL3 plasmid, were plated in 96-well microplates in density of 10 000 cells in 100 µl of maintenance medium and allowed to grow near confluence. After adding test compounds and the cytokine IL-1β, the plates were further incubated for 6-7 h without lids. Next, the combined lysis buffer and luciferin reagent (in a commercial kit) were added and the luminescence signal was read with a scintillation counter. This glow type assay was further developed into a flash type assay in 96-well format in order to reduce costing per assay because the miniaturization trial of the assay format was not successful. The cytokine induced expression enhancement of ICAM-1 using ECV304 cells assayed with the luciferase reporter gene system on a scintillation counter, was shown to be comparable with a manual assay of enhanced ICAM-1 protein expression on the surface of fixed HUVE cells, under similar conditions assayed using a cell-based ELISA system and measuring the absorbance at 490 nm with a microplate reader. This direct relationship with the actual ICAM-1 cell surface expression, distinguishes this assay format from those methods based on mRNA expression analysis. Dose-related increases in luciferase activity could be induced not only in response to IL-1β but to TNF-α, IFN-γ and LPS as well. IL-1β though, was shown to stimulate luciferase activity significantly

more (32-fold over basal) than the other stimulants tested (less than 6-fold over basal). The authors concluded that the flash-type luciferase reporter assay was able to speed up the screening process 1.6-fold, with only 1/3 of the cost per well when compared with the glow type assay. The main drawback of the assay system was that its performance was highly dependent upon the cell density within the wells. In response to cytokine stimulation, the S/B ratio greatly increased upon cell division until the cells achieved confluence, but after which point the signal diminished quickly. This was also presented as a reason for the failures in attempts to miniaturize the assay in a 384-well format. The assay system also requires a stably transfected immortal endothelial cell line and a specialized detection instrument equipped with an injector. This is an obligatory device for flash-type bioluminescence assays in general and also of great value for automated bioluminescence assays of the glow-type. Overall however, the assay format is not amenable for multiplexing and the samples are lost following the assay.

2.2.2.2 Flow cytometry-based assays

Flow cytometry (FCM) is a technique for counting and measuring large numbers of cells or microparticles in a short period (1000 – 50 000 per second) as they pass at high speed through a laser beam. The cells are incorporated within the flow as part of a fluidics system that precisely delivers the cells at the intersection of the laser beam and light gathering lens by means of hydrodynamic focusing (a single stream of cells is injected and confined within an outer stream at greater pressure). A beam of a single wavelength light (usually a laser) is directed onto this hydrodynamically focused cell stream. The beam is used to excite the fluorophore labeled cells and generates a cell-associated scattering of light. One detector is positioned in-line with the light beam and used to measure the forward scatter from the cell, while several detectors perpendicular to the light beam measure the side scatter, i.e. light reflected 90° to the laser beam. One or more further detectors collect the fluorescence emission light from the fluorescently stained cells. Forward scatter (as determined by the refractive index is proportional to the cross section of the cell. Side scatter measurement can be used to indicate the extent and state of cytoplasmic density, together with the internal shape and granularity. Although information about the cellular shape, size and molecular consistence is important, the most significant information is usually obtained from the emission light signal derived from the fluorescent dyes. Flow cytometric fluorescence detection from cells can distinguish various cell characteristics. These include surface and intracellular antigens, protein expression markers, intracellular ions and other molecules, DNA content, the redox state and cell functions such as apoptosis and cell cycle. Fluorescent probes for flow cytometry include labeled antibodies, dyes fluorescing upon binding to DNA, and dyes which respond to changes to intracellular concentrations of calcium and other ions (Sklar et al. 2007). The quantitative signal is often calculated as the mean or median fluorescence intensity (MFI) per cell or sometimes as the number of cells, positive for a specific molecular marker and indicated by specified fluorescence. Flow cytometric assays are of a homogeneous

nature, achieving up to several hundreds in nanomolar concentrations of the non-bound label (Sklar et al. 2002). They are easily automated for high-throughput format down to 384-well technology (Sklar et al. 2007) and amenable for multiplexing (Edwards et al. 2007).

Flow cytometry has been exploited widely for the quantitative analysis of cell surface molecules on cultured endothelial cells, especially in the context of immune response (Haraldsen et al. 1996, Mutin et al. 1997) and in characterization of changes in endothelial cell molecule abundance caused by various substances (Kuldo et al. 2005, Chen et al. 2006, Marttila-Ichihara et al. 2010). After treatment with defined molecules, the endothelial cell monolayers can be fixed either before or after detachment. Cells can be detached with or without the aid of trypsin/EDTA treatment. This treatment for the detaching of cells has in a number of studies been reported leading to a partial loss of cell surface antigens (Corver et al. 1995, Grabner et al. 2000). On the other hand however, it has also been a part of the standard procedures preceding flow cytometric analysis of endothelial cell adhesion molecules (Haraldsen et al. 1996, Daxecker et al. 2002). Fixation of cells for flow cytometry is not obligatory in general but is recommended, since the abundance of certain surface markers has shown to be modulated during the processing of unfixed cells (Hamblin et al. 1992, Collins et al. 1993). Following detachment (and fixation), endothelial cells are usually incubated with fluorophore-labeled monoclonal antibodies. These are targeted against the selected cell surface antigens and analyzed by flow cytometry without any further washing steps. Despite the fact that flow cytometric techniques have frequently been considered amenable for multiplexing and automation, there are no literature references on quantitative multiplexed endothelial cell surface antigen expression analysis performed with flow cytometry and neither has flow cytometry been demonstrated for automated cell-based HTS assays in search of new endothelial cell adhesion molecule expression modulators.

3 AIMS OF THE STUDY

1° To apply fluorescence polarization for estrogen receptor-ligand binding assay using two-photon excitation, in order to reveal its performance in receptor binding assays, and to compare this with the conventional one-photon excitation technique.

2° To study microplate and microscope-based assay formats for the quantitative detection of cell surface protein expression on adherent cells by immunocytochemistry, utilizing long-lifetime luminophore labeled antibodies.

3° To develop a multiplexed immunocytochemistry-based microplate assay format for the quantitative detection and time-resolved luminescence microscopy imaging of cell surface expression on the adherent cells of several proteins from the same sample, utilizing long-lifetime luminophore labeled antibodies.

4° To develop a new homogeneous single-label assay format, that utilizes long-lifetime lanthanide chelate labels for the analysis of estrogen receptor ligand binding using quenching resonance energy transfer (QRET) technology, and to compare this new assay format with the standard fluorescence polarization method.

4 MATERIALS AND METHODS

4.1 Reagents

Fulvestrant, tamoxifen, estrone, BSA, L-glutamine, RPMI-1640 medium, TNF-α, Tris, gelatine and collagenase were from Sigma-Aldrich (St.Louis, MO). 17β-estradiol was from Steraloids (Newport, RI). PBS and FBS were from Gibco (Paisley, UK). Human recombinant TNF-α was from Sigma-Aldrich (St.Louis, MO). Primary monoclonal antibodies against E-selectin and ICAM-1 were from R&D Systems (Abington, UK) and against β1-integrin was from SantaCruz Biotechnology Inc. (Santa Cruz, CA). Anti-mouse secondary polyclonal antibody was from DAKO (Carpinteria, CA). Pt-coproporphyrin isothiocyanate was from Arctic Diagnostics Oy, (Turku, Finland). Fluorescein isothiocyanate was from Calbiochem (La Jolla, CA) and syto13 stain was from Molecular Probes (Eugene, OR). The quencher molecule was from QRET Technologies (Turku, Finland). Heptadentate Eu(III) chelate was from the Laboratory of Biophysics, University of Turku, (Turku, Finland).

4.1.1 Assay buffer (immunocytochemistry)

The assay buffer (pH 8.2) used in the immunocytochemical assays (II, III) was composed of Tris 50 mM, NaCl 150 mM, Tween 20 0.01 %, BSA 0.5 %, NaN_3 10 mM.

4.2 Cell culture

4.2.1 EAhy926 cells (II)

Immortal endothelial EAhy926 cells were obtained from Professor Sirpa Jalkanen (University of Turku). The cells were grown on tissue culture dishes in a humidified incubator at 37 °C, 5% CO_2, in RPMI-1640 medium supplemented with 10% FBS and 2 mM L-glutamine and passaged with trypsin-EDTA.

4.2.1.1 Assay protocol for EAhy926 cells

Approximately one day prior to carrying out an assay, 10 000 cells were seeded onto black transparent-bottom tissue culture microplates (Greiner, Frickenhausen, Germany) in 100 µl volume of culture medium. When grown nearly to full confluence, the cell culture medium was removed by aspiration and replaced with fresh media containing variable concentrations of TNF-α. After a 24 h incubation in the humidified incubator, the medium was removed by aspiration. The cells were fixed

with 100 µl of paraformaldehyde solution (4% in PBS) for 30 min and thereafter, the fixative was removed by aspiration and the cells were briefly washed with the assay buffer. The cells were then incubated for 1 h with primary anti-ICAM-1 antibody (1 µg/ml) in assay buffer (50 µl) and washed once with assay buffer. They were then incubated for 1 h with the fluorophore-labeled (FITC, Eu-chelate or PtCP) secondary antibody (0.04 µM with respect to labels) in the assay buffer and finally, the cells were washed with the assay buffer and dried for 30 min in airflow under a laminar hood.

The plates were measured using a microplate reader (Tecan Ultra™ - Tecan AG, Austria). Optical parameters set were: FITC - excitation filter (ex) 485/(slit 20) nm, emission filter (em) 535/25 nm; PtCP - ex = 390/25 nm (uv) and 545/20 nm (vis), em = 640/20 nm, lag time = 60 µs, integration time = 100 µs; Eu label - ex = 340/35 nm, em = 612/10 nm, lag time = 400 µs, integration time = 400 µs.

4.2.2 HUVE cells (III)

4.2.2.1 Isolation of HUVE cells

Human umbilical vein endothelial (HUVE) cells were isolated according to a slightly modified method of Jaffe and co-workers (Jaffe et al. 1973). The umbilical cords were obtained from voluntary donors at Turku University Central Hospital's Maternity Ward Clinic. The cords were collected immediately after birth, put in a sterile flask by clinic staff, and stored at +4°C. The isolation process from the cords took place <15 hours post section. Before any further steps were taken, the veins were rinsed with PBS. Next, they were incubated with collagenase I (1 mg/ml) solution (PBS + 2% FBS) in 37 °C bath of sterilized water for 8 min. After the incubation period, the endothelial cells were collected in the endothelial cell culture medium (Clonetics EGM-2 Bullet Kit - Lonza, Basel, Switzerland) and centrifuged gently. Thereafter, the supernatant was discarded in order to get rid of erythrocytes and debris. Finally, the isolated HUVE cells were re-suspended in the endothelial culture medium and plated in tissue culture treated dishes (Becton Dickinson Falcon, Franklin lakes, NJ) pre-coated with gelatine (1%).

4.2.2.2 Propagation of HUVE cells

HUVE cells were grown in full EGM-2 medium on gelatine coated tissue culture dishes in a humidified incubator at 37 °C, 5% CO_2 and passaged with trypsin-EDTA. The cells were used for experiments in passages 2-4. Approximately two days before carrying out an assay, 10 000 cells were seeded onto gelatine-coated transparent 96-well tissue culture microplates (Becton Dickinson Falcon, Franklin lakes, NJ) in a volume of 100 µl and allowed to grow near full confluence.

4.2.2.3 Assay protocol for HUVE cells

On the day of the assay, cell culture medium was removed and replaced with fresh medium containing variable concentrations of TNF-α. After 4h of incubation in the humidified incubator, the medium was removed and the cells were fixed with 100 µl of paraformaldehyde solution (4% in PBS) for 20 min, then briefly washed with the assay buffer. The cells were incubated for a further 1h with a mixture of the fluorescent DNA stain syto13 and the labeled antibodies against β1-integrin, E-selectin and ICAM-1 in a volume of 50 µl of assay buffer. The antibody concentrations were 0.8 µg/ml each and the concentration of syto13 was 0.1 µM. After incubation, the cells were washed once with assay buffer and dried overnight at room temperature in the dark.

The plates were measured with a multilabel microplate reader (EnVision™ PerkinElmer - Turku, Finland). Optical parameters set were: PtCP (ICAM-1) - ex 531/(slit 20) nm, em 640/20 nm, lag time = 60 µs, integration time = 100 µs; Eu label (E-selectin) - ex 340/35 nm, em 612/10 nm, lag time = 400 µs, integration time = 400 µs; Tb label (β1-integrin) - ex 340/35 nm, em 545/25 nm, lag time = 400 µs, integration time = 400 µs.

4.3 Antibody labeling and analysis

The labeling of the antibodies with PtCP and FITC was performed as earlier described (Koskelin et al. 2002). Briefly, a 20-fold molar excess of PtCP or a 60-fold molar excess of FITC was dissolved in DMF and combined with antibody in freshly prepared carbonate buffered saline (pH 9.8) by mixing thoroughly. The labeling reaction then took place for 4 h at room temperature in the dark. The reaction was then terminated and the labeled conjugate separated from the un-reacted labeling reagent by means of gel filtration and using elution buffer (PBS, pH 7.4). Finally, the labelling degree (label / protein molar ratio) was determined photometrically.

The labelling of the antibodies with Tb-chelate (W 14016) and Eu-chelate (W 8044) were performed by PerkinElmer Life Sciences - Wallac (Turku, Finland).

The excitation and emission spectra of the labeled antibodies were recorded with a Cary Eclipse fluorescence spectrophotometer (Varian Inc., Palo Alto, CA).

4.4 Calculations

In the immunocytochemical studies (II, III), the signal-to-background (S/B) values were calculated as the ratio of signals with and without TNF-α treatment. In the ER ligand binding studies (I, IV) the S/B values were calculated as being the ratio of signals derived from the lowest and highest ligand concentration.

Relative binding affinity (RBA) is defined as the ratio of the IC_{50} value of 17β-estradiol to the IC_{50} value of the ligand; the RBA value for 17β-estradiol was set to 100% (IV).

The assay qualities were determined in terms of Z'- factor as defined as:

$$Z' = 1- [3(\sigma_s + \sigma_c)/|\mu_s - \mu_c|]$$

where σ_s and σ_c are the standard deviation of the sample and the control, respectively, and μ_s and μ_c are the mean of sample and control, respectively. A Z'- factor of > 0.5 indicates an excellent assay. An assay with $0 < Z' < 0.5$ is considered marginal but possibly suitable. Assays with a Z'- factor of < 0 are not suitable for screening purposes (Zhang et al. 1999).

4.5 Receptor binding assays

4.5.1 Two-photon excitation in fluorescence polarization-based estrogen receptor-ligand binding (I)

A comparison between two-photon and one-photon excitation in a fluorescence polarization (FP) assay was carried out using the "Estrogen Receptor Alpha Competitor Assay Red kit" of PanVera (Madison, WI). The kit included the ER Red Assay Buffer, the full-length ERα and the probe EL Red (a rhodamine-conjugated E_2 derivative). 17β-estradiol and the assay components were diluted in the screening buffer according to the manufacturer's instructions, the final concentrations of ERα and EL Red being 15 nM and 1 nM, respectively. After an incubation period of two hours at room temperature in the dark, the samples were transferred to one-photon and two-photon excitation measurement plates in a volume of 40 µl per well. The microplate used for one-photon excitation measurements was a 384-well black opaque bottom Cliniplate (Thermo electron Corp., Thermo Labsystems - Helsinki, Finland). For two-photon excitation, a transparent glass-bottom 96-well Sensoplate, (Greiner Bio-One - Kremsmuenster, Germany) was used in the macrovolume measurements with sample volumes of 40 µl and a thin COC (cyclic olefin copolymer) Topas bottom microslide (ThinXXS, Mainz, Germany) was used in the volumetric sensitivity studies with sample volumes of 1 µl. The two-photon fluorescence polarization measurements were performed with a setup built in an optical module of an ArcDia TPX (ArcDia Ltd., Turku, Finland) microfluorometer (Soini et al. 2002). In brief, a horizontally polarized laser beam (wavelength 1057 nm) was reflected by a dichroic mirror through a beam scanner and focused with a microscope objective lens through the bottom of the microwell to the sample. The fluorescence light from the sample was collected with the same microscope objective lens, directed within a range from 530 nm to 700 nm through the dichroic mirror into a broadband cube polarizing beam splitter. By the beam splitter, the fluorescence light was separated into two perpendicular polarization components and then directed to the photomultiplier tubes through 605 nm interference filters.

4.5.2 Single-label time-resolved luminescence assay for estrogen receptor-ligand binding (IV)

FP measurement in parallel with the QRET-method for ER ligand binding was carried out using the "Estrogen Receptor Alpha Competitor Assay Green kit" of Invitrogen (Madison, WI) according to the instructions of the manufacturer. The kit included the ES2 Screening Buffer, full-length ERα and the probe FITC-E_2 (ES2). The competitors were diluted in the screening buffer in a total assay volume of 40 µl, the final concentrations of ERα and FITC-E_2 being 15 nM and 1 nM, respectively. After a one-hour incubation at room temperature in the dark, the FP signals were measured with a Tecan Ultra multilabel reader (Tecan AG, Grödig, Austria) using excitation and emission filters of 485 nm and 535 nm, respectively.

4.6 Theoretical aspects of the assay methods applied

4.6.1 Basic principle of time-resolved luminescence

Long decay-time lanthanide chelates such as Eu and Tb chelates emit light at decay-times in the order of 10 – 1000s of microseconds after excitation, due to electric dipole forced transitions leading to the delayed fluorescence that is typical for lanthanide chelates (Hemmilä and Laitala 2005). The use of these chelates with exceptionally long decay times and long Stokes' shifts has been reported even in multi-parametric assays (Xu and Hemmilä 1992, Heinonen et al. 1997). Phosphorescent labels like platinum porphyrins, also hold long decay times but through a different mechanism – the slow emission from their excited triplet energy state (Gouterman et al. 1975). They also commonly have long Stokes' shifts. The long decay times compared to autofluorescence and background luminescence with decay times in the order of nanoseconds, enable efficient signal discrimination between the specific long-lifetime and the unspecific short-lifetime luminescence. This principle is illustrated in Fig. 4.

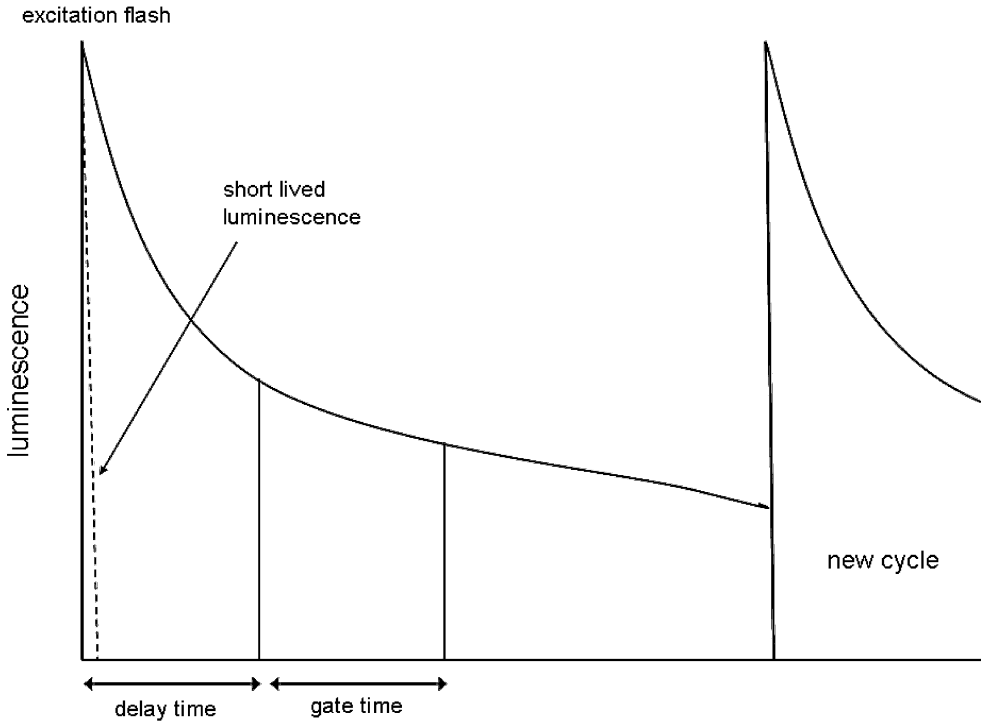

Figure 4. The principle of time-resolved luminescence detection using excitation with a pulsed flash lamp commonly used in microplate readers.

4.6.2 Principle of two-photon excitation fluorescence

In two-photon excitation the fluorophore absorbs simultaneously two photons of half the energy (and double the wavelength) of a photon needed to excite the fluorophore in conventional single-photon or one-photon excitation. This leads to an up-conversion of fluorescence emission because the wavelength of emission is smaller, when compared to the excitation light (Fig. 5 a). Two-photon excitation can be triggered using a pulsed laser illumination resulting in the generation of a confocal-like minimal illumination volume at the focal plane of the excitation light (Oheim et al. 2006). Strictly speaking, even in confocal one-photon excitation; fluorescence emission derived from fluorophores excited outside the focal plane, occurs in a substantial amount when compared to two-photon excitation where only the fluorophores at the defined focal illumination volume in the order of 1fl are able to become excited and elicit fluorescence emission (Fig. 5 b).

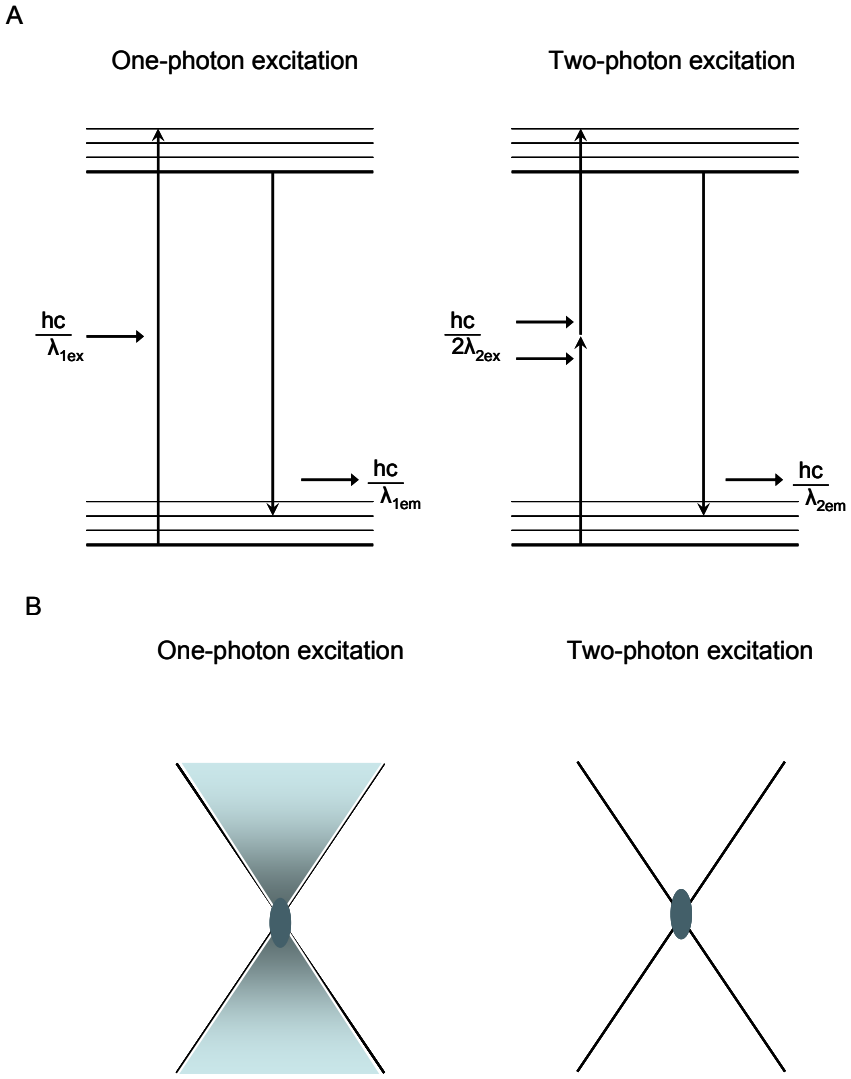

Figure 5. Essentials of two-photon excitation principle. A) Simplified Jablonski diagram of the energy conversions in one-photon and two-photon excitation. λ_{1ex} and λ_{1em} are the excitation and emission wavelengths in one-photon excitation, respectively. λ_{2ex} and λ_{2em} are the corresponding wavelengths in two-photon excitation. B) Illustration of the differences of the fluorescence emission with one–photon (confocal) and two-photon excitation.

4.6.3 Principle of the QRET technique

Quenching resonance energy transfer (QRET) is a homogeneous assay format based on the time-resolved luminescence derived from a single label. The QRET method relies on a single-labeled binding partner for a receptor, in combination with a soluble quencher molecule in the assay solution. A prerequisite for efficient quenching is that the absorption spectrum of the quencher molecule overlaps sufficiently with the emission spectrum of the luminescent label, for example the long-lifetime lanthanide (Eu, Tb) chelates. The soluble quencher molecule reduces the luminescence of the unbound labeled ligand, while the luminescence derived from the bound fraction is unaffected. The QRET method has been applied in ligand binding assay for a membrane-bound receptor (Härmä et al. 2009, Rozwandowicz-Jansen et al. 2010), in functional GTP binding assay (Rozwandowicz-Jansen et al. 2010) and cAMP assay (Martikkala et al. 2011). In addition to these, the QRET method has been compared with a homogeneous single-label prompt fluorescence polarization technique in an assay for binding of estradiol to an anti-estradiol antibody (Härmä et al. 2010). An illustration of the basic QRET principle is shown in Fig. 6.

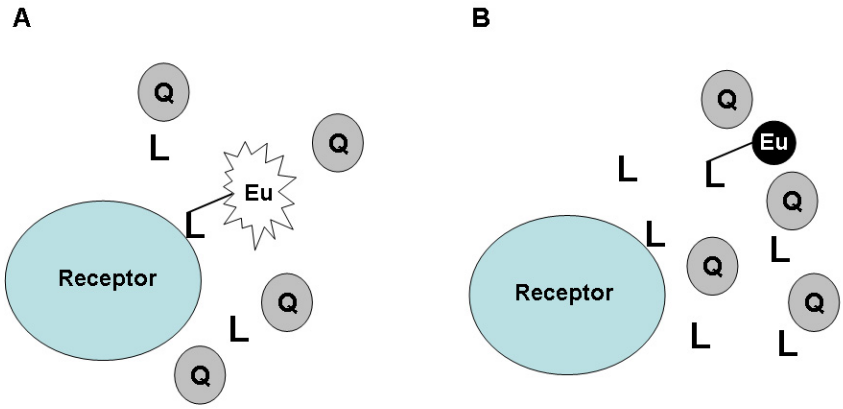

Figure 6. Principle of the QRET assay method. A) Eu-labeled ligand (L) emits light when shielded from quenchers (Q) via binding to the receptor in the presence of small amounts of the competitive unbound ligand. B) In the presence of increasing amount of the unlabeled ligand, Eu-labeled ligand is displaced and the emission quenched.

5 RESULTS AND DISCUSSION

5.1 Competitive ER ligand binding assays

5.1.1 Two-photon vs. single-photon excitation in FP measurements (I)

5.1.1.1 Background

The fraction of a fluorophore-labeled ligand bound to a given receptor in the presence of competitor compounds can often be determined in a homogeneous format by fluorescence polarization (FP). This is a ratiometric and single-step robust method for which instrumentation is commonly available in most HTS laboratories. FP is highly suitable for HTS purposes as only a single binding event between two molecules is needed. The current trend in the HTS field is towards the development of assay formats amenable for prominent miniaturization, from 96-well plates to 1536-well plates and beyond. Reducing the assay volume to a sub-microliter scale, compromises FP measurements as a result of an elevated background signal due to light scattering, autofluorescence derived from the plate plastics and a nonspecific binding of fluorescent molecules (Pope et al. 1999)

One of the most sensitive means of fluorescence detection within microvolumes is two-photon excitation fluorescence (TPEF), where two photons with equal energy are simultaneously absorbed by a fluorophore leading to up-conversion of fluorescence emission (Fig. 5 a). TPEF has been applied in fluorescence microscopy (Diaspro et al. 2005), fluorescence correlation spectroscopy (Schwille and Heinze 2001) and also in microplate format diagnostic instruments (Hänninen et al. 2000, Koskinen et al. 2005, Koskinen et al. 2006) - enabled by the introduction of low cost lasers that can be produced in the quantities required for widespread applications. The diffraction-limited focal excitation volume of the femtolitres order enables effective downscaling of the assay volume without compromising detection sensitivity and also greatly reduces the generation of out-of-focus nonspecific fluorescence emission.

5.1.1.2 Experimental and discussion

The determined binding isotherms for binding of 17β-estradiol to estrogen receptor α (ERα) are shown as a function of 17β-estradiol concentration in polarization units (I, Fig. 3) and also on a normalized scale (Fig. 7). The results show high convergence with each other in terms of the determined IC_{50} values (16 ± 2 nM for both single-photon and two-photon assays). A slight twist between the curves can be seen more clearly in the normalized isotherms (Fig. 7); this minor divergence is indicated also in the calculated Hill slope values of 1.24 and 1.68 for single-photon and two-photon

measurements, respectively. In principle, as the two parallel methods differ only in the assay instrumentation, the Hill slope values should be approximately within the same range. In this case, the differences in the stationary beds (microplates) for the assay could be suggested as a reason for this slight difference, as the single-photon measurements were carried out on plastic bottom microplates and part of the signal detected is derived from the label-receptor complexes unspecifically bound on the microplate surface. This unspecific binding of the label-receptor complex could influence its binding characteristics. In two-photon measurements, the detected signal is derived distinctly from the label-receptor complexes in the solution phase and the stationary surface appears to have no effect on the binding characteristics of the complex.

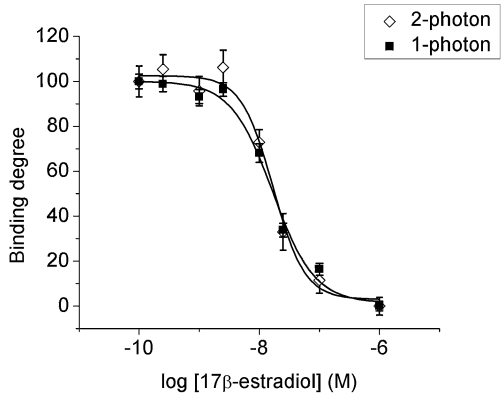

Figure 7. FP ER assay with 1-photon and 2-photon excitation shown after normalization of the signals. The binding degree achieved at the smallest 17β-estradiol concentration was set as 100.

An important aspect in the lead compound screening process is the efficacy of the assay method used in distinguishing effective molecules from inactive ones. The most often applied evaluation function for efficacy evaluation of an assay method is the Z'-factor (Zhang et al. 1999)(see also IV: Materials and Methods). For the single-photon assay, Z'-factors of 0.71 and 0.82 were achieved for 1 s and 5 s measurement times, respectively. For the two-photon assay, Z'-factors of 0.53, 0.77 and 0.85 were achieved for 1 s, 5 s and 10 s measurement times, respectively. These results are conveniently elucidated in the concentration-response curves in mP-scale (I, Fig. 4), where the performance of the two-photon excitation method in terms of S/B ratio becomes evident. Despite the fact that the two-photon assay yields signal / s levels ~ 3 orders of magnitude smaller than the single-photon assay, the two-photon assay shows a comparable Z'-factor already for a 5 s measurement time. When a 10 s time of measurement is applied for two-photon excitation, the obtained Z'-factor surpasses that obtained with a 5 s measurement time for single-photon excitation. The single-photon assay suffered from heavy bleaching during 10 s measurements and the measurements are skewed for that reason (data not shown). The measurement times are relatively long, because the probability of two photons being absorbed simultaneously (in an attosecond time window) by a fluorophore in a low

concentration solution (on average, < 1 fluorophore molecule in the excitation volume), is rather small. The higher signal deviation compared to the single-photon assay, especially in the case with unbound labeled estradiol, is compensated for by the higher S/B ratio of the two-photon assay. Presumably, the increase in S/B is due to the higher 670 mP value of the theoretical limiting polarization in two-photon excitation measurements, when compared to the theoretical limiting polarization value of 500 mP in single-photon excitation measurements. Likewise, the increased polarization contrast in two-photon assay (I, Fig. 3) is probably due to this phenomenon. The reason for the increase in the limiting polarization value is due to the fact that the photoselection process is carried out twice when two photons are absorbed, thus leading to higher selectivity in the absorption process (Jameson and Croney 2003, Lakowicz et al. 1995). This is assumed to provide improved performance for binding assays in terms of dynamic range and sensitivity. As the possibility for miniaturization offers a special advantage by the TPE technique, this was assessed through a volumetric sensitivity study using a thin-bottom COC (cyclic olefin copolymer) Topas microslide. Although COC material is designed for applications requiring glass-like clarity, it effected the polarization slightly. A volume of 1 µl manually applied onto the microslide was considered feasible with a sufficiently high reproducibility. Using a measurement time of 5 s, the Z'-factor was slightly reduced (0.73) when compared with the glass-bottom measurement of 40 µl volume (0.77). This may be due to an increased variation of polarization values, as the fluorescence signal intensities remained unchanged. This variation in polarization values was considered to be due to the microslide material and the impact of the reduced measurement volume to the assay sensitivity was negligible.

5.1.1.3 General notes and prospective

As a whole, two-photon excitation of fluorescence provides many additional advantages. These include up-converted emission (Liu et al. 2010), a large separation between the excitation and emission wavelengths, reduced photobleaching and out-of-focus excitation and a reduced scattering of excitation light in addition to minimum excitation volume (Jameson and Croney 2003, Oheim et al. 2006). Recently, two-photon excitation has emerged in FRET-based binding assays (Wahlroos et al. 2006, Liu et al. 2008, Liu et al. 2010, Liu et al. 2011) and in the application of luminescent lanthanide chelates as bioprobes (Eliseeva et al. 2010, Shao et al. 2010). Therefore FP, in addition of drug discovery related bioanalytics, has been widely used in FP immunoassay (FPIA) measurements in clinical laboratories (Baker et al. 2000), with more new FPIA applications emerging (Smith and Eremin 2008). Since the pioneering work in the field of two-photon excitation FPIA (Baker et al. 2000), no major fundamental improvements have been made. However, in the future, along with the development of liquid-dispensing technologies, FP-based two-photon excitation holds a great potential as a means for cost savings, in both drug discovery and clinical diagnostics fields. This is mainly based on the two-photon excitation techniques' extraordinarily high potential for miniaturization, though in general, the applicability

to HTS approaches is compromised due to the relatively long measurement times per assay well.

5.1.2 Paralleling FP and TRF- based single-label assays (IV)

5.1.2.1 Background

Homogeneous fluorescence-based microplate assays are widely used for HTS purposes in search of new lead compound candidates for nuclear receptors. FP and TR-FRET based assays are two standard methods commercially available for these purposes. TR-FRET based assays have been introduced to offer efficient alternatives to FP assays (Vogel et al. 2008). TR-FRET assays are not prone to common forms of assay interferences, as caused by assay medium components, such as those compounds causing inner-filter effects by absorbing either excitation or emission light, autofluorescent compounds and precipitates that scatter light (Riddle et al. 2006). However, TR-FRET assays may still be susceptible for assay artefacts, since highly fluorescent compounds in high concentrations as well as compounds effectively quenching the TR-FRET emission could further disturb the detection of the specific signal (Imbert et al. 2007). Therefore, the QRET based homogeneous assay method (Fig. 6, see Materials and Methods) for a single-label TR detection platform could offer a new option in the field of nuclear receptor lead screening.

5.1.2.2 Determination of the K_d of the Eu-E_2 probe

To assess the binding affinity of the newly synthesized long-lifetime fluorescent ER ligand, or 17α-ethinylcarboxyestradiol-1, 6-diamino hexane Eu(III) chelate (Eu-E_2) to ERα, a saturation binding isotherm was constructed by keeping the concentration of Eu-E_2 constant and varying the concentration of ERα (IV, Fig. 3). The specific signal appeared to increase with the increasing ER concentration and reached an S/B ratio of 6.5 at the receptor concentration of 200 nM. An approximated dissociation constant value (K_d) of 30 nM was yielded as a result, which is somewhat higher than what the K_d for the fluorescein-E_2 probe is (Parker et al. 2000). Therefore, Eu-E_2 was assumed a slightly weaker binder than fluorescein-E_2 but nevertheless considered strong enough to allow investigations of its performance as a fluorescent probe in ER-ligand binding assays. In order to more precisely determine the K_d of Eu-E_2 to ERα, binding assays that apply a radionuclide labeled Eu-E_2, or a label-free technique such as surface plasmon resonance, could be used.

5.1.2.3 Analysis of the binding curves

The actual applicability of Eu-E_2 as a probe in ER binding assays was tested by paralleling the developed QRET-ER assay with a commercially available and well

established homogeneous ER binding assay based on fluorescence polarization (FP) and fluorescein-E_2 as the probe (Parker et al. 2000). Four known ER binders: estradiol, estrone, fulvestrant and tamoxifen were chosen to be used in the functional evaluation of the assay. The concentration-response curves for QRET and FP formats (IV, Fig. 2 a and b, respectively) expressed in relative units, clearly show the higher S/B ratios achieved by the QRET method (4-7 fold, when compared with FP). The concentration-response curves using normalized signals (Fig. 8), show that the curve positioning with increasing ligand concentrations appears nearly in the same order in both assay methods, as indicated also by the yielded relative binding affinities (RBAs) (IV, Table 1). In the FP assay, the RBAs were 93, 2.8 and 110 for estrone, tamoxifen and fulvestrant, respectively. In the QRET assay, the RBAs were 39.4, 3.6 and 31.5 for estrone, tamoxifen and fulvestrant, respectively. The RBA for estradiol was set as 100 for both assays. Altogether, the RBAs within the assay formats (FP or QRET) stand within fairly acceptable limits as both estrone and fulvestrant have been reported to be similarly strong ERα binders as estradiol, although moderate variation in the calculated binding affinities may occur depending on the assay format applied. Comparable Z'- factors of 0.56 and 0.54 were calculated for the FP and QRET formats, respectively, indicating feasibility for HTS applications. The QRET assay had higher S/B but this was compromised by a higher signal variation, in contrast with the FP assay showing low S/B compensated by low signal variation.

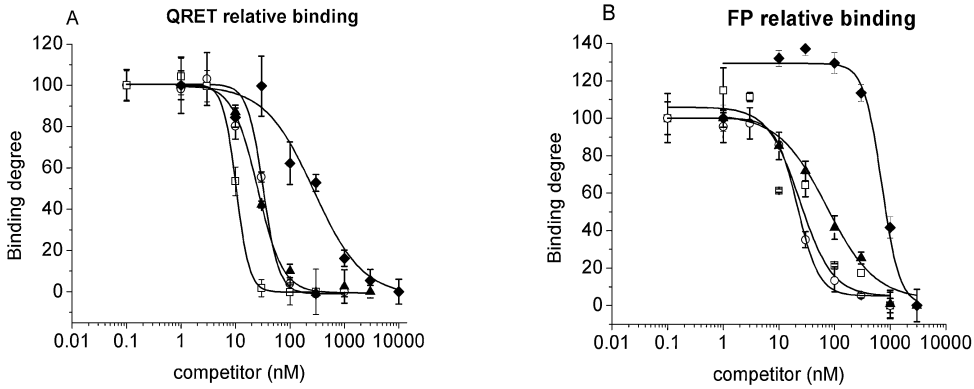

Figure 8. QRET (A) and FP (B) ER assays after normalization of the signals: estradiol (open square), estrone (solid triangle), fulvestrant (open circle), tamoxifen (solid diamond). RLU = relative luminescence units. Normalized luminescence signals were fitted to a non-linear sigmoidal model. The binding degree achieved at the smallest competitor concentration was set as 100. Result shown is a representative of two separate experiments.

The results of this study suggest a significant potential for the QRET-based assay format to be applied for HTS lead compound identification purposes in the process of nuclear receptor modulator discovery. Because the binding assay applicability of only a single aliphatic linker chain length ($[-CH_2-]_n$, n = 6) in the estradiol and Eu-chelate conjugate was surveyed in this study, it would be intriguing to explore different chain lengths, in order to elicit the influence of the linker length in the binding characteristics of the conjugate. Asai and co-workers (Asai et al. 2008) reported that

their equivalent fluorescein-estradiol conjugate with n = 6 was an average strength binder to ER, compared with conjugates with n-values of 2, 4, 8, 10 and 12 the conjugate with n = 2 being the clearly weakest binder, however, the binding affinity appeared not to be directly proportional to the length of the linker chain. Therefore the length of the aliphatic linker chain was chosen to be n = 6 as the pilot probe molecule for the development of the QRET-ER assay. Lanthanide-chelate conjugated 17α-ethynyl estradiol derivatives have been proposed as contrast agents for magnetic resonance imaging in breast cancer diagnostics and reported to retain fairly high affinity (1 μM range) to ERs (Gunanathan et al. 2007). Recently, a detailed 3D structure of the estrogen receptor α ligand-binding domain bound with a novel estradiol-derived metal complex (estradiol-pyridine tetra acetate Eu(III)), was described and suggested to be a prototype for novel Ln(III)-conjugated estradiol conjugates, for both the diagnosis and treatment of ER-associated diseases (Li et al. 2011). This also presents new perspectives for the study with Eu-E_2, as equivalent Tb(III) conjugates are achievable by similar means of synthesis and a Tb-conjugated estradiol (7-position) has already been successfully applied in a QRET immunoassay for estradiol (Härmä et al. 2010).

5.1.2.4 General notes and prospective

In an HTS approach, economics steer the selection of screening assay components and those other aspects that influence the overall labour intensity and informational content of the assay and ultimately, the total cost per assay well. In these terms, an assay method composed of only two binding components besides the test compounds is highly satisfactory. Fluorescence polarization as a ratiometric assay method with simple reaction chemistry and an amenability for automation is highly feasible for screening purposes and has already for long been a standard method when simply assaying the ligand binding to the receptor. Nonetheless, it is prone to suffer from unspecific fluorescence, derived from the assay reagents and equipment as described above. The TR-FRET method is a suggested substitute for FP-based techniques and utilizes an additional costly component in the form of an antibody targeted against the receptor which also makes the assay format more complex. A certain advantage of the TR-FRET method is that it is a ratiometric method, measuring the intensity ratio of the long-lifetime lanthanide label and a prompt fluorescent label. This advantageous feature however, makes the method vulnerable to interference by compounds that strongly quench or fluoresce at the donor or acceptor emission wavelength (Imbert et al. 2007). The QRET-ER assay format, though not a ratiometric method, was proven as being comparable with the FP based ER assay method and is not prone to interference from prompt fluorescence originating from assay reagents or equipment. Furthermore, QRET-ER also challenges the TR-FRET format with a simpler and lower-cost assay set-up; any additional quenchers in the QRET-ER assay mixture are unlikely to have a significant impact on Eu fluorescence and the method also totally lacks prompt fluorescent components and thus removes any potential vulnerability to such interference.

Beyond being applied merely in a single-label competitive ligand assay, the new ER probe, Eu-E_2 can potentially be utilized in more complex functional assays for cofactor recruitment or DNA-binding (ER-ERE-interaction). In a cofactor-binding assay of the TR-FRET type, Eu-E_2 could operate as a donor for a prompt fluorescence labeled peptide, that is targeted to bind to the cofactor-binding site. This approach could also be broadened by assaying the binding of the prompt-fluorescent labeled peptide by FP in a dual-readout (F^2) type assay. A dual-readout approach has been applied in drug screening purposes searching for Mcl-1 inhibitors in order to catch "hits" that either assay type alone would have been missed (Du et al. 2011). One could make use of the fact that the QRET-ER assay uses a full-length receptor and apply it in screening for selective inhibitors of ER binding to its target DNA. This could be achieved by combining it with an FAMA-type assay (Wang et al. 2004) in a dual assay (TR-FRET or F^2) format. Recently, FP based assays for inhibitors in ER binding to target DNA (ERE), successfully identified a potent lead compound (Mao et al. 2008) and by a subsequent cell-based screening campaign, another potent compound with a different mechanism of action (Kretzer et al. 2010). The small molecule inhibitors of nuclear receptors targeted outside the LBS are also of increasing interest (Moore et al. 2010, Shapiro et al. 2011). The binding of a ligand to the LBS of androgen receptors (AR) and ER may expose a person to hormonally regulated cancers, namely prostate cancer (in case of AR) and breast and ovarian cancers (in case of ER). Therefore, an ideal assay would probe the binding of a test compound to the LBS and to the target DNA or a cofactor simultaneously. This could prove advantageous as the binding of an antagonist to the LBS of AR or ER might increase the risk for development of antagonist resistance or hormone non-responsiveness during the medication based therapy of hormonally regulated cancers (Jordan 2001, Mao et al. 2009) – conditions characteristic of poor prognosis due to the reduced potential of pharmacotherapy.

5.2 Endothelial adhesion molecule expression assays

5.2.1 Single-parameter assay for TNF-α -induction of ICAM-1 expression on EAhy926 cells (II)

5.2.1.1 Background

In order to establish a method for assaying the expression levels of a given protein on the surface of adherent cells, we chose the stable cell line EAhy926 (a hybrid of HUVECs and A549 adenocarcinoma cells (Edgell et al. 1983)), as a model platform to quantitatively determine the cytokine-induced expression levels of the adhesion molecule ICAM-1 on the surface of these cells. The EAhy926 cells have been reported as retaining several endothelial characteristics. These include Weibel-Palade bodies harbouring von Willebrand factor (Edgell et al. 1990) and the cytokine-prone induction of ICAM-1 expression, in addition to a similar cell surface expression

pattern of a number of other cell surface antigens (Thornhill et al. 1993, Lidington et al. 1999).

5.2.1.2 Determination of the labeling degree of the secondary antibodies and their application

In general, the process of protein labelling is a cumbersome action to control effectively and the precise degree of labelling (label molecules per protein molecule) remains elusive, even when keeping the reaction conditions unchanged between separate labelling rounds. The resulting labelling degrees of the goat anti-mouse polyclonal antibody was 1.4, 5.9 and 8.6 for FITC, PtCP and Eu-chelate (W8044), respectively. These labelling degrees were determined as being suitable for use, as the fluorescein label tends to suffer from self-quenching with labelling degrees > 4. PtCP and Eu-chelate are not as sensitive to self-quenching and can readily tolerate labelling degrees as high as 10-20. As the aim was to compare the performance of the labels, the targeted degrees of labelling were not to deviate excessively from those labels used in the study.

The concentrations of primary and secondary antibodies used were chosen in order to achieve an adequate signal, along with a reasonable consumption of antibodies. In practice, both primary and secondary antibodies were used in surplus and the excess of unbound antibodies was removed in a single washing step, following antibody incubation periods. Due to an extensive reduction of washing steps when compared with similar, commonly used immunocytochemical assay methodologies (3-5 washing steps after antibody incubations), small quantities of unbound antibodies might remain in the assay well. However, the signal-to-background ratio achieved was at a satisfactory level, despite the use of only a single washing step following each round of antibody incubation. The extent of unspecific binding of the primary antibody was not specified and the unspecific binding of the secondary antibody was assessed to be relatively small in terms of assay performance.

5.2.1.3 Analysis of the results from Z' –factor and concentration-response experiments (II)

The signal-to-background ratio (S/B) was determined as the ratio of signals obtained with and without TNF-α treatment (II, Fig. 6). Both the primary antibody and the labeled secondary antibodies were introduced to all wells treated with or without TNF-α in order to measure the real enhancement in protein expression levels triggered by cytokine treatment. The S/B-value for FITC was 2.5 when measured immediately and 2.7 when measured repeatedly after 7 months storage. The corresponding values for Eu-chelate were 16.0 and 15.6. For PtCP(uv) the values were 7.3 and 9.8 and for PtCP(vis) they were 8.8 and 10.6 (uv and vis are referring to the excitation in the uv and visible wavelength ranges, respectively). The enhanced S/B-values after prolonged storage using PtCP may be due to a continued process of dehydration, since

PtCP luminescence is sensitive to quenching by molecular oxygen and dehydrated conditions help preserve the luminescence properties of PtCP. With FITC and Eu-chelate, the change in S/B after prolonged storage is practically negligible.

The Z'-factors for FITC were 0.61 when measured immediately and 0.11 when re-measured after 7 months. The corresponding Z'-factors for Eu-chelate were 0.77 and 0.72,. For PtCP(uv), Z'-factors were 0.55 and 0.59 and for PtCP(vis), they were 0.50 and 0.56. As above, the enhanced Z'-factors after prolonged storage using PtCP could be due to a continued process of dehydration. With Eu-chelate, the reduction seems due to the signal variation, especially with respect to the background signal, as is the case also with FITC. The fundamental reasons for the increased variation in the background signals of Eu-chelate and FITC after a prolonged period of storage however, remains inexplicable.

Titration experiments with TNF-α showed a clearly positive concentration-response relationship with all labels (Fig. 9). Similar EC_{50} values of 0.22, 0.26 and 0.21 nM for FITC, Eu-label and PtCP were calculated, respectively. As the corresponding Hill slope values (1.24, 1.23 and 1.11 for FITC, Eu-label and PtCP respectively) showed high similarity, all three labels were considered to exhibit congruity in these terms. The detection limit determined in terms of TNF-α concentration was 0.04, 0.02 and 0.01 ng/ml for FITC, PtCP and the Eu-label, respectively.

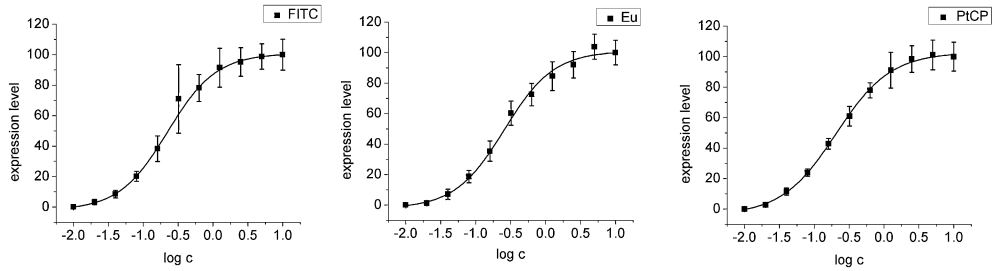

Figure 9. ICAM-1 expression as **a** function of TNF- α titre after signal normalization. The binding degree achieved at the highest TNF-α concentration was set as 100. Uniform shapes of the fitted concentration-response curves are clearly visible. C = concentration of TNF-α (ng/ml).

5.2.1.4 Microscopy analysis

Visual evaluation through time-resolved fluorescence microscope inspection revealed the significant potential of long-lifetime fluorophores in *in-situ* analysis of adhered cells, following the preceding microplate reader measurements in order to elicit additional detailed information of the localization of the target cell surface proteins. The EAhy926 cells possessed the characteristic classical swirling cobblestone morphology (Edgell et al. 1990) clearly observed with long-lifetime fluorophores, while with FITC, the visual inspection became more complicated due to strong cellular autofluorescence (II, Fig. 4).

Treatment with a 0.01 ng/ml concentration of TNF-α was adequate for the detection of ICAM-1 expression using Eu-chelate and PtCP (Fig. 10). No detectable difference, with respect to varying TNF-α concentration, could be seen using the FITC label for imaging. A striking observation with respect to the detection limit of microscopy, was that it was of the same (in the case of Eu) and approximately the same (in the case of PtCP) order of magnitude as that achieved using a microplate reader. In general, microscopic analysis is a more sensitive method of detection. Resultantly, in the case of TRF imaging, longer exposure times are necessary in order to achieve a satisfactory level of specific signal.

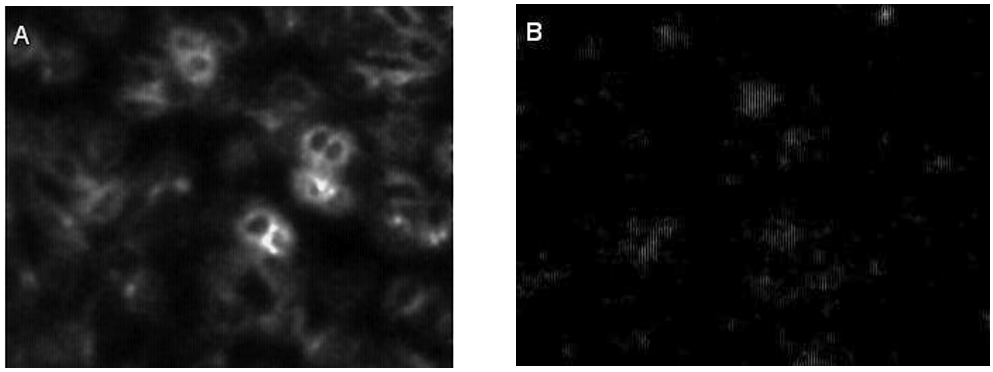

Figure 10. A) Minimum expression of ICAM-1 treated with 0.01 ng/ml TNF-α visualized with Eu-labeled antibody. With this minimum concentration of TNF-α ICAM-1 appears to be localized on the cellular boundaries. B) Minimum expression of ICAM-1 treated with 0.01 ng/ml TNF-α visualized with PtCP-labeled antibody. The appearance of the cells is visualized substantially faintly.

5.2.2 Multi-parameter assay for TNF-α -induction of β1-integrin, E-selectin and ICAM-1 expression on HUVE cells (III)

5.2.2.1 Background

For the development of a multi-parametric method for the quantitative determination of the protein expression levels on the surface of adherent cells, HUVE cells were chosen as a model platform. In HUVE cells, the expression levels of E-selectin and ICAM-1 were known to be substantially regulated through cytokine-initiated response (Bevilacqua et al. 1994, Meager 1999, Zerwes et al. 2002) while β_1-integerin was known to be abundantly expressed in these cells with no primary effect due to cytokine action (Defilippi et al. 1992). The discrimination of the long-lifetime labels is based on the use of suitable filters, as all three labels hold distinct spectral properties in the form of narrow emission peaks and long Stokes' shifts. Furthermore, PtCP has an additional excitation peak (Soret band) in the visible region of the spectrum (III, Fig. 1).

Many of the general aspects concerning the single parametric protein expression assay described above (II), also hold for the multi-parametric approach.

5.2.2.2 Optimization of time-course for TNF-α treatment

In order to evaluate the optimal TNF-α exposure time, the time course of β1-integrin, E-selectin and ICAM-1 was surveyed applying the methodology published earlier (II). The induced enhancement in the expression level of ICAM-1 was nearly approaching the maximum level after a 4 h treatment with TNF-α, while the enhancement in expression level of E-selectin reached the maximum after 4h of treatment (III, Fig. S2). The expression level of β_1-integrin was not dependent on the concentration of TNF-α but remained at a constant level. Accordingly, a time of 4 h was selected for TNF-α treatments in the multi-parameter assay approach.

5.2.2.3 Labeling degrees of the antibodies and application

The labelling degrees of the primary antibodies were 9.9, 7.1 and 2.7 for anti-β_1-integrin-Tb, anti-E-selectin and anti-ICAM-1, respectively. The antibodies were each applied at 40 ng per well in the label mixture used for cell staining. Any normalization of antibody or label concentrations was not required in the multi-parametric approach in order to quantify the changes in the expression levels of single species of target protein.

5.2.2.4 Analysis of the results from Z' –factor and concentration-response experiments

The signal enhancement values (S/B) due to TNF-α treatment were 9.9 and 2.9 for E-selectin (Eu) and ICAM-1 (Pt), respectively. The corresponding Z'-factors were 0.82 and 0.72 for E-selectin (Eu) and ICAM-1 (Pt) (III, Fig. 4). Due to the fact that the total fluorescence signal counts may vary significantly between separate assays (III, Fig. S3 a, c), it is advantageous to normalize the signals for the comparison of individual experiments. A highly practical approach for normalization of the signal is to process the fluorescence response as a ratio between the counts in the wells treated with TNF-α and the counts in the wells not treated (i.e. signal to background, S/B) (III, Fig. S3 b, d). Thus, the relative enhancement or decrease in the expression level can be effectively traced. The durability of label performance was assessed by re-measurement of the plates after 7 months of storage in the dark. The Z'-factor and S/B values after prolonged period of storage remained nearly unchanged with respect to both E-selectin and ICAM-1 expression analysis. Because the expression level of β1-integrin was not responsive to treatment with TNF-α, calculation of S/B or Z'-factor was not considered to be reasonable as it would give information of minor importance. These results with respect to E-selectin and ICAM-1 are in fair concordance with a DELFIA-based multi-parametric adhesion molecule assay (Zerwes et al. 2002) taking into account the differences in antibodies, labels and labelling degrees: in the DELFIA assay anti-E-selectin antibody was labeled with a Eu-chelate and anti-ICAM-1 antibody was labeled with a Sm-chelate emitting in around same wavelength region as

PtCP. The DELFIA assay however, does not provide any possibility for site-specific or morphological inspection, nor does it allow for postponed analysis after prolonged storage.

A clearly positive concentration-response relationship was found in the TNF-α titration experiments with respect to E-selectin and ICAM-1, while β_1-integrin was not responsive as expected (III, Fig. 3). A detection limit of 1 pg/ml of TNF-α was obtained for both E-selectin and ICAM-1 expression. This is a quite satisfying result, as a specific immunoassay using a Eu-chelate for TNF-α detection has previously been reported to achieve a detection limit of 0.1 pg/ml (Xie et al. 2008).

5.2.2.5 Microscopy analysis

Micrographs taken on syto13, Eu, Pt and Tb channels, clearly reveal the distinct localization patterns of each target protein and the positions of the nuclei in relation to the target proteins and other nuclei (III, Fig. 5). The cell boundaries were not distinguishable as expected; however by choosing for example PECAM-1 as a target protein, the cell boundaries might become more readily visualized (Scholz et al. 1996). Any significant cross-over between the channels was not observed and the labels did not otherwise appear to interfere with each other's performance. At least theoretically, the labels might stack on each other when brought into the dry state and thereby produce anomalous observations due to unpredictable short-distance interactions between the piled-up set of heterogeneous label molecules. These theoretical interferences might be due to the issue, that after the advisedly minimized number of washes following the staining step, residual counts of free and unspecifically bound labeled antibody conjugates and DNA stain might still remain in the wells. No indication of such bothersome effects on the assay performance was observed and the assay appeared to be feasible, with satisfactory results.

5.2.3 General notes and prospective

Image-based high-content screening (HCS) is gaining increasing attraction in the field of drug discovery (Ramm 2005), including the high-throughput methods for siRNA-based target identification and validation (Giuliano et al. 2004, Rantala et al. 2011). HCS methods are often based on automated microscopy which enables the observation of cellular aggregates or single cells as point sources (on low magnification) and even subcellular details can be resolved (on high magnification). Concurrently, multicolour fluorescence applications of cellular targets are emerging in fields of basic cell biology and pathology (Buchwalow et al. 2005, Ma et al. 2006, Schieker et al. 2007, Sweeney et al. 2008). HCS equipment is very expensive and large cellular descriptor data sets are yielded requiring rather complex data manipulation algorithms to find biological relevance. On average however, high multiplexicity (manifold assay parameters) and high throughput levels are the main benefits (Feng et al. 2009). The high autofluorescence of cells, especially when fixed

(Aubin 1979) and often overlapping excitation and emission spectra of prompt fluorescence labels, challenge the effective quantification of fluorescence. In microscopy, these difficulties have been challenged by utilizing long-lifetime (lifetimes on the order of 10s and 100s of μs) luminophores, such as lanthanide chelates (Seveus et al. 1992, de Haas et al. 1996) or phosphorescent platinum porphyrins (de Haas et al. 1996, de Haas et al. 1997, Soini et al. 2003) as labels, including a multicolour application (Soini et al. 2003) and the analysis of histological samples (de Haas et al. 1999, Siivola et al. 2000, Väisänen et al. 2000). Time-resolved luminescence-based microscopy has been applied for the quantitative analysis of histological biomarkers (Bjartell et al. 1999, Siivola et al. 2000) but the dynamic range of measurement is limited when compared with a microplate reader and the analysis of expression levels for screening applications is cumbersome.

In drug discovery applications, the value of suppressing the cellular autofluorescence in immunocytochemical assays is emphasized because; in addition to the usage of cancer cell lines in developing drugs against various cancers, cell lines immortalized with oncogenes are commonly used in biomedical research and in screening applications. Even though these immortalized cell lines are not necessarily able to induce tumour formation when introduced in animals, they probably possess several tumoural phenotypes, including the differential appearance and distribution of molecules causing cellular autofluorescence and reflecting the physiological state of the cells (e.g. Provenzano et al. 2008). This might lead to distorted and biased information in fluorescence applications applying exogenous fluorophores (as in immunocytochemistry), especially in co-culture applications comprising of cells with differing properties. Recently, applications based on cellular autofluorescence have also been identified as diagnostic tools (Monici 2005, De Giorgi et al. 2009, Masilamani et al. 2011).

Since a HCS instrument utilizing long-lifetime fluorophores for imaging has not been introduced yet, a HTS-applicable method for the combination of a standard plate reader and time-resolved microscopy in both single parameter (II) and multi-parameter (III) modes for cell surface expression analysis was developed. Earlier, single parameter (Trinh et al. 1999) and multi-parameter methods (Zerwes et al. 2002) based on dissociative DELFIA technology were introduced, but they were compromised with respect to the loss of spatial and morphological information. The methods developed in this work (II, III) provide a quasi-quantitative (O'Hara et al. 2011) approach with added information value, provided through time-resolved luminescence microscopy inspection. The methods enable amongst other things, the detection and analysis of the clustering of the cell adhesion molecules, an event described in several contexts of adhesion molecule research (Wójciak-Stothard et al. 1999, Bouzin et al. 2007, van Buul et al. 2010). In other cellular systems, clustering or de-clustering might be of importance in secondary screening, in order to reveal possibly occurring adverse or other off-target events in the early phase of the drug discovery process. Time-resolved microscopy is a constantly developing technique and more effective ways for precise time-gated signal acquisition through an electronically controlled shutter connected to a CCD camera have been introduced

(Hanaoka et al. 2007, Gahlaut and Miller 2010). These technical improvements together with advancing label technologies suggest extraordinary possibilities for future research.

6 SUMMARY AND CONCLUSIONS

1) Comparable performance between two-photon and one-photon excitation in fluorescence polarization based detection was established by studying ligand binding to estrogen receptor α in a competitive assay format. Since the optical setups differed between the assays, the conclusions drawn remain suggestive. Using a short measurement time of 1 s, one-photon excitation gave a clearly better Z'-factor value than that of two-photon excitation. Nearly similar Z'-factor values were achieved using measurement time of 5 s per well, even though the one-photon excited samples suffered from some photobleaching. Due to the scarce acquisition of emissive photons, the two-photon excitation mode reaches and surpasses the one-photon excitation mode in performance only when using longer measurement times. However, the signal-to-background values (polarization contrast) for two-photon excitation were clearly higher than for one-photon excitation as expected, based on the fact that the photoselection process is enhanced in two-photon excitation, when compared with that of one-photon excitation. The results suggest that the applicability of the two-photon excitation technique in fluorescence polarization assays in HTS environment is limited due to the long measurement times per assay well needed to achieve reliable screening results. The proven amenability to miniaturization suggests that two-photon excitation methods could be used for highly valuable samples in special cases, where cost saving is deemed more essential than the degree of the assay throughput.

2) Proof-of-concept development and validation of a cell-based functional screening assay platform for the quantitative immunocytochemical analysis of cytokine (TNF-α) induced enhancement in cell surface protein expression levels on endothelial cells was established. In the single parameter approach, two long-lifetime (Pt-coproporphyrin and Eu-chelate) fluorophores and one prompt fluorescent (fluorescein) fluorophore were compared in assay performance in a platform applying fixed endothelial cells of an immortal cell line and fluorophore-labeled secondary antibodies against the unlabeled primary anti-ICAM-1 antibody. The heterogeneous assays were successfully carried out using a minimum number of washing steps and the final measurements of the samples took place in a dry state, leading to sufficiently reliable results. Eu-chelate showed the best performance in both Z'-factor determination and TNF-α titration experiments, whilst Pt-coproporphyrin and fluorescein gave a slightly lower performance, but altogether all labels had Z'factor values indicating an excellent screening assay. After prolonged storage for 7 months, the performance of both long-lifetime fluorophores was preserved, while the fluorescein's performance collapsed, probably due to the deterioration of the unspecifically bound labeled antibody. In contrast, the fairly satisfactory Z'-factor for fluorescein originally observed in the immediate measurements was most probably due to the same unspecifically bound labeled antibody, which generated a high background signal with limited signal deviation, and thus a reasonable Z'-factor was obtained. Microscopy analysis definitely proved the benefits of the long-lifetime fluorophores in the subsequent protein localization analysis. The wells without

cytokine treatment remained practically dark at the Pt and Eu channels, while at the fluorescein channel, the untreated cells were clearly visible due to extensive autofluorescence. Finally, as the localization pattern of ICAM-1 was tractable using long-lifetime fluorophores but not using fluorescein, it is reasonable to conclude that Eu-chelate and Pt-coproporphyrin could be used as immuno-labels in microplate-based screening efforts, with increased informational content for searching modulators of TNF-α induced adhesion molecule expression. The applicability of fluorescein for similar purposes however, remains questionable.

3) On the basis of the successful results from the preliminary pilot study (II), an immunocytochemistry-based assay platform for analyzing the cell surface expression of multiple target proteins on primary HUVE cells was developed. TNF-α was applied to induce the expression levels of E-selectin and ICAM-1, although it had no influence on the expression of β_1-integrin as expected on the basis of literature. The developed multi-parametric model assay platform turned out to be practically insensitive to spectral interference from channel-to-channel crosstalk between the different fluorophores, in both the microplate reader and microscopy analysis. The method produced excellent Z'-factors and a remarkably low detection limit of TNF-α for both E-selectin and ICAM-1 expression. Subsequent microscopy analysis revealed a clear and distinctive localization pattern for each target protein and the nuclei though the detailed cellular morphology of the individual cells remained indefinable, as expected for the type of staining used. Prior to the study, it was not obvious how the desiccation of the sample plates after the assay procedure would influence the fluorescence properties of the label molecules, as the dyes (at least theoretically) might stack up on each other in an unpredictable pattern when brought into the dry state. This appears not to have formed any obstacle for the assay development. It can be concluded therefore, that a multi-parametric approach for cell surface protein expression analysis in a microplate format and combined with high-content image analysis, is feasible.

4) A new homogeneous competitive estrogen receptor (ER) ligand binding assay method, based on the QRET-principle was developed. A Eu-chelate labeled estradiol derivative Eu-E_2 was synthesized as a probe for the assay. The approximated K_d value of 30 nM for the binding of the luminescent probe Eu-E_2 to ERα suggests a reasonable feasibility for the development of a new competitive assay. The method developed is the first of its kind in applying a lanthanide-conjugate-labeled probe targeted to the ligand-binding site of ERα. The QRET method was paralleled with a homogeneous fluorescence polarization (FP) assay applying a fluorescein-labeled estradiol derivative in order to compare the assay performance with known binders to the ERα ligand binding site. The QRET method produced an improved signal-to-background ratio, with comparable relative binding affinities of the competitors and a similar Z'-factor with the FP assay suggesting the potentiality of the new methodology within the screening field. The developed QRET assay is a robust and simple format, amenable for multiplexing, miniaturization and automation with the benefits of time-resolved luminescence detection.

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