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GANESH BABU MANOHARAN

Combining chemical and genetic approaches for photoluminescence assays of protein kinases

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Combining chemical and genetic approaches for photoluminescence assays of protein kinases

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Institute of Chemistry, Faculty of Science and Technology, University of Tartu, Estonia.

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LIST OF ORIGINAL PUBLICATIONS

The current thesis is based on the following original publications, referred to in the text by corresponding Roman numerals:

I Ramesh Ekamabram, Erki Enkvist, **Ganesh babu Manoharan**, Mihkel Ugandi, Marje Kasari, Kaido Viht, Stefan Knapp, Olaf-Georg Issinger & Asko Uri (2014). Benzoselenadiazole-based responsive long-lifetime photoluminescent probes for protein kinases. *Chemical Communications*, 50, 4096–4098.

II **Ganesh babu Manoharan**, Erki Enkvist, Marje Kasari, Kaido Viht, Michael Zenn, Anke Prinz, Odile Filhol, Friedrich W. Herberg & Asko Uri (2015). FRET-based screening assay using small-molecule photoluminescent probes in lysate of cells overexpressing RFP-fused protein kinases. *Analytical Biochemistry*, 481, 10–17.

III Ramesh Ekambaram, **Ganesh babu Manoharan**, Erki Enkvist, Kadri Ligi, Stefan Knapp & Asko Uri (2015). PIM kinase-responsive microsecond-lifetime photoluminescent probes based on selenium-containing heteroaromatic tricycle. *RSC Advances*, 5, 96750–96757.

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Author's contribution

I The author participated in planning of the experiments, performed the optical and biochemical characterization of the compounds and wrote the respective part of the manuscript. Compounds used in the study were synthesized by co-authors.

II The author planned and performed most of the molecular biological experiments and all the biochemical experiments and wrote the manuscript. Compounds used in the study were synthesized by co-authors.

III The author participated in planning the experiments and performed most of the optical and biochemical characterization of the compounds and wrote the respective part of the manuscript. Compounds used in the study were synthesized by co-authors.

IV The author planned and performed the all molecular biological and biochemical experiments and wrote the manuscript. Compounds used in the study were synthesized by co-authors.

ABBREVIATIONS

Ahx	6-aminohexanoic acid moiety
ARC	conjugate of a heterocyclic fragment and a peptide or peptide analogue
ARC-Fluo	ARC-probe labeled with a fluorescent dye
ARC-inhibitor	ARC-based inhibitor
ARC-Lum	ARC-probe possessing protein-induced signal with microsecond-scale lifetime
ARC-Lum(–)	ARC-Lum probe lacking a fluorescent dye
ARC-Lum(Fluo)	ARC-Lum probe incorporating a fluorescent dye
ARC-Lumi4Tb	ARC-probe incorporating terbium cryptate Lumi4Tb
ARC-PF647	ARC-probe incorporating PromoFluor-647 dye
ARC-Photo	ARC-probe incorporating a photoluminescent label
ARC-probe	ARC-based inhibitor possessing luminescent properties
ARC-TAMRA	ARC-probe incorporating 5-TAMRA dye
ATP	adenosine 5'-triphosphate
avGFP	green fluorescent protein from the jelly fish *Aequorea victoria*
BRET	bioluminescence resonance energy transfer
cAMP	cyclic adenosine 3',5'-monophosphate
CK2α	catalytic subunit of protein kinase CK2, subtype α
CYP	cyan fluorescent protein
EGFP	enhanced green fluorescent protein
FA	fluorescence anisotropy
FI	fluorescence intensity
FITC	fluorescein isothiocyanate
FP	fluorescent protein
FRET	Förster-type resonant energy transfer
GFP	green fluorescent protein
GOI	gene of interest
His6	hexa-histidine
HTS	high-throughput screening
IC_{50}	concentration of inhibitor that causes 50% reduction of enzymatic/catalytic activity of the enzyme in the assay conditions
K_D	equilibrium dissociation constant determined from direct binding assay
K_d	equilibrium dissociation constant determined from displacement assay
K_i	equilibrium dissociation constant determined from inhibition assay
MW	molecular weight
PF555	PromoFluor-555
PF647	PromoFluor-647

PK	protein kinase
PKA	cAMP-dependent protein kinase A or protein kinase A
PKAc	catalytic subunit of PKA
PKAr	regulatory subunit of PKA
POI	protein of interest
QY	quantum yield
RFP	red fluorescent protein
TAMRA	5-carboxytetramethylrhodamine
TGL	time-gated luminescence
TR	time-resolved
TRF	time-resolved fluorescence
UV	ultra-violet
YFP	yellow fluorescent protein

INTRODUCTION

Phosphorylation reaction is an important post-translational protein modification procedure in cells, which is carried out by protein kinases (PKs). This modification leads to increase in the diversity of the proteome that influences various aspects of normal and pathological physiology. Dysregulation of protein phosphorylation balances, caused by the aberrant activity of PKs is a cause or consequence of several complex diseases such as cancers, inflammatory disorders, cardio-vascular diseases and diabetes [1]. Therefore PKs have become important drug targets in the 21st century [2]. In recent 15 years, 30 small-molecule PK inhibitors have been approved for use in clinical practice [3].

More than 500 PKs, constituting almost 2.5 % proteins coded by the human genome, are carrying out the phosphorylation of various proteins [4]. In addition to being a potential drug targets, PKs also serve as biomarkers for cancers and other diseases, as altered expression level of various PKs is observed in a variety of malignancies [5]. Thus there is high demand for analytical methods that enable determination of expression and activity levels of specific PKs in clinical samples, such as bodily fluids and cancerous tissues. Thus high throughput assays for screening PK inhibitors also form an important component of drug development pipeline.

During the last decade, it has been understood that merging of two disciplines, synthetic chemistry and protein engineering, is needed to construct molecules and their complexes with new functionalities that can create novel opportunities for biomedical research, drug development and disease diagnostics [6, 7].

In this thesis combination of chemical and genetic approaches was used for the development of analytical tools for the characterization of PKs and PK inhibitors. PKs (PKAc, CK2α, and PIM kinases) were fused with fluorescent proteins (FPs). On the other hand, ARC-based small-molecule PK inhibitors were developed into protein binding-responsive ARC-Lum probes possessing unique photoluminescent properties. Joint application of PKs fused with fluorescent proteins and ARC-Lum probes enabled the construction of sensor systems that can be used for specific and sensitive determination of PKs in biological samples and as research tools for mapping and monitoring PK activity in living cells.

LITERATURE OVERVIEW

1. Protein kinases

The protein phosphorylation plays a crucial role in intercellular communication during development, in physiological responses and in homeostasis, and in the functioning of the nervous and immune systems. Phosphorylation has been found to be a common post-translational modification with 500,000 potential phosphorylation sites in the human proteome and 25,000 phosphorylation events described for 7000 human proteins [8]. Protein phosphorylation is carried out by PKs which belong to the transferase class of enzymes. PKs associate with a substrate protein and a nucleoside triphosphate [usually adenosine 5'-triphosphate (ATP)] and transfer the γ-phosphoryl group of the latter to specific amino acid residues, such as serine, threonine and tyrosine of the substrate proteins. PKs mediate most of the signal transduction cascades in cells by modifying of substrate protein activity. PKs also control many cellular processes, such as metabolism, transcription, cell cycle progression, differentiation, and apoptosis [9].

In 2002, Manning *et al.* [9], classified the PK complement of human genome (kinome) into nine groups of PKs based on the phylogenetic analysis [1]. They reported 518 PKs, comprising of 478 genes and 40 atypical genes. However, a very simple way for classification of PKs is based on the target amino acid on the substrate protein which is phosphorylated by the PK. During the course of phosphorylation, the phosphate group from the nucleotide is transferred to the side chain of an amino acid of the substrate protein. The human PKs phosphorylates, hydroxyl group of the side chain of the amino acids of a target protein, follows O-phosphorylation. However, N-phosphorylation of residues with amine-containing side chains (histidine, lysine, and arginine) are not prevalent in human kinome [10]. These phosphorylated residues are asymmetrically distributed (85% serine, 11.8% threonine, and 1.8% tyrosine residues) [4]. Based on the phosphorylatable residue PKs can be classified as follows,

- Serine/Threonine kinases: catalyze the phosphorylation of the hydroxyl group of serine or threonine in the substrate protein (*e.g.,* AGC kinases, CAMK kinases, *etc.*)
- Tyrosine kinases: catalyze the phosphorylation of the hydroxyl group of tyrosine in the substrates protein (*e.g.,* ABL kinases, SRC kinases, *etc.*)
- Dual specific kinases: can act as both serine/threonine and tyrosine kinases (*e.g.,* MAPK)

PKs recognize certain consensus sequence surrounding the phosphorylatable residue of the substrate protein and specifically carry out the phosphorylation reactions. Thus, based on the substrate consensus sequences or the specificity determinants, the serine/threonine kinases can be classified into three categories [11],

- Basophilic PKs, prefer basic and hydrophobic amino acids as the determinants; majority of PKs of the AGC group and some CAMK group of PKs belong to this category;
- Proline-directed PKs, prefer basic amino acids in substrate recognition sequence but additionally require a proline residue at position P+1; members of CMGC group fall in this category;
- Acidophilic/phosphate-directed PKs, comprise carboxylic acids and phosphorylated residues in their consensus sequence.

1.1. Basophilic kinases

Basic amino acids arginine and lysine serve as the specificity determinants for PKs that belong to this category, they target serine and threonine residues of substrate protein surrounded by arginine and lysine residues. PKs PKA, PKB, PKC, PKG, ROCK, PIM, MSK, *etc.* belong to this category [12].

Protein kinase A (PKA) or cAMP-dependent protein kinase was first characterized in 1968 and its mechanism of action well understood, thus it serves as the proto-type kinase for the study of other PKs [13]. PKA was the first PK whose crystal structure was solved already in 1991 [14]. PKA holoenzyme exits as an inactive tetramer composed of two catalytic subunits (PKAc) and a dimer of regulatory subunits (PKAr). The activity of PKA is controlled by cyclic adenosine 3',5'-monophosphate (cAMP), which is produced by cyclisation of ATP, catalyzed by adenylyl cyclases. On the other hand, cAMP is degraded by phosphodiesterase. The activity of adenylyl cyclase is controlled by G-protein coupled receptors which are responding to external stimuli or by direct activators such as forskolin. When adenylyl cyclase is activated, its triggers the conversion of ATP to cAMP. The PKA tetramer is dismantled in response to the elevation of cAMP concentration in cells. Two molecules of cAMP bound to each of the PKAr subunits leading to the release PKAc subunits. Then PKAc subunit catalyzes the phosphorylation of the target proteins.

Though PKA has been suggested to participate in the genesis and progression of various tumors [15], the knowledge about its ubiquitous expression and hundreds of established substrate proteins [12] has reduced its potential as a drug target. Still, a novel oral ATP-competitive multi-AGC kinase inhibitor (also inhibiting PKAc) AT13148 has been taken to clinical trials as a cancer drug candidate [16]. Only recently, it has been established that a mutation (L205R) in PKAc abolishes the binding of PKAc to PKAr and the resulting increased catalytic activity of PKAc is associated with the onset of cortisol-secreting adrenocortical adenomas [17–19]. Thus, there is a potential option for the development of drugs based on inhibitors that are specifically targeting the mutated form of PKAc.

Three constitutively active PKs PIM1, PIM2, and PIM3 form an important family (PIM kinases, possessing highly similar kinase domain with 60% homology) of basophilic serine/threonine kinases [20]. The PIM family of kinases

are named for their mode of discovery as proviral common integration sites in moloney murine leukemia virus-induced lymphomas. PIM kinases belong to the calcium/calmodulin-dependent (CAMK) group of PKs. Although all PKs of the PIM family participate in regulation of key biological processes, such as cell proliferation, differentiation, survival, and apoptosis, their relative expression level can be vary in cells of different origin and the expression level depends on the physiological conditions [21, 22]. PIM kinases lack obvious regulatory domains, the regulation of their activity occurs at the level of transcription, translation, and degradation [23]. Overexpression of PIM kinases has been found in human myeloid and T-cell leukemias and lymphomas, but also in numerous solid tumors (*e.g.,* pancreatic and prostate cancers, non-small-cell lung cancer, squamous cell carcinoma, gastric carcinoma, liver carcinoma, liposarcoma, *etc.*) [24]. Different isoforms of PIM kinases have been observed in cells, these isoforms are encoded from the PIM gene by alternate translation initiation codon. For example, in mice and humans, a single PIM2 transcript gives rise to three isoforms of the protein (MWs of 34, 37, and 40 kDa) that share an identical catalytic site but differ at their N-termini, due to in-frame alternative translation initiation sites [25]. In leukemic cells, the three PIM2 isoforms have very short half-lives, thereby the longest isoform is significantly more stable than the shorter isoforms [26].

High expression level of PIM2 isozyme of the PIM family has been reported for several hematologic malignancies [24]. For example, increased expression of the human PIM2 is observed in chronic lymphocytic leukemia and non-Hodgkin lymphomas. PIM2 also known to promote the cell survival and inhibit programmed cell death [27]. Therefore, in addition to being a potential drug target for hematologic malignancies, PIM2 kinase is also of interest as biomarkers for diagnosis of these diseases [21].

1.2. Acidophilic kinases

Acidophilic PKs phosphorylate serine/threonine residues flanked by acidic amino acids such as aspartic acid and glutamic acid [28]. Protein kinase CK2 (the acronym being derived from the misnomer “casein kinase 2”) is a ubiquitous acidophilic kinase which effectively contributes to the human phosphoproteome [29].

PK CK2 is a highly conserved acidophilic serine/threonine protein kinase ubiquitously expressed in all eukaryotic cells [30]. CK2 is a pleotropic PK that is known to phosphorylate more than 300 substrates, it effectively uses both ATP and Guanosine-5'-triphosphate (GTP) as phosphate donors. A consensus sequence for CK2 catalyzed phosphorylation contains acidic amino acids (Ser/Thr-X-X-Glu/Asp/pSer) [31]. CK2 may also be a dual-specific kinase that can also phosphorylate tyrosine in some special cases [32].

CK2 is a tetramer composed of two catalytic (α and α′) and two regulatory (β) subunits. The catalytic subunits, CK2α (MW = 42–44 kDa) and CK2α′

(MW = 38 kDa), are products of individual genes in mammals with more than 90% sequence identity in their N-terminal 330 amino acids, and entirely unrelated C-termini. A third catalytic isoform, CK2α′′ is also found in human cells. CK2α′′ is practically identical to CK2α, except for the last 32 amino acids of its C-terminus. However the regulatory CK2β subunit (25 kDa) has no extensive similarity to other proteins [33]. In contrast to many signaling PKs, CK2 is constitutively active, independent of second messengers or phosphorylation events, and the mechanism of regulation of CK2 activity in cells is still unclear [34].

A large number of substrates which are involved in gene expression and cell growth are phosphorylated by CK2. The expression level of CK2 has been found to be upregulated in many of the cancers. Overexpression of CK2 has been reported in several inflammatory diseases, cardio-vascular disorders and neurodegenerative disorders. So, CK2 is targeted for the treatment of various diseases [35].

2. Photoluminescence

Due to their high sensitivity and productivity photoluminescence-based techniques form the basis of many research tools used for high throughput biochemical assays, live cell imaging and luminescence-based sensors for analysis of proteins in complex biological solutions such as bodily fluids and cell lysates *etc*. Luminescence is the generic term for the emission of light and generally divided as fluorescence and phosphorescence (luminescence does not include emission of light that occurs from substances heated to high temperature). The luminescence arising from direct photoexcitation of the emitting species is called photoluminescence [36].

So, the radiative emission of photon from excited energy level to ground level is called as photoluminescence. When a molecule is excited to higher energy level (singlet state, S_1 or S_2) by absorbing a photon, it returns to the ground state (S_0) by emitting a photon or by internal conversion (Figure 1). In case of fluorescence, the excited molecule returns to the ground state from the singlet excited state by emitting a photon. The energy of the emitted photon is less compared to that of the absorbed photon, thus the emission results in longer wavelength. The difference between the positions of band maxima of the absorption and emission spectra is called the Stokes shift. Several other relaxation process compete with fluorescence emission such as non-radiate dissipation of energy as heat, and energy transfer to other molecule (quenching) and intersystem crossing. During the intersystem crossing, the excited molecule goes to first excited triplet state (T_1) and then returns to the ground singlet state by emitting a photon, the phenomenon is called phosphorescence. The transition of excited molecule from S_1 to T_1 state is a forbidden process. Usually luminophores comprising of heavy atoms facilitate this process [37]. Typically phosphorescence emission lasts for few micro-seconds to seconds and fluorescence lasts for nanoseconds.

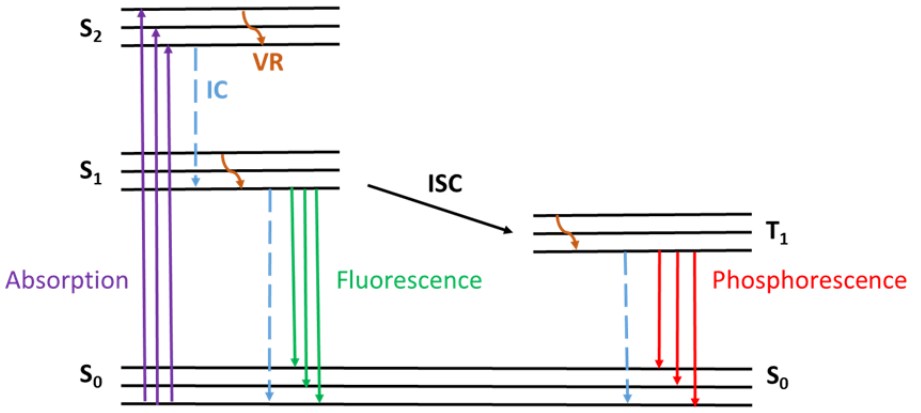


Figure 1. A simplified Jablonski diagram for photoluminescence, adopted from [37]. When a luminophore absorbs light, it is excited from its ground state (S_0) to excited state (S_1 and S_2). The molecule can rapidly relax to the lowest vibrational level of the lowest excited state by vibrational rotation (VR). By internal conversion (IC), the molecule can relax to S_1 from S_2. The molecule then returns to ground state by emitting energy as fluorescence or by IC. Additionally, molecules in S_1 state can undergo a spin conversion to first excited triplet state (T_1) *via* intersystem crossing (ISC) and then relaxes to ground state by phosphorescence.

The average lifetime that a molecule spends in the excited state before returning to the ground state is called luminescence lifetime. The ratio between the number of emitted and absorbed photons is called as the quantum yield (QY). Luminophores possessing high quantum yield, display brighter emission. Thus the brightness of a luminophore is the product of its molar absorption coefficient (ε) and QY. The QY of the fluorophore is related to the processes that compete with fluorescence for depopulation of the excited state. Therefore, the fluorescence intensity (FI) depends on the initial population of excited state multiplied by QY [38].

In general photoluminescence-based measurement can be performed either in steady-state or time-resolved (TR) mode. In case of steady-state measurement the sample is illuminated with continuous beam of light or pulses of light and the emission is recorded simultaneously. As the fluorescence decay is very quick, in fluorescence-based measurements the steady-state is reached almost immediately. In case of TR measurements, the sample is exposed to pulse of light, whereas the pulse width is shorter than the decay time of the luminophore [37]. In case of complex samples, such as live cell assays and cell lysates, TR measurements are advantageous as the background autofluorescence that arises from the cellular components can be avoided.

Photoluminescence-based detection measures changes in the luminescence parameters such FI or luminescence intensity, fluorescence anisotropy/ polarization (FA) and luminescence lifetime of a luminophore.

2.1. Förster-type resonant energy transfer

Förster-type resonant energy transfer (FRET, also used as Förster resonance energy transfer) is a non-radiate distance-dependent energy transfer from a donor molecule to an acceptor molecule provided that the emission spectrum of the donor luminophore overlaps with the absorption spectrum of the acceptor luminophore and these luminophores are positioned in closer proximity [39]. The FRET does not involve emission of light from the donor, rather the donor and acceptor are coupled by dipole–dipole interaction. The resonance energy transfer from excited state of donor, $^1D^*$ to the excited state, $^1A^*$ of acceptor molecule, results in the decreased luminescence lifetime and intensity of the donor luminophore. At the same time, the sensitized emission of the acceptor molecule occurs that leads to increase in the emission intensity of the acceptor.

The FRET is dependent on many criteria, such as the distance between the donor and acceptor molecules, the spectral overlap between the emission spectrum of donor and the absorption spectrum of acceptor. The efficiency (E) and the rate of energy transfer [$K_T(r)$] are related by the following equations,

$$K_T(r) = \frac{1}{\tau_D}\left(\frac{R_0}{r}\right)^6 \qquad \text{(Equation 1)}$$

$$E = \frac{{R_0}^6}{{R_0}^6 + r^6} \qquad \text{(Equation 2)}$$

where, r is the actual distance between donor and acceptor, τ_D is the decay time of donor in the absence of acceptor, and R_0 is the Förster distance at which the energy transfer is 50%.

The Förster distance depends on a number of factors, including the fluorescence QY of donor, refractive index of the solution and the spectral overlap integral of donor and acceptor. The typical Förster distance is ranges from 1 to 10 nm, and FRET is useful to study the biological macromolecules, as this distance is comparable to the diameters of many proteins and thickness of biological membranes [40].

In FRET-based sensors, energy transfer represents a dynamic type of quenching of the donor fluorescence and simultaneous increase of the acceptor fluorescence. Therefore, FRET efficiency can be detected in two modes [41]. In steady-state or stationary mode, a decrease of the donor's FI, an increase of the acceptor FI and by change of the ratio of donor and acceptor FIs in dual channel mode measurements (at two wavelengths) are used to measure the FRET efficiency. In TR mode, decrease of the donor luminescence lifetime is measured. FRET efficiency measurement using luminescence lifetime change is the most accurate and reliable method, since the luminescence lifetime is independent of concentration of fluorophores, photo bleaching, changes in the intensity of the excitation light, and light scattering.

FRET efficiency is defined as proportion of the donor molecules that have transferred excitation state energy to the acceptor molecules, which increases

with decrease in the intermolecular distance between the luminophores [42]. In experimental setup, the FRET efficiency can be calculated by measuring the change in the luminescence lifetime and intensity of the donor, using the following equations,

$$E = 1 - \frac{I_{DA}}{I_D} \qquad \text{(Equation 3)}$$

where, I_{DA} is luminescence intensity of donor in the presence of the acceptor that leads to FRET and I_D is luminescence intensity of donor in the absence of the acceptor.

$$E = 1 - \frac{\tau_{DA}}{\tau_D} \qquad \text{(Equation 4)}$$

where, τ_D is luminescence lifetime of donor and τ_{DA} is luminescence lifetime of donor and acceptor complex.

Since FRET efficiency is dependent on the distance between two fluorophores, it is a very promising spectroscopic tool to study interaction of biological molecules. For example, protein-protein interaction, protein-ligand interaction and conformational change of the proteins can be studied using the principle of FRET [43]. Especially after the availability of genetically encoded fluorophores such as fluorescent protein (FP), various FRET-based sensors comprising of FPs pairs were constructed. FRET-based sensors are very widely used in various fields, for molecular biology applications [44, 45], high-throughput screening assays [46], cancer imaging [47] and as an indicator of intracellular signaling molecules [48], *etc.* to name few. FRET pairs may be established utilizing non-fluorophore acceptors (quenchers), such as BHQ [49], and quenching of the donor fluorescence can be used to monitor the interaction between the donor and acceptor. Since FRET-based measurements are less interfered by non-target interactions, they are suitable for measurements in cell lysates and live cell based high-content screening assays [50].

Although, FRET measurements are widely used in biochemical, biological, biomedical and medical studies, the measurements are often disturbed by the autofluorescence of biological samples. In that case, organic phosphorescent materials would be useful [51]. However non-organic, lanthanide based complexes such as terbium and europium chelates, possessing long lifetime luminescence (in milliseconds range) are widely used in FRET studies [52]. The application of lanthanide complexes as partners in a FRET-pair and a TR measurement mode allows the elimination of short-lived background signals associated with the autofluorescence of biomolecules and scattered light with a time delay [41]. In addition, measurements with time delay detect only sensitized fluorescence of the acceptor, and solve the problem of simultaneous fluorescence excitation of the donor and acceptor, thereby increasing the dynamic range of measurements and the accuracy of the FRET efficiency determination.

3. Combining chemical and genetic approaches

Interdisciplinary approaches, combining synthetic chemistry and protein engineering are needed to develop novel opportunities for biomedical research, drug development and disease diagnostics [6, 7]. Thus chemical genetics has emerged as an important approach to study the biological systems. Chemical genetics can be defined as the study of biological systems using small-molecules [53]. Classical genetics uses mutagenesis to elucidate the relationship between genes and phenotypes, whereas chemical genetics additionally employs small-molecule compounds [54]. Small-molecule compounds that intervene the biological systems by perturbing enzyme function or protein-protein interaction are used as tools in chemical genetics. Various protein modification techniques have been described to develop protein – small-molecule interfaces [55, 56]. This includes labeling a protein with a fluorescent dye [57], fluorescent protein (FP) tagging [6] , tetra cysteine motif-based labeling [58], enzyme mediated peptide tags, SNAP-tag, CLIP-tag and HaloTag [59, 60], labeling using trimethoprim conjugates [61] and introduction of unnatural amino acids [62] *etc.*

All the protein modification techniques have their own pros and cons. Perhaps the simplest technique is the FP tagging. Though fluorescently labeled proteins are widely used in various applications, the chemical labeling of a target protein in *in vitro* is difficult. Using genetic engineering techniques, a FP or a FP-tagged protein can be expressed in cells very easily. Though small, genetically encoded non-FP tag such as tetra cysteine motif-based FlAsH and ReAsh tags [63] are available, but the application of such tags is minimal in recent times, compared to the use of FPs, in spite of challenges such as huge size and the oligomeric nature of FPs. The major advantage of FPs is that they do not need any external chemicals to become fluorescent. The fluorescence of the FP is originated from interaction of amino acid residues in the FP, which leads to the formation of the chromophore responsible for fluorescence, once the FP is functionally folded. This in contradiction with genetically encoded tetra cysteine-based tags which require additional chemicals to be supplied into the cell for the fusion protein to become fluorescent and to be followed by thorough washing steps to remove the non-labeled substances.

Chemical genetic approaches have been applied to PKs for various reasons [64], including the establishment of the interaction between a PK and a substrate, the development of target specific inhibitors, the assignment of specific function to a PK in a signaling pathway, *etc.* A simple and reliable fluorescence-based technique for studying the interaction between PKAc and ligands using modified PK was demonstrated already 20 years ago. A mutated PKAc (N326C) was chemically labeled with acrylodan dye and fluorescence-based techniques were applied for studying the binding of PKAc with nucleotides and inhibitors [65]. In a classical chemical genetics approach [64], a gate keeper amino acid which is important for the binding of a substrate or a specific inhibitor, is replaced by a bulky amino acid. The point-mutated 'analogue – sensitive' PK is specific only to a particular set of inhibitors. On the other hand,

substrates and inhibitors are designed in such a way that they recognize only the modified PK and not the wild-type PK [66].

In this study, a combination of chemical and genetic approaches was used for construction of sensor systems for analysis of PKs. Small-molecule organic probes with unique optical properties were developed and used together with PKs that were fused with complementary fluorescent proteins.

4. The chemical approach: Development of small-molecule ARC-based photoluminescent probes

Small-molecules are defined as carbon-based compounds whose molecular weight is usually under 500 Da and always less than that of macromolecules such as DNA, RNA, and proteins [54]. Small peptides refer to peptides with less than 30 amino acids or a molecular weight of less than 3500 Da [67]. ARCs (that are conjugates of a heterocyclic fragment and peptide or peptide analogue) possess MW of less than 2000 Da. Therefore, in line with the classification that has been accepted for peptides, the term 'small-molecules' is also used for ARCs.

4.1. Inhibitors of PKs

Inhibitors of PKs have great practical value. Inhibitors of PKs are now widely used as drugs in clinical practice, but PK inhibitors are also used as research tools to study the regulation of cellular protein phosphorylation balances.

Considering the importance of the pathophysiological roles of PKs, various pharmaceutical companies are involved in the process of developing inhibitors against the PKs. Since the approval of imatinib (Glivec®) by Food and Drug Administration of United States of America (FDA) in 2002, the number of approved kinase inhibitors has increased to 33, with many other PK inhibitors still in preclinical development. More than 130 kinase inhibitors are reported to be in phase-2 and phase-3 clinical trials [3]. Among them Glivec® dramatically improved the prognosis of patients with Chronic myeloid leukemia which is caused by constitutively active Bcr-Abl tyrosine kinase [68]. As most of the approved kinase drugs are active against more than one specific PK, only a few of them have been used for the treatment of non-oncological indications.

Inhibitors that bind to the active site of a PK can be classified as inhibitors that are binding to the ATP-site, substrate protein-site or bisubstrate inhibitors which simultaneously associates with both substrate sites of the PK. The ATP binding site is conserved among all the PKs. Not only more than 500 PKs, but almost 1500 other proteins are known to bind purine nucleotides. Therefore, developing inhibitors that selectively target the ATP-binding site of a PK is challenging [69, 70]. On the other hand, the intracellular concentration of ATP is 1–10 mM, hence the inhibition potency of an ATP-competitive inhibitor

should be relatively high in order to compete with such a high concentration of ATP [71]. An inhibitor that possesses similar K_i values against multiple kinases will inhibit more potently those kinases in cells that have a higher ATP K_m value[71].

Since the ATP-site is conserved among the PKs, substrate protein binding-site offers more selectivity for development of inhibitors of PKs. Small-molecule inhibitors of PKs targeting this site have been derived from both natural sources as well as by synthetic methods [72]. Pseudo-substrate inhibitors that mimic the consensus sequence of substrate proteins of PKs are also available. For example, the heat-stable protein kinase inhibitor, PKI and PKAr are the physiological inhibitors of PKA [73].

Despite the difficulties, development of ATP-competitive inhibitors for various PKs is still progressing. For example, SGI-1776 is a potent inhibitor of PKs of PIM family, has been shown to be active against acute myeloid leukemia [74] both in *in vivo* and *in vitro* studies. A potent and selective inhibitor for CK2, targeting its ATP-site, namely CX-4945 has been shown to be active towards many forms of cancer in mice xenografts [75]. But still, these inhibitors have been shown to affect some off-target PKs to certain level. Thus making an inhibitor selective towards a single target PK is very difficult. For example, inhibitors that are comprising halogenated benzimidazole moiety as the ATP-binding ligand inhibit both PK CK2 and PIM kinases [76]. In that case making them selective towards one PK can be accomplished using the bisubstrate approach.

4.2. The bisubstrate inhibitors of protein kinases: ARC-probes

Bisubstrate inhibitors simultaneously bind to both substrate binding sites of the PK, hence they are conjugates of two fragments that associates to ATP-binding site and substrate protein binding-site of the PK. The bisubstrate character of an inhibitor towards a PK can be proved with the following methods [77]:

1. X-ray analysis of PK/inhibitor co-crystals
2. Kinetic analysis of the competitiveness of inhibitor against both substrates
3. Displacement of the inhibitor from its complex with a PK by either ATP- or protein substrate-competitive inhibitors

On the basis of ARC-compounds bisubstrate inhibitors of PKs have been developed in our research group [77]. ARCs are comprised of a heterocyclic fragment (targeted to the ATP pocket of the PK) and a peptide or peptide analogue (targeted to the substrate protein-binding site of the PK), connected by a flexible linker. The acronym ARC is derived from the combination of words *Adenosine analogue (A) and arginine (R)-rich peptide conjugate (C)*, representing the structural features of the first generation of ARC-based bisubstrate inhibitors [78]. Later on, various aromatic fragments and peptide analogues have been

introduced into the compounds, the acronym ARC has been retained for the historical perspective.

The crystallographic studies enabled the optimization of the design of ARC compounds [79, 80]. This resulted in ARC compounds with improved selectivity and affinity towards a variety of PKs. The ARC inhibitors targeting basophilic PKs, such as PKAc, PIM, ROCK, PKB, *etc.,* usually comprise D-arginine residues in the peptidic part, to achieve selectivity and resist proteolytic cleavage in the intracellular milieu [78, 81, 82]. Nevertheless, the peptidic part can be modified according to the origin of the target PK with other amino acids, based on the substrate consensus sequence of the PK.

The ARC-compounds targeting the acidophilic PK CK2α comprise of an ATP-targeting heterocyclic moiety and an oligo-aspartate, the latter mimics the acidic substrate phosphorylated by the CK2α. Both D- and L-isomers of oligo-aspartates have been utilized for developing ARCs targeting CK2α [83], as well as non-chiral peptoid analogue has been used in the peptide part [84]. Bisubstrate inhibitors of CK2α incorporating oligo-glutamates were recently reported by another research group [85]. The ARC-compounds targeting mitotic PK Haspin, which phosphorylates histone H3 at Thr3, a peptide mimicking the N-terminus of histone H3 was used was used as fragment of ARC [86]. Thus the ARC compounds have been widely modified at both the ATP-targeting and peptide mimetic parts. The simultaneous targeting of the ATP-binding site and substrate binding site of the PK by ARC compounds resulted in bisubstrate inhibitors possessing high affinity (K_d values in picomolar to nanomolar range) as well high selectivity.

In addition to being potent inhibitor of PKs, the ARC-compounds that are labeled with luminophores are successfully applied as small-molecule probes in the High-throughput screening (HTS) of PK inhibitors using luminescence-based detection methods [87].

4.2.1. ARC-Photo probes

ARC-Photo probes are ARC-compounds which incorporated a photoluminescent label such as a fluorescent dye (*e.g.,* TAMRA, PromoFluor-647) or a lanthanide chelates (*e.g.,* Lumi4Tb). ARC-Photo probes are used in luminescence-based binding/displacement assays. Luminescence-based detection methods are safe, environment friendly and convenient, compared to the traditional radioligand techniques. Inhibition assays using a fluorescently labeled substrates (*e.g.,* TAMRA-labeled kemptide) were also developed which used thin layer chromatography for separation of the phosphorylated and non-phosphorylated fluorescent substrates [88].

FA-based assays for the characterization of affinity of PK inhibitors have been developed using fluorescently labeled ARC-photo probes (ARC-Fluo probes). Here, the association of the PK and ARC-Fluo probes is measured by means of change in fluorescence anisotropy/polarization (FA) and/or fluores-

cence intensity (FI), hence it is a binding assay. The ARC-Fluo probe is used in FA-based displacement assay for the determination of dissociation constants (K_d) of inhibitors. FA-based binding/displacement assays using ARC-photo probes for inhibitors of basophilic kinases such as PKAc, ROCK [89], PKGα [90] and PIM [91] as well as acidophilic kinases CK2α [83] have been reported. Sometimes, the emission intensity of a PK-bound ARC-Photo probe is different compared to its free form. The ratio between the emission intensity of bound form and non-bound form of an ARC-Fluo probe is represented as the Q value and it is dependent on the structure of the ARC-probe, the conjugated fluorescent dye, and the PK used in the assay. ARC-Fluo probes, possessing higher Q values ($Q > 2$) were shown to be useful for the determination of dissociation constants of inhibitors towards cGMP-dependent protein kinase (PKGIα) in FI-based measurements [90] using both a fluorescence plate reader and a spectrofluorometer.

For FA-based assays, choice of the fluorescent dye is critical, as the affinity of the probe can be altered by the nature of the fluorescent dye. In FA-based assay, the range of resolvable affinities of compounds under evaluation is limited by the affinity of the fluorescent ligand [92]. The non-specific binding of fluorescent dye to the plastic or glass surface of the microtiter plate is a usual problem encountered in FA-based assay. For the same reasons, FA-based detection methods are restricted to biochemical assay and not widely used in cell lysates and live cell-based assays, although few exceptions point to wider applicability of FA-based assays [93-95].

ARC-Photo probes possessing fluorescent label (*e.g.,* Alexa Fluor 647 *etc.*) or luminescent label (*e.g.,* Lumi4Tb cryptate, europium cryptate *etc.*) are used in steady-state or TR FRET-based assays in combination with a genetically tagged PK (*e.g.,* His6 tag) and an antibody (*e.g.,* an anti-His6 antibody). Such multiplexed assays with various possible detection modes (FA, steady-state and TR FRET) are used for the characterization of protein-protein interaction between the PK and an antibody as well as the affinity of an antibody towards a PK.

4.2.2. ARC-Lum probes

ARC-Lum probes are organic, non-metal photoluminescent probes possessing protein-induced long-lifetime luminescence property [96]. ARC-Lum probes are ARC-type inhibitors of PK that produces micro-second scale photoluminescence upon binding to the PK in response to a near-UV radiation (300–370 nm). The fixation of the sulfur or selenium-containing heterocyclic ring of the ARC-Lum probe to the ATP pocket of PK, results in protein-induced long-lifetime luminescence signal when the complex is excited with the flash of UV radiation, whereas the free probe produces very weak or no such signal. Sulfur and selenium are heavy atoms that support intersystem crossing of electrons in the excited state leading to the triplet activated state that may emit light as

phosphorescence. In contrast to the free (unbound) state of the probe, the hydrophobic and shielded environment in the ATP-binding pocket of the PK protects the exited triplet state from quenching by oxygen and restricts molecular movements, leading to increased phosphorescence and PK-binding responsiveness of ARC-Lum probes.

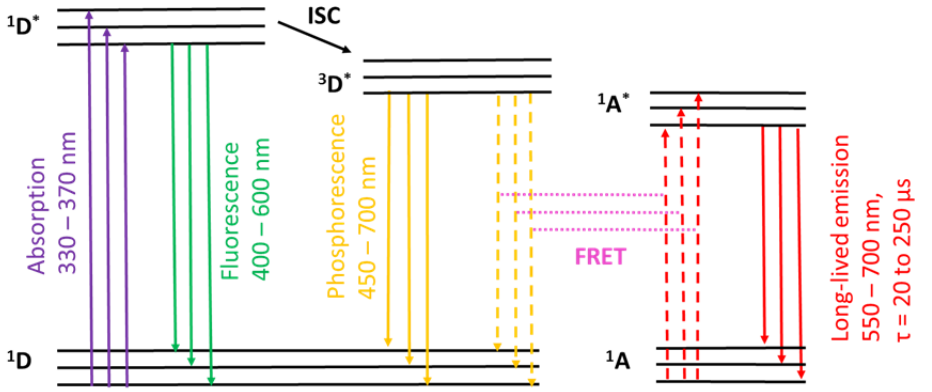


Figure 2. Mechanism of FRET in ARC-Lum probes illustrated by a Jablonski diagram adopted from [37, 39]. The donor luminophore of the ARC-Lum probe (sulfur or selenium-comprising aromatic fragment), absorbs light at 330–370 nm and goes to the singlet excited state $^1D^*$ from the ground state ^1D and emits part of its energy as fluorescence. Then the molecule undergoes intersystem crossing (ISC) to the triplet excited state $^3D^*$ and emits energy as phosphorescence or transfers energy to the singlet excited state of the acceptor fluorophore $^1A^*$ by FRET. Then the time-delayed emission from $^1A^*$ state of donor fluorophore occurs.

Two types of ARC-Lum probes were disclosed. If an ARC-Lum probes lacks a fluorescent dye, it is designated as an ARC-Lum(–) probe. ARC-Lum(–) probes, when associate with PKs possess a weak phosphorescence signal with microsecond-long decay time in the wavelength range from 450 to 650 nm. If an ARC-Lum probe additionally incorporates a fluorescent dye that is covalently attached to the peptide moiety of its preceding ARC-Lum(–) counterpart, the probes is designated as an ARC-Lum(Fluo) probe. The conjugation of fluorescent dye to ARC-Lum(–) probe, if the absorption spectrum partly overlaps with the phosphorescence emission spectrum of the complex PK/ARC-Lum(–), leads to immense sensitization (up to a 1000-fold) of the luminescence signal. This effect is the result of intramolecular FRET (Figure 2) from the excited triplet state $^3D^*$ of sulfur- or selenium-comprising heteroaromatic system (phosphorescence donor, D) to the conjugated fluorescent dye (acceptor fluorophore, A) leading to singlet excitation state of the acceptor $^1A^*$ and slow emission of light from acceptor fluorophore. Thus the emission spectrum of the tan-

dem dye coincides with that of the fluorescent acceptor dye [97], but the decay of the signal is slow as it follows prohibited energy transfer from $^3D^*$ of donor to the singlet state of the acceptor. The acceptor fluorescent dye can also be conjugated to the interacting PK (*e.g.,* PromoFluor-647-labeled PKAc) [96] provided that the acceptor fluorophores are located close enough to the donor phosphor of the ARC-Lum(–) probe to enable the intermolecular FRET to occur.

Some features of ARC-Lum probes are

- ARC-Lum probe comprises a heavy atom such as sulfur or selenium which supports the intersystem crossing serves as the phosphor or the phosphorescent donor
- The near-UV excitation of the luminophore of ARC-Lum probe leads to a fluorescence emission in both unbound (free) form as well as in bound form with PK that ranges from 400 nm to 600 nm
- An ARC-Lum probe emits phosphorescence at 450 to 700 nm only in its bound form with PK upon exciting with near-UV radiation and the unbound (free) form of the probe does not produce any phosphorescence
- The time-delayed emission spectrum of ARC-Lum(Fluo) probes coincides with the fluorescence emission spectrum of the conjugated dye
- ARC-Lum(Fluo) probes possesses shorter luminescence lifetimes than their ARC-Lum(–) counterparts. The intramolecular FRET efficiency depends on choice of the dye. The dye with high molar absorption coefficient and good spectral overlap leads to efficient FRET
- The time-gated luminescence (TGL) signal intensity of PK/ARC-Lum probe complex is irrespective of the affinity of the probe to PK
- As ARC-Lum(Fluo) probes are conjugated with a fluorescent dye, technically they are ARC-Fluo probes which can be also used in FA-based assays

Since the discovery the ARC-Lum probes, their suitability for characterization of inhibitors of PK in biochemical assays [97] as well as cell lysate and live cell-based assays [96], analysis of biomarkers in blood plasma samples [98], mapping and monitoring PK activity in living cells by time-gated luminescence microscopy [99] has been demonstrated.

5. The genetic approach: Labeling protein kinases with a fluorescent protein tag

Manipulations involving FPs are done at the genetic level using gene engineering techniques, in contrast to other types of fluorophores where fluorophores are labeled to the protein of interest (POI) by an *in vitro* chemical coupling step [39]. The gene that encodes FP is ligated to either 5'-end (N-terminus of POI) or 3'-end (C-terminus of POI) of the gene that encodes POI without any inter-

vening stop codons in an expression plasmid. Then the recombinant plasmid is introduced to the expression host by transformation (in case of a bacterial expression system) or transfection (in case of a mammalian expression system). The resulting translated gene product is a chimera of fusion protein that is covalently linking the POI and FP. As techniques are available for purifying FP-tagged proteins [100, 101], the fusion proteins can be studied not only in complex systems like cells and cell lysates, but also in homogeneous biochemical systems.

Though monomeric FPs are preferred for fusions with POIs, the resulting oligomeric nature of the obtained fusion proteins is rather unpredictable and depends on the POI itself and the level of expression of the fusion protein. The tagging of the POI with a FP that is considered to be 'silent', may still lead to disruption of the normal function and localization of the POI. The positioning of the FP tag is another important aspect in producing the fusion protein chimera as the positioning of the FP tag is known to affect the localization of the target protein [102].

5.1. Fluorescent proteins

Fluorescent proteins (FPs) are class of homologues proteins having the ability to form a visible wavelength fluorophore from their own amino acid residues [39]. The discovery of green fluorescent protein (GFP) form the jelly fish *Aequorea victoria*, gained much attention by the scientific community after it has been shown that GFP can be expressed in *Escherichia coli* and *Caenorhabditis elegans* with its intact fluorescence properties [103]. The ancestor of many widely used FPs is originated from the jelly fish *Aequorea victoria* [104]. Interestingly, red shifted FPs such as DsRed were derived from non-bioluminescent reef corals of Anthozoa class [105]. Since then a quest for FPs from different natural sources has been explored. This resulted in a panel of FPs which covers almost the visible light region of electromagnetic spectrum from near ultra-violet (UV) to far infra-red (IR). The advancement in molecular biology techniques, such as error prone polymerase chain reaction (PCR) and site directed mutagenesis, enable the modification of the genes of native FPs, which in turn resulted in FPs with different spectral properties. For example, modifications in the native avGFP gene, resulted in variants of FPs such as cyan, enhanced green and yellow FPs [106]. Together avGFP and Anthozoan FPs such as DsRed served as the progenitors of numerous engineered variants of FPs that are have been used in various applications. The pioneering works of Osamu Shimomura, Martin Chalfie and Roger Y. Tsien, for the *discovery and development of the green fluorescent protein, GFP,* were marked by the 2008 Nobel prize award in chemistry [107]. FPs expressed with the POI by genetic engineering techniques enabled the scientific community to visualize the synthesis, localization and function of a protein in cells in real time using fluorescence microscopy.

The FPs (derived from avGFP and DsRed like FPs) are usually proteins of 220 to 240 amino acids with a molecular weight of approximately 27 kDa [108]. The FPs are folded as an 11-standraded antiparallel β-sheet structure that is often referred as a β-barrel. The chromophore in avGFP is formed by a tripeptide S65-Y66-G67 [109] which is a part of a helix, located in the geometric center of the β-barrel structure of GFP. The tripeptide undergoes a cyclization – oxidation – dehydration reaction to form the chromophore, p-hydroxybenzylideneimidazolidinone. The enhanced GFP (EGFP) contains two mutations, S65T [110] and F64L [111], the former was to improve the spectral characteristic of the chromophore and the latter was to improve the folding at warmer temperature [106]. EGFP possesses excitation/emission maxima at 489 nm/509 nm with an ε value of 55,000 $M^{-1}cm^{-1}$ and a QY of 0.6 [112]. In case of DsRed, the chromophore is formed by a similar tripeptide Q65-Y66-G67 [113]. The chromophore formation of DsRed and FPs derived from it, undergoes an additional oxidation that forms an acylimine (C=N–C=O). This reaction is followed by an isomerization step that extended the π-conjugated electron system of the chromophore which is responsible for red-shifted absorbance and emission [113–115].

In live cell imaging, as a golden rule, red shifted FPs are better for biological studies [116]. Many of the RFPs are derived from DsRed, which is an obligate tetramer [117]. In fact, all FPs exhibit some degree of oligomerizations from weak head-to-tail dimerization (avGFP) to tetramerization (Anthazoan FPs). avGFP and FPs derived from it, are less prone to dimerization, hence the overexpression of these proteins in bacterial or mammalian expression systems do not cause any aggregation or misfolding (avGFP is generally monomeric, but tends to form dimer with a K_d value of 110 μM [118]). But still RFPs with different oligomeric nature were derived from DsRed, thanks to protein engineering techniques [119], such as DsRed express [120], DsRed-monomer [115, 121], E2-crimson [122], mRFP1 [123]. The mFruit series [124] that includes numerous variants such as mOrange, tdTomato, mCherry and mPlum were also derived from DsRed. Today, color diversity of monomeric FPs covers almost the whole visible spectrum [112], from violet (*e.g.,* Sirius [125]) to far-red (*e.g.,* mKate [126]). The wider choice of monomeric FPs with various spectral properties, enabled to perform multi-parameter imaging of biological processes in living systems. However despite the availability of palette of red shifted FPs, none of them have achieved same level of widespread application compared to EGFP [127].

5.2. The red fluorescent protein, TagRFP

TagRFP [128] was chosen as the fluorophore for genetically tagging the PKs in the present study. TagRFP is a bright, pH stable, fast maturing, orange FP with desirable photophysical properties, generated from the wild-type RFP from sea anemone *Entacmaea quadricolor*. TagRFP is a 26.7 kDa (237 amino acids)

monomeric protein, however, partial dimerization of the protein at higher micromolar concentrations has been shown recently [129]. TagRFP has the pKa value of 3.8. TagRFP possess the QY of 0.48, ε value of 100,000 $M^{-1}cm^{-1}$ and a fluorescence lifetime of 2.3 ns with excitation/emission maxima at 555 nm/584 nm. Some photophysical properties of TagRFP is very similar to the widely used chemical dye 5-carboxytetramethylrhodamine (TAMRA), hence the detection of TagRFP is possible with filter sets of many fluorescence devices. TagRFP is about three times brighter fluorophore than another widely used RFP, mCherry [124], which makes it the one of the brightest commercially available monomeric RFPs.

The chromophore formation of TagRFP is very similar to that of DsRed. The fluorophore of TagRFP originates from the tripeptide M67-Y68-G69 that forms 5-[(4-hydroxyphenyl)-methylene]-imidazolone chromophore [130]. The DsRed-like chromophore in TagRFP is converted into the GFP-like chromophore under acidic or alkaline conditions. This property has been observed for DsRed [113] itself and in purple-blue chromoprotein from the anemone coral *Goniopora tenuidens* [131].

Nevertheless, TagRFP has been widely used in many FRET-based sensors [132]. For example, the genetically encoded TagGFP–TagRFP FRET pair is one of the most efficient green/red FRET pairs available [133]. The high extinction coefficient of TagRFP makes it a preferable FRET acceptor for green FPs and green dyes, such as fluorescein isothiocyanate (FITC) and HiLyte488. The emission spectrum of TagRFP overlaps with the absorption spectra of number of red dyes, such as Alexa Fluor 647 and Cy5, which makes TagRFP an efficient donor fluorophore for various red dye-labeled acceptors. The higher quantum yield of TagRFP is beneficial for acceptor based ratiometric FRET studies. The FRET between TagRFP fusions and Lumi4Tb-labeled partners in a TR measurement of FRET also shown previously [41, 134]. Thus TagRFP has a great potential as a fluorophore for both steady-state and TR FRET applications.

5.3. Tagging of protein kinases with fluorescent proteins

The tagging of PKA with FPs was started along with the development of FPs themselves [106]. The applicability of FRET detection for PKA activity measurement was demonstrated in two conceptual papers. Firstly, a sensor was designed for PKA in which the PKAc and PKAr subunits were each labeled with a different fluorescent dye, fluorescein and rhodamine, respectively capable for FRET in the holoenzyme complex [48]. Secondly, a cAMP sensor was engineered by fusing a blue-emitting FP to the PKAr subunit and a green-emitting FP to the PKAc subunit [135]. Thus, when the concentration of cAMP is low, majority of the two fluorophores are in close proximity and generate FRET, whereas increasing concentration of cAMP leads to progressive reduction of FRET as the two fluorophores diffuse farther apart. An improved cAMP

sensor of cyan fluorescent protein (CYP)-fused PKAr and yellow fluorescent protein (YFP)-fused PKAc pair was developed later [136].

The generation of FP-tagged PKAc turned out to be very challenging as it required a lot of optimization of the structures of fused proteins [106]. A lot of research was carried out for optimization of expression procedures for GFP-fused PKAc [106, 137, 138]. The construction of an intermolecular FRET-based fluorescent sensor, incorporating an interacting protein tagged with a FP, is normally a trial and error process, given because of the difficulty of reliable prediction how the chromophores fused to the sensor will orient themselves in space relative to each other [139].

PKs that are tagged with N- or C-terminal FP exhibit different behavior, concerning their localization and activity. The N- and C-terminally GFP-fused PKAc reveals different localization [140], where the former is evenly distributed in cytoplasm and is excluded from the nuclear compartment, and the latter is found in the form of fluorescent aggregates in cytoplasm. Nuclear pores are thought to allow passage of globular proteins with molecular weight of less than 45–60 kDa [141], thus the localization of fusion proteins is mainly dependent on the size, function, and oligomeric nature of POI. The study of interaction between the GFP-tagged PKAc and *Renilla reniformis* luciferase-tagged PKAr that was analyzed by intermolecular bioluminescence energy transfer (BRET) between the luminophores, shows that the BRET efficiency depends on the positioning of the FP tag on PKAc [138]. The positioning of not only FP tag, but also other purification tags that are attached to PKs, have huge impact on the FRET efficiency between the tagged PK and interacting partner. Tyrosine kinases possessing N- and C-terminal positioning of biotin tag, His6 tag and GST tag have been shown to have different FRET efficiency in biochemical assays involving the FRET between europium cryptate-labeled anti-tag antibodies and Alexa Fluor 647-labeled inhibitors [142]. These facts point to the importance of positioning of FP tag to PK not only for its effect on FRET efficiency but also for activity of the PK.

6. Combining small-molecule ARC-probes and fluorescent protein-tagged protein kinases

In this study genetically modified PKs and ARC-probes are combined together to develop FRET-based sensors for various applications. One hand, small-molecule ARC-probes incorporating various aromatic systems and possessing diverse luminescent properties are developed. On the other hand, PKs with genetically fused FP tags are developed. The combination of these two entities led to the construction of FRET sensors and their application for screening of PK inhibitors is described.

FRET between the fluorophores of FP-fused PKs and ARC-Photo probes has been previously demonstrated for biochemical assays and live cell experiments. The application of ARC-Photo probes for monitoring the activity of PKA using

the FRET between C-terminally YFP-fused PKAc and TAMRA-labeled ARC-Photo probe (ARC-TAMRA) has been described using C9H6 [135, 139] cell line that is stably expressing PKAr-CFP and PKAc-YFP [143]. The FRET between the fluorophores of PKAc-YFP and cell permeable ARC-TAMRA probe was detected in live cells and the effect of cAMP and H89 on FRET was analyzed using a fluorescence microscope. Thereafter an *in vitro* assay was developed in HTS format which employed a fluorescent plate reader. However, in this study, cell-to-cell signal heterogeneity resulted in lower signal-to-noise ratio in the plate reader assay compared to the detection of FRET in individual cells. In another study, the FRET between PKGα fused with a pH-insensitive YFP, citrine [144] and ARC-TAMRA was demonstrated [90]. The purified fusion protein, cygnet 2.1 [145] which consists of a mutant PKGα with N-terminal ECFP and C-terminal citrine, was used as the FP-fused PK. The FRET between citrine of cygnet 2.1 and an ARC-TAMRA probe was established and the system was used to characterize the affinity of inhibitors using a spectrofluorometer. However the difficulties in purifying FP-tagged PKs and the instability of purified fusion proteins restrict the application of such a FRET sensor for HTS of inhibitors of PK.

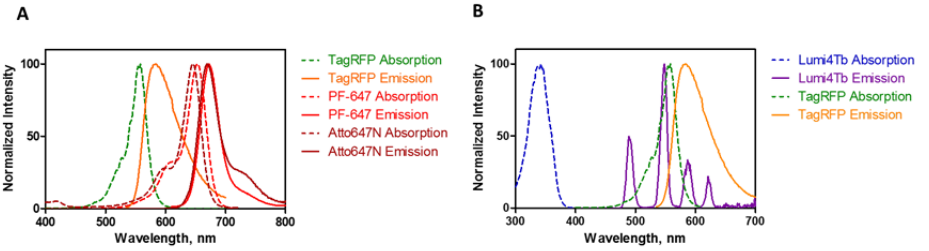


Figure 3. Spectral overlap between the excitation and emission spectra of TagRFP with fluorescence dyes (A) and Lumi4Tb terbium cryptate (B).

In order to achieve the selectivity towards the target PK in a complex biological solution like cell lysate, the PK was tagged with a FP, TagRFP. Then FRET between ARC-Photo probes and TagRFP-fused PKs was established. Shifting the emission wavelengths of FRET pairs towards the red region of the spectrum reduces of the background cellular autofluorescence and generally increases the FRET efficiency due to increased R_0 values [146] . Hence it was decided to combine TagRFP-fused PKs and ARC-Photo probes that are labeled with red dyes for construction of a steady-state FRET sensor. For TR version of FRET, ARC-Photo labeled with terbium cryptate, Lumi4Tb and TagRFP-fused PKs were used.

The emission spectrum of TagRFP overlaps with the absorption spectra of various red dyes such as PromoFluor-647 (PF647), Alexa Fluor647, Cy5, and

Atto647N (Figure 3A). Hence TagRFP fused to PK can be used as a donor fluorophore for ARC-Photo probes with these red dyes. Thus, the FRET assays involving steady-state FI measurement between the donor and acceptor fluorophore were developed. On the other hand, the absorption spectrum of TagRFP overlaps with the emission peaks of Lumi4Tb (Figure 3B). Hence, the TR measurement of FRET is also possible with TagRFP. Also the absorption spectrum of TagRFP overlaps with the phosphorescence emission spectra of some ARC-Lum(–) probes. Hence the triplet–singlet FRET between the luminophores of sulfur/selenium-comprising aromatic fragment of ARC-Lum(–) probe and TagRFP-fused PKs is possible provided that the direct phosphorescence emission of PK/ARC-Lum(–) complex can be discriminated from the long-lived emission of TagRFP. Thus TagRFP plays a multiple role in various FRET sensors based on their application in combination with ARC-probes.

The complementation between the genetically fused PKs and small-molecule ARC-probes helped to improve our understanding of PKs. This supported the development of structurally diverse ARC-probes and ARC-inhibitors which can be used for biochemical and biomedical research.

AIMS OF THE STUDY

The general aim of the study was the development of photoluminescence-based assay systems for PK analysis by combining genetic engineering techniques for expression of PKs fused with FPs in mammalian cells and chemical methods for synthesis of photoluminescent ARC-probes for the characterization of PKs and their inhibitors in cell lysates. The specific aims of the study are:

- Cloning of recombinant plasmids that encode PKs, PKAc, PIM and CK2α together with a genetically encoded FP tag, TagRFP, and optimization of their expression in mammalian cells
- Development of small-molecule ARC-Lum probes for PKs PKAc, PIM and CK2α and establishing their optical and biochemical properties
- Development of photoluminescence-based assays using TagRFP-fused PKs and ARC-probes in cell lysates
- Establishing multiple FRET mechanisms between luminophores of ARC-Lum probes and the TagRFP-fused PKs
- Analyzing the possibilities of ARC-Lum probes for determination of PIM kinases in cell lysates.

METHODS

1. Cloning of protein kinase genes

The genes that encode human PKAc, CK2α, PIM1 and PIM2 were obtained with custom designed restriction sites from Genewinz Inc. in pUCKan57 vector as shown in Table 1. These plasmids were used as the donor plasmid for the recombinant plasmids. The pTagRFP vectors contain Kozak [147] consensus translation initiation site (gccacc) at 5' of the TagRFP gene, and the same Kozak consensus sequence was used at 5' of the GOI.

Table 1. Custom-designed donor plasmids

Plasmid	Design	Encoding PK
pUCKan-PKAc	NheI-Kozak-*PKAc*-SacI	PKAc (40 kDa)
pUCKan-CK2α	NheI-SalI-Kozak-*CK2α*-HindIII-Stop-BamHI	CK2α (45 kDa)
pUCKan-PIM1	NheI-Kozak-*PIM1*-HindIII	PIM1 (35.7 kDa isoform)
pUCKan-PIM2	NheI-BglII-Kozak-*PIM2*-HindIII-Stop-PstI	PIM2 (34.2 kDa isoform)

Using a pair of restriction enzymes, each one from 5' and 3' primes, the GOI was excised from the donor plasmid and cloned into various vector plasmids. Thus the custom designed gene can be used a source plasmids for various re-combinant plasmids. In case of C-terminal fusion of TagRFP, the gene was excised without a stop codon, whereas for N-terminal fusion of TagRFP the GOI was excised with a stop codon. Recombinant plasmids produced in this study are listed in Table 2 and Table 3.

Table 2. Recombinant plasmids produced for mammalian expression systems

Donor plasmid	Vector plasmid	Restriction sites		Recombinant plasmid	Encoded protein
		5'	3'		
pUCKan-PKAc	pTagRFP-N	NheI	SacI	phCa-TagRFP	C-terminally TagRFP-fused PKAc *via* 17 amino acid linker (68.6 kDa)
phCa-TagRFP	pDsRed1-N1	NheI	HindIII	phCa-DsRed1	C-terminally DsRed1-fused PKAc *via* 21 amino acid linker (68.9 kDa)
pUCKan-PIM1	pTagRFP-N	NheI	HindIII	pPIM1-TagRFP	C-terminally TagRFP-fused PIM1 *via* 17 amino acid linker (64.5 kDa)
pUCKan-PIM2	pTagRFP-N	NheI	HindIII	pPIM2-TagRFP	C-terminally TagRFP-fused PIM2 *via* 17 amino acid linker (63 kDa)
pUCKan-PIM2	pTagRFP-C	BglII	PstI	pTagRFP-PIM2	N-terminally TagRFP-fused PIM2 *via* 7 amino acid linker (61.8 kDa)
pUCKan-PIM2	pTagRFP-C	NheI	PstI	pCMV-PIM2	Full-length native PIM2 (34.4 kDa)
pUCKan-CK2α	pTagRFP-N	NheI	HindIII	pCK2α-TagRFP	C-terminally TagRFP-fused CK2α *via* 19 amino acid linker (74 kDa)
pUCKan-CK2α	pTagRFP-C	SalI	BamHI	pTagRFP-CK2α	N-terminally TagRFP-fused CK2α *via* 17 amino acid linker (73.8 kDa)
pUCKan-CK2α	pcDNA3.1(+)	NheI	BamHI	pcDNA-CK2α	Full-length native CK2α (45.4 kDa)

Table 3. Recombinant plasmids produced for expressing the PKs with a His6 tag or as a fusion with TagRFP or with both tags in *E.coli*

Donor plasmid	Vector plasmid	Restriction sites		Recombinant plasmid	Encoded protein
		5'	3'		
pTagRFP-N	pET28a	SacI	NotI	pET-HisTagRFP	TagRFP tagged with N terminal His6 tag (33 kDa)
phCa-TagRFP	pET28a	NheI	HindIII	pET-HisPKAcHis	PKAc tagged with N and C terminal His6 tag (45 kDa)
phCa-TagRFP	pET28a	NheI	NotI	pET-HisPKAcRFP	PKAc tagged with N terminal His6 tag and C terminal TagRFP (72.3 kDa)
pUCKan-PIM1	pET28a	NheI	HindIII	pET-HisPIM1His	PIM1 tagged with N and C terminal His6 tag (39.8 kDa)
pPIM1-TagRFP	pET28a	NheI	NotI	pET-HisPIM1RFP	PIM1 tagged with N terminal His6 tag and C terminal TagRFP (67 kDa)
pUCKan-PIM2	pET28a	NheI	HindIII	pET-HisPIM2His	PIM2 tagged with N and C terminal His6 tag (38.6 kDa)
pPIM2-TagRFP	pET28a	NheI	NotI	pET-HisPIM2RFP	PIM2 tagged with N terminal His6 tag and C terminal TagRFP (66 kDa)
pCMV-PIM2	pET28a	NheI	SalI	pET-HisPIM2	PIM2 tagged with N terminal His6 tag (37.3 kDa)
pUCKan-CK2α	pET28a	NheI	HindIII	pET-HisCK2αHis	CK2α tagged with N and C terminal His6 tag (49.5 kDa)
pUCKan-CK2α	pET28a	SalI	BamHI	pET-HisCK2α	CK2α tagged with N terminal His6 tag (48.2 kDa)
pUCKan-CK2α	pET-HisTagRFP	NheI	HindIII	pET-HisCK2αRFP	CK2α tagged with N terminal His6 tag and C terminal TagRFP (76.8 kDa)

2. Transfection

Transfection is a method by which a foreign DNA, such as a plasmid DNA is introduced into mammalian cells. The cells that are transfected are called as trasnfectants [148]. The introduction of a plasmid DNA into bacterial cells is called transformation. Transfection is used to express a foreign protein or over-express a protein of interest (POI) in the mammalian cells. Transfection can be classified stable or transient transfection. In case of stable transfection, the foreign DNA is integrated into the genome of host. An antibiotic is used to select cells that contain the foreign DNA and they are established as stable cell line that expresses the POI. In case of transient expression, the foreign DNA is not integrated into the genomic DNA of host, but the genes are expressed for a specific period of time (24–96 h).

For transient expression of a plasmid DNA, various transfection reagents and techniques are available. Transfection reagents includes lipid-based reagents (*e.g.,* Lipofectamine™), cationic polymers (*e.g.,* TurboFect™, diethylaminoethyl-Dextran), calcium phosphate, *etc*. Electroporation, microinjection and laserfection are mechanical means of delivering the foreign DNA into cells. Transfection methods using viral vectors are also in practice. However, it is not guaranteed that a particular reagent or method will work for all cell lines.

Various factors can affect the transfection including, the passage number of the cells, confluency of the cells during transfection, presence of serum in the growth medium and the quality of the plasmid DNA. The successful expression of PKs and fusions of PKs in mammalian cells may need a lot of optimization. In addition to common problems with the transfection reagents, other criteria may influence the PK expression, for example overexpression of the PK may be toxic to cells. The efficiency of transfection and expression of a particular PK greatly vary among various cell lines (*e.g.,* cancerous or non-cancerous).

3. Determination of dissociation constant of inhibitors

The binding efficiency between an enzyme and a ligand is characterized by the value of the dissociation constant K_D. Lower K_D value represents higher affinity between a ligand and enzyme. The K_D value is expressed as follows,

$$K_D = \frac{[E][L]}{[EL]} \qquad \text{(Equation 5)}$$

[E], [L], [EL] are equilibrium concertation of an enzyme, a ligand and their complex, respectively.

If the equilibrium dissociation constant between a PK and an ARC-Photo probe is determined from a direct binding assay with FA or TGL read-out [89, 97], the constant it is denoted as K_D.

The affinity of a non-labeled inhibitor is determined from FA or TGL-based displacement assay, using an ARC-Photo probe. In this case, the IC_{50} value

which represents the concertation of the inhibitor at which 50% of the ligand (ARC-Photo probe) is displaced from its complex with a PK is measured. Form the IC_{50} value, the equilibrium dissociation constant (K_d) of a non-labeled inhibitor is obtained using the following Cheng-Prusoff equation [149],

$$K_d = \frac{IC_{50}}{1+\frac{[L]}{K_D}} \qquad \text{(Equation 6)}$$

K_d is the dissociation constant of the complex of PK and the inhibitor, K_D is the dissociation constant of the complex of PK and the ARC-Photo probe and [L] is the concentration of the ARC-Photo probe used in the assay.

RESULTS AND DISCUSSION

1. Production of protein kinases with genetically encoded tags (Paper II, IV and unpublished data)

Gene engineering techniques were applied for producing genetically tagged PKs. This study is focused on PKs of the PIM family, PKAc, and CK2α. Recombinant plasmids that encode fusion proteins of PKs with TagRFP and His6 tag were produced for their expression in both mammalian expression system and *E.coli*.

1.1. Expression of TagRFP-fused PKs

The genes of PKs were cloned into pTagRFP vectors to produce recombinant plasmids encoding fusion proteins of PKs with TagRFP in mammalian expression system. Plasmids were introduced into mammalian cells by transfection and the recombinant proteins were transiently expressed. The PKAc fusions were expressed in HeLa cells whereas fusion proteins of PIM kinases and CK2α were expressed in NIH/3T3 cells.

The TagRFP-fused PKs were expressed in two forms, with N- and C-terminal positioning of TagRFP. The FI of lysates of cells overexpressing TagRFP-fused PKs was compared with FI of recombinant TagRFP or an ARC-TAMRA probe, ARC-669. In general the expression level of N-terminally TagRFP-fused PKs was higher than that of C-terminally TagRFP-fused PKs (*e.g.,* PKAc and CK2α fusions). However in case of PIM2 fusions, both forms of the fusion protein were expressed very well.

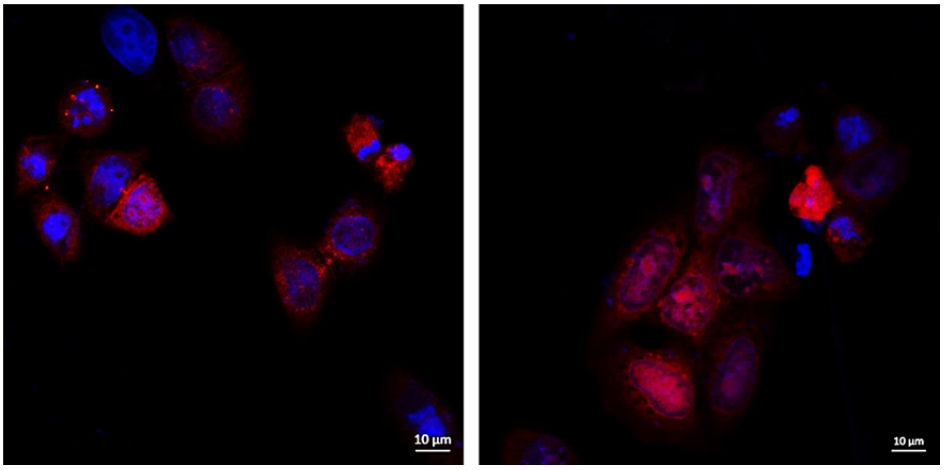


Figure 4. Fixed cell imaging of PKAc-TagRFP (left) and TagRFP-PKAc (right) in HeLa cells using a confocal microscope. 63X oil objective. Pseudo colored images with DAPI channel (blue) and TagRFP channel (red).

Lysates of cells overexpressing TagRFP-fused PKs were subjected to Western blot analysis using an anti-TagRFP antibody (Paper II, Figure S2 and Paper IV, Figure S2). MWs of all fusion proteins found from Western blots were slightly smaller than their predicted molecular weights. Also, two bands were observed for the TagRFP-fused PKs at the predicted MW, with the MW difference of approximately 8 kDa. This has been reported to result from the cleavage of acylimine band in the DsRed-like chromophore structure of TagRFP (M67-Y68-G69) during the heat denaturation [113, 131, 133]. Lysates were analyzed using anti-PK specific antibody to compare the level of endogenous expression of a PK in host cells (*e.g.,* PKAc and CK2α) and the over expression of TagRFP-fused PK (Paper II, Figure S3 and Paper IV, Figure S3). HeLa and NIH/3T3 cells expressed native PKAc and CK2α at significantly high level. But there was almost no endogenous expression of PIM2 in NIH/3T3 cells. It is interesting to note that attempts to overexpress PIM2 fusions in HeLa and PC3 cells failed, while it was successfully expressed in NIH/3T3 cells, a non-cancerous cell line, which is lacking endogenous PIM2 expression [27].

PKs that are genetically tagged with N- and C-terminal FP, in present case TagRFP, should be treated as two different proteins. Both the expression level as well as the intracellular localization of FP-tagged PKs was dependent on the positioning of the FP in the fusion with the PK [140]. The localization of TagRFP-fused PKs was studied using fluorescence microscopy techniques (Figure 4 and Paper IV, Figure S1). TagRFP-fused PKs are distributed all over the cell and localized both in cytoplasm as well as in nucleus. In general, no difference in the localization of N- and C-terminally TagRFP-fused PKs were observed for PKAc, CK2α and PIM2 in the current study.

1.2. Expression of fusion proteins of PKs in bacteria

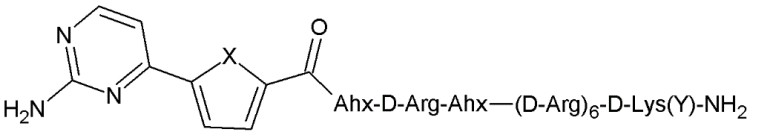
The genes of PKs were cloned into pET expression vector in order to produce PKs with His6 tag at N-terminus, C-terminus or at both termini of PKs in *E.coli*. Recombinant plasmids encoding PKs with N terminal His6 tag and C-terminal TagRFP fusion were also produced. This allowed the production and purification of His6-tagged PKs in large quantities. The tagged proteins were further purified using nickel affinity columns.

The recombinant purified His6 tagged PKs were used in biochemical assays together with ARC-Photo probes and antibodies. FA-based multiplexed assays utilizing ARC-Photo probes, anti-His6 antibody and His6 tagged PKs are under development in the research group. These assays could be converted to steady-state or TR FRET format using ARC-Photo probes (with a fluorescent label or a photoluminescent label with long decay time, such as terbium and europium cryptate) and fluorescently labeled anti-His6 antibodies or terbium or europium cryptate-labeled anti-His antibodies. Thus the His6-tagged PKs are potential tools for the development of multiplexing assays. These assays are expected to

be also useful for the characterization of protein-protein interactions, *e.g.,* between antibodies and a PKs.

2. Development of ARC-Lum probes for protein kinases (Paper I, III, IV and unpublished data)

Since the discovery of protein binding-induced long-lifetime luminescence property of sulfur- and selenium-comprising aromatic systems, various heteroaromatic rings have been utilized for the construction of ARC-Lum probes. The very first ARC-Lum probes were comprised of two different monocyclic aromatic rings connected by a rotatable single bond. These bicyclic compounds comprised of either a thiophene or selenophene ring as the phosphorescence donor [96]. For example, the ARC-Lum probe ARC-1063, comprised of an amino pyrimidine ring and a thiophene ring connected by a C–C bond (Figure 5). The protein binding-induced long-lifetime photoluminescence is stronger in case of selenophene-comprising compounds compared to thiophene-comprising compounds. For example, ARC-1139 is a selenophene-comprising counterpart of thiophene-comprising probe ARC-1063. The TGL intensity of ARC-1139/PK complex is about 5–20-fold higher than that of ARC-1063/PK complex. Hence the selenium-comprising aromatic fragments are more promising ring systems for the development of ARC-Lum probes.



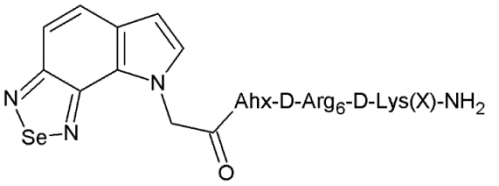
Compound	X	Y
ARC-668	S	H
ARC-669	S	TAMRA
ARC-1182	S	PF647
ARC-1063	S	Alexa647
ARC-1138	Se	H
ARC-1139	Se	PF647

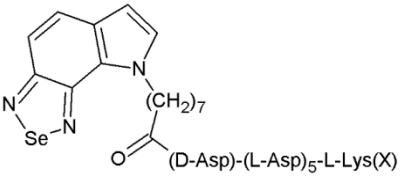
Figure 5. Structure of ARC-Lum probes targeting basophilic kinases.

The selenophene-containing ARC-Lum probe ARC-1139 gave the protein-induced long-lifetime signal with many basophilic PKs, including PIM kinases.

Though, ARC-1139 is 250–800-fold more affine towards PKAc, than to PIM kinases, ARC-1139 was still successfully used for the characterization of PIM kinase inhibitors using a TGL-based displacement assays [91]. Hence the construction of ARC-Lum probes possessing high selectivity and affinity towards PIM kinase was challenging.

2.1. ARC-Lum probes comprising benzoselenadiazole moiety





Compound	X
ARC-3131	H
ARC-3132	PF647

Compound	X
ARC-3138	H
ARC-3141	PF647

Figure 6. Structures of benzoselenadiazole-comprising ARC-Lum probes.

A new aromatic scaffold based on benzoselenadiazole fragment was introduced for ARC-Lum probes. Benzoselenadiazole is a fused tricyclic ring system, which is more rigid compared to the previous ARC-Lum probes, thus the positioning of this fragment to the PK is more predictable. Also the synthesis of ARC-Lum probes with benzoselenadiazole scaffold is relatively easier, hence various substitutions are possible.

To develop PK-specific ARC-Lum probes that incorporate benzoselenadiazole fragment, bisubstrate inhibitor approach was applied. The benzoselenadiazole moiety was connected to an oligo-arginine or an oligo-aspartate peptide *via* a flexible linker (Figure 6). The oligo-arginine conjugated benzoselenadiazole (ARC-3131) was an ARC-Lum(–) probe targeting basophilic PKs, including PKAc and PIM kinases, and an oligo-aspartate conjugated benzoselenadiazole compound ARC-3138 was an ARC-Lum(–) probe for an acidophilic PK CK2α.

The phosphorescence emission maximum of these compounds is at 600–650 nm, hence red fluorescent dye PF647 was chosen as the phosphorescence acceptor. The luminescence lifetime (τ) of ARC-3131 and ARC-3138 in complex with PKAc and CK2α was 55 μs and 26 μs, respectively. ARC-3131 and ARC-3138 were labeled with PF647 to yield ARC-Lum(Fluo) probes ARC-3132 and ARC-3141, respectively. The labeling of ARC-Lum(–) probes with the acceptor dye PF647 led to the amplification of TGL signal by 250-fold and 400-fold for PKAc complex and CK2α complex, respectively (Paper I, Figure

S5). The luminescence lifetime of ARC-3132 and ARC-3141 in complex with PKAc and CK2α was 45 μs and 20 μs, respectively (Paper I, Figure S3 and S4). This corresponds to approximately 20% of intramolecular FRET efficiency. The K_D values of ARC-3132 and ARC-3141 towards PKAc and CK2α were 46 nM and 25 nM, respectively (Paper I, Figure 1). The probe, ARC-3141 was the first ARC-Lum(Fluo) probe constructed for the acidophilic PK CK2α. The developed probe was used for the determination of CK2α concentration in cell lysates.

However the probe ARC-3132 failed to produce long-lifetime luminescence signal with PIM kinases. On the other hand, the binding of ARC-3132 to PIM kinases resulted in increase in FA value (Paper I, Figure 1). The binding curve matched to K_D values of 6 nM, 54 nM and 35 nM for PIM1, PIM2 and PIM3, respectively. The TGL signal of ARC-3132/PKAc complex was 300-fold stronger than that of ARC-3132/PIM2 complex. This result was quite unexpected as several previously reported ARC-Lum probes were capable of producing luminescence with many basophilic kinases, including kinases of the PIM family. The reduced TGL signal of complexes of ARC-3132 with PIM kinases could be resulting from the unique structure of the hinge region of PIM kinases, all possessing an amino acid residue proline at the position 123 in the hinge region, where other PKs have a valine residue. Proline residue lacks the hydrogen bond donor properties for binding ligands [150].This interaction seems to be important for stabilization of the triplet electron state of the donor phosphor. CK2α that is possessing a more common Val116 residue in this position is able to give the required hydrogen bond. Because of its unique hinge region [20], construction of specific probes for PIM kinases was challenging.

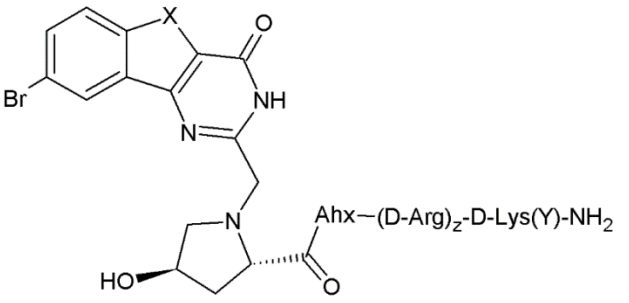
2.2. ARC-Lum probes comprising benzoselenopyrimidine moiety

Bisubstrate inhibitors comprising of benzothienopyrimidine and an oligo-arginine peptide were developed for PIM kinases [91]. Conjugation of benzothienopyrimidine and hexa-arginine *via* a flexible linker yielded ARC-3104 (Figure 7) possessing 4000–8000-fold higher affinity towards PIM kinases compared to PKAc. The compound was labeled with PF647 to yield the ARC-Lum(Fluo) probe ARC-3117 which was used in assays with PIM kinases using FA readout. This probe had subnanomolar affinity towards PIM kinases and high selectivity (500-fold) over PKAc. However the probe produced very weak or no luminescence signal with PIM kinases and therefore was not developed further as an ARC-Lum probe.

We hypothesized that replacing sulfur atom in benzothienopyrimidine with heavier selenium atom could improve the phosphorescence properties of the donor phosphor (Paper III, Figure 1). Thus ARC-Lum probes containing benzoselenopyrimidine moiety were developed. The sulfur atom of ARC-3104 was replaced with selenium, to yield ARC-3157 (Figure 7). As expected the ARC-Lum(–) probe ARC-3157 produced 100-fold stronger long-lifetime lumines-

cence signal if bound to PIM kinases as well as PKAc than its sulfur counterpart. These probes possessed 500-fold higher affinity towards PIM kinases compared to PKAc.

ARC-3157 produced 3–7-fold stronger TGL signal with PIM2 kinase compared to other kinases of PIM family (Paper III, Figure 4). But this probe possessed a stronger TGL signal intensity with PKAc also. The luminescence lifetime of ARC-3157 in complex with PIM2 and PKAc was 84 ± 10 μs and 132 ± 22 μs respectively. However, these probes had more than 100-fold selectivity for PIM kinases compared to PKAc. For example, ARC-3158 (ARC-3157 conjugated with PF647 dye), possessed a K_D value of 0.7 ± 0.3 nM and 187 ± 58 nM towards PIM2 and PKAc, respectively. So at low PK concentration ARC-3158 generates a PIM-2-selective signal, but at higher (micromolar) concentration PKAc (and probably some other PKs of the AGC group) give rise to comparable TGL signal intensity (Paper III, Figure 7).



Compound	X	Y	Z
ARC-3104	S	H	6
ARC-3117	S	PF647	6
ARC-3157	Se	H	6
ARC-3158	Se	PF647	6
ARC-3159	Se	PF555	6
ARC-3160	Se	H	9
ARC-3161	Se	PF647	9
ARC-1451	Se	TAMRA	6
ARC-1452	Se	FITC	6
ARC-1454	Se	Atto647N	6

Figure 7. Structures of ARC-Lum probes targeting PIM kinases

2.2.1. Effect of dye on intramolecular FRET efficiency

The phosphorescence emission of ARC-3157/PIM2 complex was ranging from 450 to 600 nm and possessed a maximum at 500 nm (Figure 8). Thus fluorescent dyes possessing strong absorption in green region of visible spectrum, such as PromoFluor-555 (PF555) and TAMRA were found to be suitable acceptors for the phosphor of ARC-3157.

The phosphorescence emission from the phosphor of ARC-3157 complex is amplified by the fluorescent acceptor dye that is incorporated into ARC-Lum(Fluo) probes derived from ARC-3157 in complexes with PKs. The magnitude of signal amplification depends on the spectral overlap between the absorption spectrum of the dye and phosphorescence emission spectrum of phosphor as well as other photophysical properties of the acceptor fluorophore, such as molar absorption coefficient and QY (Table 4).

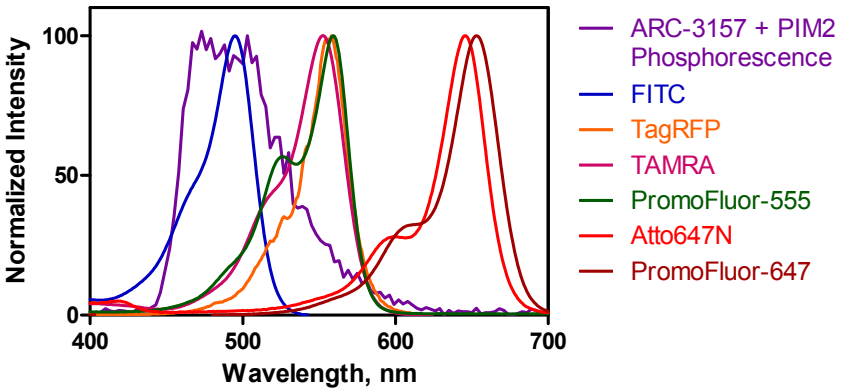


Figure 8. Overlap of the phosphorescence emission spectrum of ARC-3157/PIM2 complex and absorption spectra of various fluorescent dyes and TagRFP.

Table 4. Optical and biochemical characters of some ARC-Lum(Fluo) probes derived from ARC-3157.

Compound		**Properties of dye**		**Tau (μs)**	**FRET efficiency**	**K_D (nM)**	**Q value**
Code	Conjugated dye	ε value ($M^{-1}cm^{-1}$)	QY				
ARC-3158	PF647	250,000	0.4	67 ± 10	20%	0.7 ± 0.3	1.5
ARC-3159	PF555	150,000	0.135	51 ± 4	40%	1.8 ± 0.5	1.3
ARC-1451	TAMRA	80,000	0.3	47 ± 5	45%	2 ± 1.5	1.8
ARC-1452	FITC	75,000	0.7	45 ± 10	46%	5 ± 2	1.5
ARC-1454	Atto467N	150,000	0.65	74 ± 5	12%	3 ± 1.5	1.6

The FITC-labeled compound ARC-1452 resulted in 46% of intramolecular FRET efficiency though the ε value of the dye is smaller. This efficient energy transfer resulted from the good overlap of the absorption spectrum of FITC with the phosphorescence emission spectrum of ARC-3157/PIM2 complex (Figure 8). The energy transfer to PF647 (E = 20%) labeled compound ARC-3158 is higher compared to Atto647N (E = 12%) labeled compound ARC-1454. This indicates that energy from selenium-comprising heteroaromatic system (phosphorescence donor) is more efficiently transferred to the acceptor fluorophore PF647 than to Atto647N Although the absorption spectra of both fluorophores have similar overlap with the phosphorescence emission spectrum of ARC-3157, the more efficient energy transfer may reflect the higher molar absorption coefficient of PF647 compared to that of Atto647N.

2.2.2. Effect of dye on the binding of ARC-probe with PK

The ARC-Lum(–) probe ARC-3157 has K_d value of 0.4 nM towards PIM2 kinase. Various ARC-Lum(Fluo) probes were derived from ARC-3157 by labeling of the latter with different dyes. The attached dye altered the binding of the probe to PIM2 which resulted in different K_D values. The TAMRA-labeled probe, ARC-1451 revealed high Q value that points to the nonspecific binding of the probe to surface of microplate. The binding of Atto647N-labeled compound ARC-1454 with BSA was detected in an assay with FA read-out. Titration of the probe with BSA revealed the K_D value of 1.7 μM (Paper IV, Figure S6). Atto647 dye possesses nonspecific binding to different proteins and sticks to glass and plastic surfaces because of its high lipophilicity and low polarity [151]. Therefore, Atto647N was not used for labeling of probes for cell lysate-based assays. PF647-labeled probe ARC-3158 is well suitable for measurements based on the FA read-out (Paper IV, Figure S11) also. Although both probes are usable in TGL-based measurements, PF647 is still a better dye for the construction of photoluminescence probes for measurements in biological solutions and cells as it shows less intense nonspecific interactions with lysate components and plastic and glass surfaces.

2.3. Cellular delivery of ARCs comprising an oligo-aspartate peptide using a transfection reagent

Peptides that are rich in cationic amino acids have been shown to be cell permeable and used widely as vehicles to deliver cell plasma membrane-impermeable compounds [152, 153] as well as nucleic acids [154] into cells. The ARC-probes that are conjugated with oligo-arginines are effectively taken up cells and their application for studying PKs in live cells has been demonstrated [143]. Because of their effective cellular uptake, live cell *in vitro* assays employing oligo-arginine comprising ARC-probes have been developed [96].

However, peptides and peptide conjugates comprising several aspartate residues are poorly penetrating the cell plasma membrane due to their negative charge. Thus the development of *in vitro* assays for monitoring the activity of PK CK2 using oligo-aspartate comprising ARC-probes is challenging. In that case, masking of the negative charge of the oligo-aspartate moiety is necessary, which is shown to be accomplished by prodrug approach or ester-loading technology [84].

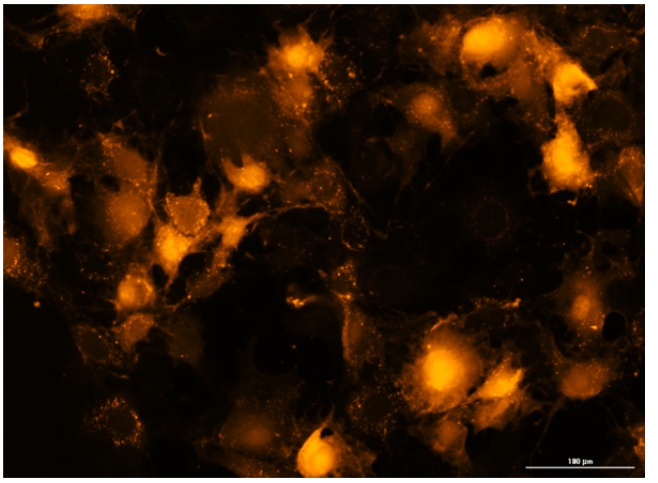


Figure 9. An ARC-Photo probe incorporating an oligo-aspartate and TAMRA dye (ARC-1505) delivered into NIH/3T3 cells using TurboFect transfection reagent. Live cell imaging, 20X magnification, TAMRA channel (pseudo colored image).

During the course of optimization of transfection of plasmid DNA using TurboFect reagent, it was found that transfection reagents can be used to deliver conjugates of oligo-aspartates into living cells. TurboFect is a cationic polymer, which forms complex with the negatively charged plasmid DNA and delivers the complex into the cells. We hypothesized that ARC compounds with negatively charged oligo-aspartates (inhibitors of CK2α) would also be delivered into cells using TurboFect reagent.

The cell-impermeable ARC-Photo probe, ARC-1505, labeled with TAMRA dye complexed with TurboFect and was successfully delivered into cells. ARC-1505 was widely distributed all over cells. ARC-1505 was even transported into the nucleus of the cell. This is an easy way to deliver the ARC-probes with oligo-aspartates into cells. The activity of delivered compounds can be further monitored by their retarding effect on the phosphorylation of CK2α substrates (*e.g.,* CDC37) using phospho-specific antibodies in Western blot analysis [84].

This makes cellular delivery of oligo-aspartate comprising ARC-probes possible without any chemical modification. The technique can be used for

developing *in vitro* live cell-based assays for CK2 holoenzyme and CK2 inhibitors using TagRFP-fused CK2α and ARC-Photo probes.

3. Determination of concentration of PIM kinases in cell lysate using ARC-Lum probes (Paper III and IV)

PIM kinases are emerging as biomarkers for the early diagnosis of various types of cancer [23]. Therefore techniques are in need for determination of concentration of PIM2 in clinical samples. Previously, ARC-Lum probes have been successfully used for measurement of the concentration of extracellular PKAc (ECPKA) in blood plasma samples [limit of quantification of the method was11 pM (220 attomoles) of PKAc in the sample] of healthy persons and cancer patients [98].

Now the ARC-Lum probe ARC-3158 was successfully used for the determination of PIM2 kinase in cell lysates. ARC-3158 possesses K_D value of 0.7 nM and has over 400-fold selectivity towards PIM2 over PKAc. However the produced TGL signal is comparable for PK/ARC-Lum complexes with both kinases (Paper III, Figure 7). Thus at low PK concentration ARC-3158 generates a PIM-2-selective signal, but at higher concentration PKAc give rise to comparable signal intensity.

The PIM-selective ARC-Lum(Fluo) probe ARC-3158 was used to determine the concentration level of PIM2 in cell lysates (Paper IV, Figure 9). For this purpose, lysate of NIH/3T3 cells transfected with pCMV-PIM2 was produced. To avoid the non-specific signal that could arise from the off-target binding of the probe to other PKs in the cell lysate (*e.g.,* PKAc), a PIM-selective inhibitor SGI-1776 was used as a PIM-selective inhibitor. In case of NIH/3T3 cells the endogenous expression of PKAc is high (Paper IV, Figure S3), therefore ARC-3158 could bind to PKAc as well [155].

The titration of ARC-3158 with the lysate of non-transfected NIH/3T3 cells produced a weak TGL signal, which resulted from the binding of ARC-3158 to other PKs, including PKAc. The addition of SGI-1776 to the samples did not reduce the TGL signal, this result indicated that there was no native PIM present. Western blot analysis also revealed the absence of PIM2 protein in the lysate of native NIH/3T3 cells. The results demonstrate the applicability of ARC-Lum(Fluo) probes for the determination of the concentration of active PIM2 kinase in cell lysates . These techniques can be adapted for determining the level of PIM2 in lysates of cancerous cells or tissues.

4. FRET between TagRFP-fused protein kinases and ARC-probes (Paper II, IV and unpublished data)

In general, three types of intermolecular FRET are possible between luminophores of ARC-Lum(Fluo) probes and TagRFP-fused PKs.

- The steady-state FRET (singlet–singlet FRET) between the fluorophore of TagRFP-fused PKs and fluorophores of ARC-Lum(Fluo) probes that incorporate fluorescent dyes emitting green light (*e.g.,* Hilyte488, FITC) or red light (*e.g.,* PF647, Atto647N)
- The TR FRET (triplet–singlet FRET) between the luminophores of sulfur/selenium-containing ARC-Lum(–) probes and fluorophore of TagRFP-fused PKs
- The TR FRET between luminophores of TagRFP-fused PKs and ARC-Photo probes that are labeled with Lumi4Tb lanthanide cryptate

The three types of intermolecular FRET-based sensors systems described here are specific between the luminophore of the ARC-probe and fluorophore of TagRFP-fused PK, thus the interaction of only genetically tagged PK and ARC-probe is monitored. In case of an ARC-Lum(Fluo) probe, the intramolecular triplet–singlet FRET between the sulfur/selenium fragment and the fluorescent dye taking place, when the ARC-Lum(Fluo) probe is complexed with a PK. In this case, the PK is need not to be genetically tagged, thus the ARC-Lum(Fluo) probes have the selectivity issue, like other fluorescent probes of PKs.

4.1. The intermolecular FRET between the ARC-Lum(–) probe and TagRFP-fused PKs

After excitation of the selenium-comprising heteroaromatic fragment of ARC-Lum(–) probes in complex with a PK with a flash of near-UV radiation at 330 nm, part of energy is emitted from donor as fluorescence from the singlet excited state $^{1}D^{*}$ and part of energy as phosphorescence from the triplet excited state $^{3}D^{*}$with microsecond-scale decay time (Figure 2). In case of ARC-Lum(Fluo) probes energy from $^{3}D^{*}$ is also transferred non-radiatively by the FRET mechanism to the fluorophore attached to its peptidic part [if the absorption spectrum of the fluorophore at least partly overlaps with the phosphorescence emission spectrum of the donor]. In complex with a PK, FRET is also possible from ARC-Lum(–) probes to labels covalently attached to the protein if conditions for FRET between the luminophores are fulfilled, for example, the distance between the luminophores does not exceed two Förster distances of the luminophore pair. However, intramolecular FRET is usually more efficient as the distance between the phosphorescence donor and the acceptor fluorescent dye is shorter.

The phosphorescence spectrum of ARC-3157/PK (PK = PIM2 or PKAc) complex with emission in the range of 450 to 600 nm (Figure 10) well overlaps with the absorption spectrum of the acceptor fluorescent protein TagRFP (Figure 8). The phosphorescence emission of ARC-3157/PK complex is very weak at the emission maximum of TagRFP (590 nm), thus the direct phosphorescence emission from the complex at 520 nm could be discriminated from the TagRFP-

mediated signal at 590 nm resulting from the energy transfer from the $^3D^*$ state of ARC-3157/PK complex to singlet state of acceptor fluorophore TagRFP and emission from $^1A^*$ of TagRFP. In case of bicyclic ARC-Lum(–) probes, such as ARC-1138, the phosphorescence emission ranges from 400 nm to 750 nm with a wide peak at 550 nm to 650 nm (Figure 10) [97]. Therefore, the discrimination of the time-delayed phosphorescence emission from $^3D^*$ of ARC-1138/PK complex and retarded fluorescence emission from $^1A^*$ of ARC-1138/TagRFP-PK was not possible.

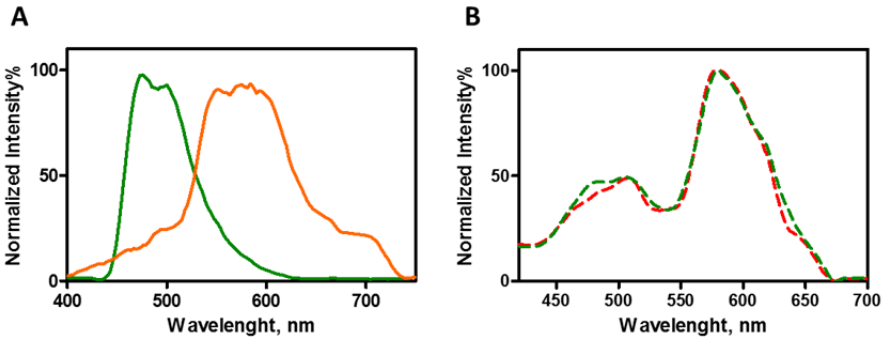


Figure 10. The intermolecular FRET between ARC-Lum(–) probe and TagRFP-fused PKs. (A) The phosphorescence emission spectrum of ARC-Lum(–) probes, ARC-3157 (green) and ARC-1138 (orange) in complex with PK (PKAc or PIM2). (B) The time-delayed emission spectra of complex of ARC-3157 with TagRFP-PKAc (green) or TagRFP-PIM2 (red) in lysates of HeLa and NIH/3T3 cells, respectively.

TGL emission spectra (excitation at 340 nm, delay 50 μs) of ARC-3157 in complexes with TagRFP-PKAc and TagRFP-PIM2 in diluted lysates were taken with Cytation 5 (Biotek) multi-mode reader (Figure 10). For the complex ARC-3157/PIM2 or ARC-3157/PKAc, the delayed emission spectrum of the lysate possessed a maximum at 500 nm [155] and no emission was detectable at 590 nm. The delayed emission spectrum of the complex of ARC-3157 with TagRFP-PIM2 and TagRFP-PKAc had two maxima, with peaks at 500 nm and 590 nm. The latter peak is the result of intermolecular energy transfer from $^3D^*$ of ARC-3157 to the fluorophore of TagRFP-fused PKs and fluorescence emission from TagRFP, microsecond-scale decay being caused by slow prohibited energy transfer from $^3D^*$ to the singlet state of the acceptor. The obtained result is the first indication that a phosphorescent ARC-Lum(–) probe can be used together with a PK that is genetically tagged with a FP (for example, TagRFP-fused PIM2) for construction of biosensor systems possessing photoluminescence with microsecond-scale decay time for analysis of PKs and their inhibitors.

Next, the direct triplet–singlet energy transfer between the luminophores of selenium-comprising fragment of ARC-3157 and TagRFP-fused PIM2 was compared in a displacement assay (Figure 11). The disruption of the complex ARC-3157/TagRFP-fused PIM2 by recombinant PIM2 led to the association of ARC-3157 with recombinant PIM2, thus the phosphorescence emission of ARC-3157 from $^3D^*$ state at 520 nm is recovered. As a result of the interruption of intermolecular FRET between the phosphorescent donor and acceptor TagRFP-fused PIM2, the microsecond-timescale emission from $^1A^*$ of TagRFP at 590 nm is decreased.

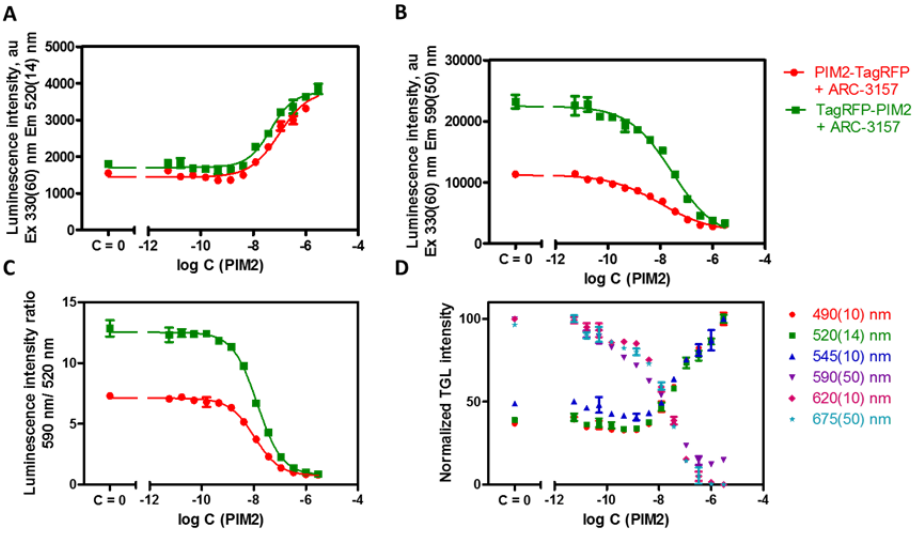


Figure 11. The intermolecular FRET between the selenium-comprising fragment of an ARC-Lum(–) probe and TagRFP-fused PIM2 (Paper IV, Figure 4). TGL intensity measured for disruption of complexes of ARC-3157 with PIM2-TagRFP and TagRFP-PIM2 by recombinant PIM2. The luminescence intensity measured at (A) 520 nm and (B) 590 nm (B) and their ratio (C). (D) The normalized TGL intensity of ARC-3157/ PIM2-TagRFP complex measured at all the wavelengths.

The displacement was monitored in wavelength range from 485 nm to 700 nm using different TRF modules of the plate reader (Figure 11D). The TGL signal measured at the phosphorescence emission range at 480 to 570 nm [where the energy from $^3D^*$ of selenium fragment is predominant], led to a binding curve, due to the association of ARC-3157 with recombinant PIM2. But the TGL signal measured at the fluorescence emission range of TagRFP at 580 nm to 700 nm [where emission from $^1A^*$ of TagRFP takes place] led to displacement curve because of the disruption of the FRET between selenium-comprising phosphor and TagRFP.

4.1.1. Comparison of intermolecular and intramolecular FRET

ARC-1451 is an ARC-Lum(Fluo) probe that derived from ARC-3157 through attachment of the fluorescent dye TAMRA. TAMRA possess photoluminescent properties (QY = 0.3, ε = 80,000 $M^{-1}cm^{-1}$ and similar spectral overlap with the phosphorescence emission of ARC-3157/PIM2 complex) that are similar to these of fluorescent protein TagRFP, thus this probe can be directly compared to ARC-3157/TagRFP-fused PIM2 complex.

The luminescence lifetime of ARC-1451 in complex with PIM2 was 47 ± 5 μs, this points to the intramolecular FRET efficiency of 40%. The luminescence lifetime ARC-3157/TagRFP-fused PIM2 complex was about 77 μs, which corresponds to less than 10% intermolecular FRET efficiency. Also the TGL signal intensity of ARC-1451//PIM2 complex was 10–20-fold stronger than ARC-3157/TagRFP-fused PIM2 complex. This shows that the intramolecular FRET is more efficient as the distance between the phosphorescence donor and the acceptor fluorescent dye, in this case TAMRA, is shorter.

Smaller FRET efficiency of the system with intermolecular positioning of luminophores, ARC-3157/TagRFP-fused PIM2, points to more distinct positioning of the luminophores in the complex. The improvement of FRET efficiency could be achieved *via* increase of the Förster distance for the interacting luminophores, for example by increasing the phosphorescence QY of the donor phosphor, selenium-comprising aromatic system. Taking into consideration the feasibility of genetic introduction of fusion proteins into cells and efficient cellular uptake of ARC-Lum probes, the intermolecular version of the assay (as exemplified through ARC-3157/TagRFP-fused PIM2 here) would have great potential for studies in living cells an additional dimension is given to the measurements *via* the signal with microsecond-scale lifetime, mediated by the FP. This signal is easily separable from background fluorescence of cells and fluorescent reagents, including FPs that do not participate in the interaction [96].

4.2. The effect of positioning of the FP in TagRFP-fused PIM2 on the FRET efficiency

The effect of positioning of TagRFP on FRET efficiency was studied using TagRFP-fused PIM2 and PIM specific ARC-Lum probes. Both N- and C-terminally TagRFP-fused PIM2 proteins were expressed in NIH/3T3 cells and their concertation in cell lysate was sufficient for photoluminescence measurements. In case of PIM2-TagRFP protein, a 19 amino acid-long linker was used to fuse the proteins. In the other version, TagRFP-PIM2, the proteins were connected by a short, 7 amino acid-long linker.

The triplet–singlet FRET between the luminophores of selenium-comprising aromatic system and TagRFP was discussed in the previous section. The TGL signal intensity of ARC-3157/TagRFP-PIM2 complex was approximately 2.5-fold stronger than ARC-3157/PIM2-TagRFP complex (Figure 11B). Thus the intermolecular FRET from the phosphorescent donor, selenium-comprising

aromatic system to TagRFP is more efficient in case of TagRFP-PIM2 compared to PIM2-TagRFP. This shows that in case of TagRFP-PIM2, the acceptor luminophore TagRFP is positioned more closely to the phosphorescence donor in the active site of PIM2 than in case of the other fusion protein.

Then singlet–singlet FRET between the fluorophores of TagRFP-fused PIM2 and ARC-PF647 probes was compared. Two ARC-PF647 probes that structurally differed in length of the peptide part were chosen to compare the FRET efficiency from the donor fluorophore TagRFP. ARC-3158 comprised a hexa-arginine and ARC-3161 a nona-arginine peptide moiety. These spacers positioned the acceptor fluorescent dye PF647 at different distances from TagRFP. The affinity of the probes towards PIM2 was quite similar (K_D values 0.5–0.7 nM) [155]. FRET efficiency was calculated by measuring the change in the FI of donor fluorophores of TagRFP-fused PIM2, in the presence of PF647-labeled ARC-3158 or ARC-3161.

The FRET efficiency between the TagRFP donor fluorophore of PIM2-TagRFP and PF647 acceptor fluorescent dye of ARC-3158 in ARC-3158/PIM2-TagRFP complex was about 40% whereas that for the same fluorophores in ARC-3158/TagRFP-PIM2 complex was about 30% (Paper IV, Figure 5). Thus the positioning of fluorophores is closer to each other in ARC-3158/PIM2-TagRFP complex. The application of a structurally similar luminescent probe ARC-3161, possessing more arginine residues in the peptide moiety in FRET studies resulted in well comparable results. The FRET efficiency was about 30% in ARC-3161/PIM2-TagRFP complex and about 20% in ARC-3161/TagRFP-PIM2 complex.

It is remarkable that singlet–singlet FRET efficiency between fluorophores of TagRFP-fused PIM2 with PF647 of ARC-3158 is more efficient in case of the complex ARC-3158/PIM2-TagRFP than in case of ARC-3158/PIM2-TagRFP, that is contrasting the result of the triplet–singlet FRET efficiency between the luminophores of ARC-3157 and TagRFP-fused PIM2. This result points to different positioning of two interacting luminophores. In case of steady-state FRET assay the acceptor fluorophore of the ARC-Lum(Fluo) probe PF647 is out of the active site of PIM2 and more close to the fluorophore of TagRFP in complex ARC-3158/PIM2-TagRFP than in ARC-3158/PIM2-TagRFP. Differently, the phosphor of ARC-3157 that is sitting in the ATP pocket of PIM2 is more close to TagRFP in complex ARC-3157/TagRFP-PIM2 than in the complex ARC-3157/ PIM2-TagRFP.

4.3. FRET-based assays in cell lysates using ARC-Photo probes and TagRFP-fused PKs

The binding/displacement process between the complexes of ARC-Lum(Fluo)/TagRFP-fused PK was monitored in either steady-state mode, TR mode or in both the modes in parallel. The application of the binding/ displacement assay with steady-state and/or TR FRET read-out enabled the determination of displacement IC_{50} values for inhibitors in cell lysates.

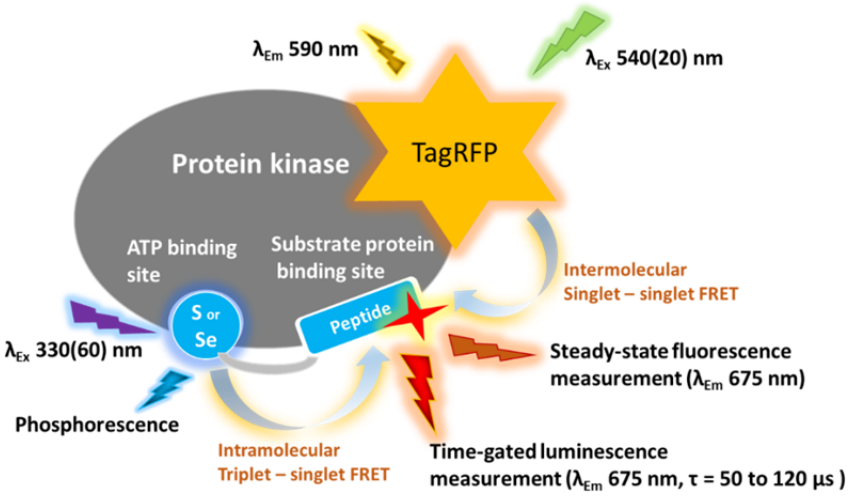


Figure 12. Schematic representation of interaction of an ARC-Lum(Fluo) probe labeled with PF647 dye and and TagRFP-fused PK. Steady-state and TR modes of FRET measurement between the donor and acceptor luminophores are represented in the figure.

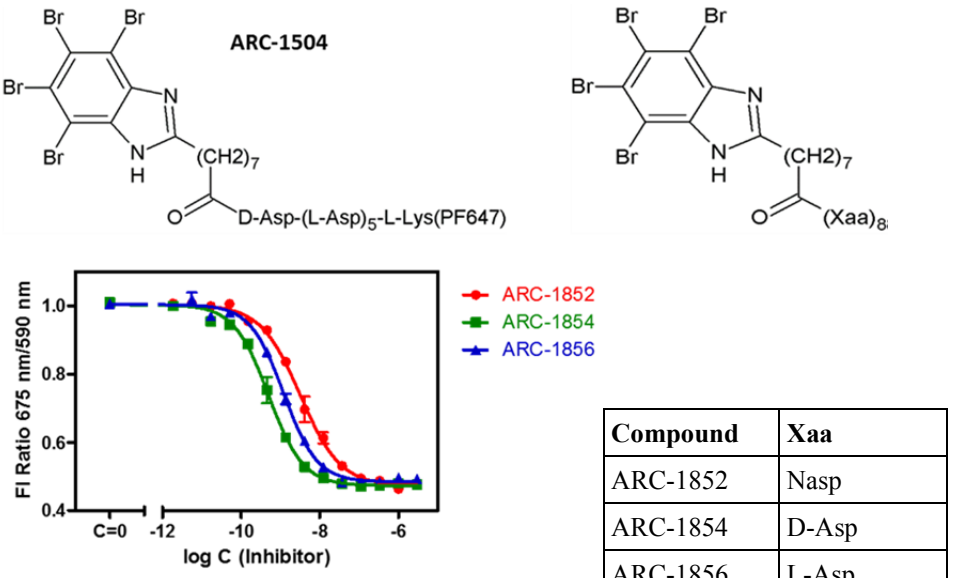
Occurrence of FRET between PF647-labeled ARC-Photo probes, ARC-1182, ARC-1504, and ARC-3158, from one side and TagRFP-PKAc, TagRFP-CK2α and TagRFP-fused PIM2, from other side, was established. ARC-1182 and ARC-3158 are ARC-Lum(Fluo) probes that can be used for both the steady-state and TR measurements, whereas ARC-1504 is a fluorescent probe that was used only in steady-state measurement of FRET.

4.3.1. Steady-state measurement of FRET between ARC-PF647 probes and TagRFP-fused PKs

The disruption of ARC-PF647/TagRFP-fused PKs complex by ATP-competitive and substrate protein-competitive inhibitors was monitored by change in the emission intensities in the acceptor and donor channels (675 and 590 nm, respectively). The ratio of acceptor and donor emission intensities ($FI_{675\ nm}/FI_{590\ nm}$) was plotted against the concentration of inhibitor to obtain displacement curves. Better signal to background ratio was obtained upon converting the emission intensities of donor and acceptor channels into their ratio. At least 50% decrease in the value of FI ratio was obtained as the result of full displacement of the probe from the complex with TagRFP-fused PKs (Paper II, Figure 5 and Paper IV, Figure 6).

The IC_{50} values determined in the FRET-based displacement assays were well correlated with the K_d values or K_i values of inhibitors measured using purified recombinant PKs in biochemical binding or inhibition assays (Paper II, Figure S12). The steady-state FRET system enabled the determination of IC_{50}

values of inhibitors of PKAc, CK2α and PIM2 kinases in a cell environment-like conditions.



Compound	Xaa
ARC-1852	Nasp
ARC-1854	D-Asp
ARC-1856	L-Asp

Figure 13. A displacement assay involving steady-state FRET measurement between fluorophores of TagRFP-fused CK2α and ARC-1504.

In the example presented in Figure 13, structurally closely related inhibitors of CK2α were distinguished by FRET-based assay using the ARC-Photo probe, ARC-1504 and TagRFP-CK2α fusion protein in lysate of NIH/3T3 cells. The complex of ARC-1504/TagRFP-CK2α was disrupted by three inhibitors of CK2α, which were constructed by conjugating 4,5,6,7-tetrabromo-1H-benzimidazole (TBBi) moiety and carboxylate-comprising oligomers. The peptidic part of ARC-1852, ARC-1854 and ARC-1856 comprised 8 peptoidic residues with carboxylic acid groups, 8 L-aspartic acid residues or 8 D-aspartic acid residues, respectively. The compounds possessed K_d values of less than 0.1 nM. In spite of very high affinity of these inhibitors, the proposed system well differentiated the IC_{50} values of the compounds.

The established steady-state FRET assay can be easily adapted for other PKs fused with TagRFP or other RFPs, such as mCherry and DsRed. To test the applicability of the proposed system to other RFPs, DsRed2 was used in the role of a FRET partner. Differently from monomeric TagRFP, DsRed2 is a tetrameric protein [117] possessing a high QY of 0.55 [120] and somewhat lower brightness than that of TagRFP [128]. FRET between fluorophores of N-terminally DsRed2-fused CK2α (DsRed2-CK2α) and the fluorescent probe

ARC-1504 was established (Figure II Figure 7) and used for the determination of affinity of CK2α inhibitors. This shows that the method described in this study can be adapted to a wide variety of RFPs, regardless of their oligomeric nature. Still, the tetrameric nature of DsRed2 leads to the polymeric occurrence of the fused kinases that can lead to more complicated interpretation of optical effects and different binding kinetics of inhibitors.

4.3.2. TR measurement of FRET between ARC-Lum(Fluo) probes and TagRFP-fused PKs

In case of a displacement assays employing ARC-Lum(Fluo) probes, the binding/displacement can be monitored in parallel to steady-state FRET measurements also with TR measurement. A problem in TGL-based assays employing ARC-Lum(Fluo) probes could be non-specific binding of the probes to other, non-target PKs present in cell lysate. Although the concentration of TagRFP-fused PK in the lysate is high, the concentrations of other PKs and that of the endogenous untagged PK are unknown. But in case of the steady-state FRET measurement the obtained signal is pure in case of excess of the probe compared to the total molar amount of PKs, meaning that FI measured is the result of the interaction of the donor fluorophore (TagRFP) fused to the PK and acceptor fluorophore conjugated to the ARC-Lum(Fluo). But still, the IC_{50} values calculated from FRET-based measurements were very well correlated with IC_{50} values obtained in measurements with TGL read-out (Paper IV, Figure 6). In case of PIM2 fusions, both versions of the fusion protein were successfully used in the displacement assay to determine the IC_{50} values of inhibitors and the calculated K_d values were similar (Paper IV, Figure S9). Lysate containing overexpressed native PIM2 along with ARC-3158 was also used for the determination of IC_{50} values of the inhibitors and the calculated K_d values well correlated with the K_d values measured using recombinant PIM2 in other biochemical assays (Paper IV, Figure S12).

The application of bisubstrate ARC-Lum(Fluo) probes (that are displaced from the PK complex with both ATP-competitive and protein substrate-competitive inhibitors [77]) in binding/displacement assays (Paper IV, Figure 6) led us to the discovery that an oligo(D-arginine) peptide, nona(D-arginine) amide [$(D\text{-}Arg)_9\text{-}NH_2$], binds to PIM kinases with high affinity. The peptide revealed K_d value of 150–200 nM with PKs PIM1 [91] and PIM2. $(D\text{-}Arg)_9\text{-}NH_2$ is very efficiently taken up by cells as an acknowledged arginine-rich transport peptide [156]. Its inhibitory effect towards PKs is not ATP-competitive, therefore its PIM inhibitory potential may be very high in living cells. Taking into account high (1–5 mM) concentration of ATP in cells and very low ATP K_m value (K_m = 4 μM) for PIM2 catalyzed reactions [157], at equal intracellular concentrations $(D\text{-}Arg)_9\text{-}NH_2$ inhibits PIM2 kinase as potently as ATP-competitive inhibitors possessing subnanomolar K_i values. Thus the application of $(D\text{-}Arg)_9\text{-}NH_2$ as a transport peptide at usual 10 μM concentration may cause unexpected

cellular effects *via* inhibition of PIM2 and other PKs of the PIM family. Differently from some other transport peptides (*e.g.,* TAT peptide) whose inhibitory potential towards PKs PKA and PKC has been also shown previously [158], $(D\text{-}Arg)_9\text{-}NH_2$ is proteolytically very stable and may retain its inhibitory effect for long time in cells and bodily fluids. Additionally, $(D\text{-}Arg)_9\text{-}NH_2$ is a potent inhibitor of a proprotein convertase furin (K_i = 1.3 nM) [159]. All these specific effects of $(D\text{-}Arg)_9\text{-}NH_2$ make the application of this peptide as a vehicle for the transport of a biologically active cargoes into cells (*e.g.,*[160]) a rather risky activity and the obtained results should be interpreted with care.

In cell lysate-based displacement assays with the application of ARC-Lum(Fluo) probes, 2 nM concentration of the probe was used together with 0.1 to 0.5 µl of cell lysates. 100 µl of cell lysate was obtained by lysing transfected cells from a confluent well of a 6-well plate. Thus this lysate is sufficient for making a displacement assays in 200 to 1000 wells of a 384-well plate. The assay system enables the application of non-purified PKs for determination of affinity of inhibitors. Cell lysates containing the over-expressed TagRFP-fused PKs were used as the source of PK for the FRET-based assays. The developed assay system has the potential for testing of inhibitors of all PKs, provided that the expression of the PK as a fusion with TagRFP is successful and a red dye-labeled probe is available that possesses sufficient affinity towards the PK. The cell lysate-based assay enables the application of non-purified PKs for determination of affinity of inhibitors. In this assay there is no need for capricious and expensive antibodies labeled with luminophores. A fresh batch of the target PK-fused with TagRFP can be produced cheaply every week. Moreover, genes of different PKs can be easily introduced into plasmids instead of these of PKAc or CK2α thus adapting the assay for other PKs.

4.3.3. Disruption of FRET in TagRFP-fused PK/ARC-Lum(Fluo) complex by recombinant PKs

Different types of energy transfer take place in a FRET sensor that is comprised of a TagRFP-fused PK and an ARC-Lum(Fluo) probe. One is intermolecular steady-state fluorescence FRET (singlet–singlet) between the fluorophore of TagRFP and PF647. Secondly, the intramolecular and intermolecular TR FRET (triplet–singlet) between selenium-comprising phosphor of ARC-Lum probe to fluorophores of TagRFP and PF647, respectively. Thus a disruption of complex of ARC-Lum(Fluo) and TagRFP-fused PK by recombinant PK leads to the changes in different optical parameters.

The complexes ARC-1182/TagRFP-PKAc and ARC-3158/TagRFP-fused PIM2 was disrupted by the addition of recombinant PKAc and PIM2 protein, respectively. The binding process was monitored in FA, FI and TGL measurements.

Re-association of ARC-1182 with un-tagged PKAc led to displacement of TagRFP-PKAc from the complex (Figure 14). FA read-out showed increase in

anisotropy values due to the additional binding of the free probe with recombinant PK. The displacement led to the disruption of singlet–singlet FRET between donor TagRFP and acceptor PF647. The steady-state FRET measurement resulted in up to 50–60% decrease in FI ratio (Paper II, Figure S13). The TGL measurement at the emission maximum of PF647 and TagRFP led to a binding curve due to the association of probe to the recombinant PK, resulting from intramolecular triplet–singlet FRET between the sulfur-containing fragment and PF647 dye.

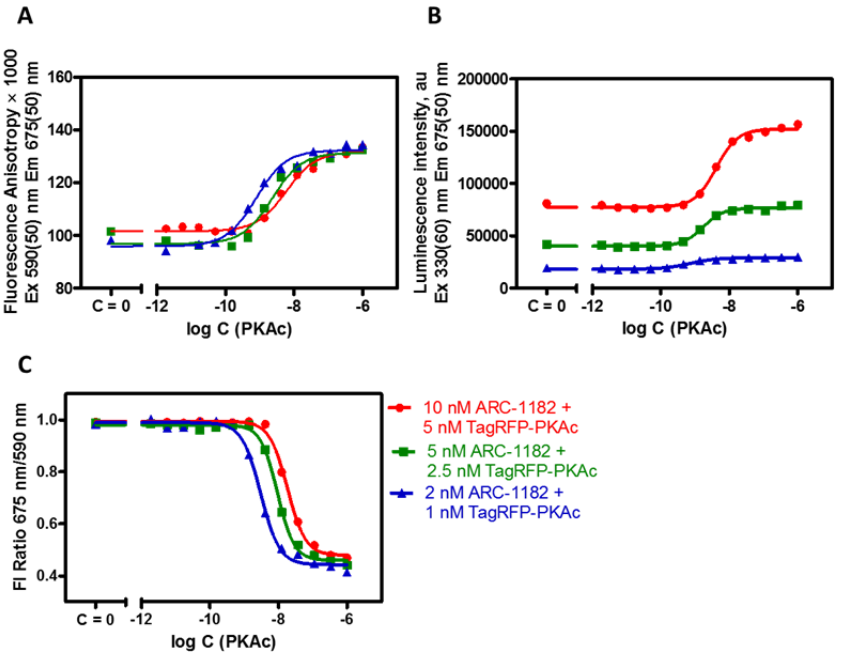


Figure 14. Disruption of complex ARC-1182/ TagRFP-PKAc by recombinant PKAc. (A) Fluorescence anisotropy measurement (B) TGL measurement (C) steady-state FRET measurement.

Interestingly in case of ARC-3158/TagRFP-fused PIM2 complex, TGL measurement taken at the fluorescence emission maximum of TagRFP at 590 nm resulted in a displacement curve (Paper IV, Figure 7). Displacement of ARC-3158/TagRFP-fused PIM2 by PIM2 led to the termination of energy transfer from selenium-containing fragment to PF647. This points to the intermolecular energy transfer taking place from the selenium-comprising heteroaromatic system (phosphorescence donor) to the acceptor fluorophore TagRFP even in the presence of another acceptor, PF647 which is located in closer proximity. The TGL intensity measured at the emission of the acceptor fluorophore PF647 at 675 nm resulted in a binding curve, due to the association of ARC-3158 with

the recombinant PIM2. This led to efficient energy transfer from the selenium-comprising phosphor of ARC-3158 to PF647 dye of ARC-3158. In parallel, the association of the selenium-comprising phosphor and TagRFP-fused PIM2 was disrupted that led to the displacement curve due to decrease in intermolecular FRET from selenium-comprising phosphor to TagRFP. These experiments show that the intramolecular energy transfer from the selenium-comprising phosphor to the fluorescent dye incorporated into ARC-Lum(Fluo) probes is not complete as there is still a partial energy intermolecular energy transfer to the TagRFP fused to PIM2.

This effect could not be observed in case of ARC-1182/TagRFP-PKAc complex. The phosphorescence emission maximum of the preceding ARC-Lum(–) probe, ARC-668 is at 550–650 nm (Figure 10) which overlaps with the fluorescence emission maximum of TagRFP. Hence the triplet–singlet emission of ARC-668/TagRFP-PKAc complex at 590 nm could not be discriminated from the phosphorescence emission of ARC-668/PKAc complex. In case of ARC-3157, the phosphorescence emission maximum is at 500 nm, hence emission at 590 nm, resulting from the triplet–singlet FRET between phosphor of ARC-3157 to TagRFP, could be very well discriminated from the phosphorescence of ARC-3157/PIM2 complex.

4.3.4. TR measurement of FRET between ARC-Lumi4Tb probes and TagRFP-fused PKs

The excitation spectrum of terbium cryptate Lumi4Tb has a maximum in near-UV region of spectrum at 340 nm [161]. The emission spectrum of Lumi4Tb comprises four sharp peaks positioned at 490, 545, 590 and 620 nm [162], with distinct silent regions between the peaks (Figure 3). The emission spectrum of Lumi4Tb at least partially overlaps with absorption spectra of green, orange and red fluorophores, therefore all these dyes can be used as FRET partners (acceptor luminophores) with Lumi4Tb donor luminophore. Not only small organic fluorophores but also FPs such as different forms of GFP, YFP and RFP have been used as FRET partners for terbium chelates [163]. TR FRET assays for protein kinases have been previously reported using terbium and GFP-fused peptide substrates [164]. The application of Lumi4Tb in FRET measurements together with RFPs whose absorption spectra even better overlap with emission spectra of terbium cryptates than those of GFP or YFP has also been described [134]. As the absorption spectrum of TagRFP overlaps with the major emission peaks of terbium, the TR version of FRET assay is possible with TagRFP.

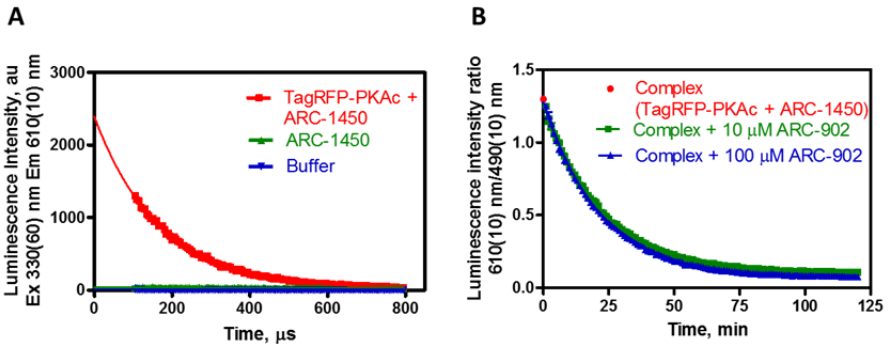


Figure 15. Characterization of complex of ARC-Lumi4Tb and TagRFP-PKAc. (A) Determination of emission half-life of complies of ARC-1450 and TagRFP-PKAc (Paper II, Figure S9). (B) Determination of dissociation half-life of complex of ARC-1450 and TagRFP-PKAc (Paper II, Figure S15).

The emission spectrum of TagRFP is quite wide, possessing intense emission at 570 and 610 nm, these regions well fit into the silent regions of the terbium emission spectrum. Therefore the emission of the acceptor luminophore TagRFP was measured using narrow band-pass filters 570(10) and 610(10) nm. The donor emission was measured at 490 and 545 nm. As Lumi4Tb luminophore possesses very long luminescence lifetime (>2 ms) [161] the luminophore can be used for TR FRET measurements. In this case the donor luminophore is excited with a pulse of electromagnetic radiation (near-UV light, *e.g.,* at 300–370 nm using a xenon flash lamp or at 337 nm with a nitrogen laser) and the signal is measured after delay of 50–200 µs for 100–500 µs (acquisition time or gate time).

The TR FRET-based sensors for PK inhibitors have been developed using TagRFP-fused PKs and Lumi4Tb labeled ARC-Photo probe ARC-1450. ARC-1450 is derived by conjugating ARC-1411 with Lumi4Tb label. The FRET sensor, ARC-1450/TagRFP-PKAc, possessed long emission half-life (110–120 µs) resulting from efficient energy transfer to TagRFP as determined from the decay curves (Figure 15A). As the parent compound ARC-1411 possesses slow dissociation kinetics, the dissociation half-life of ARC-1450 was determined. The dissociation half-life of the ARC-1450/TagRFP-PKAc complex was found to be 15 min (Figure 15B). After the characterization of FRET between these two partners, the FRET system was successfully used for the characterization of inhibitors of PKs (Paper II, Figure 6 and Paper IV, Figure 8). An incubation time of 60–120 min was used to achieve the complete disruption of the FRET complex (Paper II, Figure S16) by the inhibitors of PKAc. The application of the TagRFP-fused PKs and ARC-1450 was demonstrated in displacement assays for the characterization of inhibitors of PKAc and PIM2 kinase. The ratio of luminescence intensity calculated from all emission channels had sufficient measurement window and high signal to background ratio.

SUMMARY

In this study, a combination of chemical and genetic approaches was used for construction of sensor systems for analysis of protein kinases.

Synthetic ARC-Lum probes possessing unique photoluminescence properties were developed for protein kinases PKAc, CK2α and PIM kinases. Bisubstrate inhibitor approach was applied for achieving high affinity and selectivity of ARC-inhibitors towards the target protein kinases. Various heteroaromatic fragments such as benzoselenadiazole and benzoselenopyrimidine were introduced into ARC-compounds for construction of photoluminescent ARC-Lum(Fluo) probes.

To develop FRET-based assays, protein kinases (PKAc, CK2α, and PIM kinases) were overexpressed in mammalian cells as fusions with red fluorescent protein, TagRFP. Assays were developed for measurements in cell lysates by combining TagRFP-fused kinases and ARC-probes that were labeled with complementary luminophores, such as PromoFluor-647 and Lumi4Tb. Photoluminescence assays using both steady-state and time-resolved read-outs were developed. Application of Lumi4Tb-labeled ARC-probes enabled to perform these assays with very high signal-to-noise ratio.

The mechanism of energy transfer between interacting luminophores was extensively studied in this work. The protein-induced long-lifetime luminescence property of ARC-Lum(Fluo) probes is based on intramolecular energy transfer from the excited triplet state of the selenium-comprising heteroaromatic system to the fluorescent dye, leading to long-lived (τ = 50–120 μs) emission in the region of the fluorescence emission spectrum of the dye. Suitability of different dyes for efficient FRET-based ARC-Lum(Fluo) was determined.

Considering the emerging importance of kinases of the PIM family (PIM1, PIM2 and PIM3) as potential drug targets as well as cancer biomarkers, the development of research tools for these kinases has especially great importance. Therefore the applicability of different ARC-Lum probes as FRET partners for PIM2-fused with TagRFP was determined. Fusions of PIM2 with N- or C-terminally positioned fluorescent proteins (TagRFP-PIM2 and PIM2-TagRFP, respectively) were expressed in mammalian cells. This study systematically evaluated the dependence of efficiency of FRET on structure of the probe and architecture of the fusion protein. The study revealed that in case of same interacting partners (the probe and the protein kinase) measurement of FRET efficiency according to different energy transfer mechanisms (including triplet–singlet and singlet–singlet energy transfer) may give complementary information for structural studies and construction of novel sensor systems for kinase research.

The study demonstrated for the first time that phosphorescence of selenium-comprising aromatic systems can be transferred by FRET mechanism to fluorescent labels of genetically tagged protein kinases. This opens a new possibility for the application of cell plasma membrane-permeable ARC-Lum probes in combination with genetically modified kinases for construction of sensor

systems for monitoring and mapping of protein kinase activity in living cells. Considering the long-lived (microsecond-scale) photoluminescence of the novel sensor systems, such sensors produce signals that are well separable from auto-fluorescence of cells and chemical compounds. It follows that the measurements can be performed with high signal-to-noise ratio and good three-dimensional resolution using time-gated luminescence microscopy techniques.

SUMMARY IN ESTONIAN

Keemiliste ja geneetiliste meetodite ühitamine proteiinkinaaside fotoluminestsentsanalüüsiks

Doktoritöös kasutati geneetilisi ja keemilisi meetodeid sensorsüsteemide loomiseks proteiinkinaaside analüüsiks.

Proteiinkinaasid katalüüsivad valkude fosforüülimist, see on oluline reaktsioon valkude modifitseerimisel, mille kaudu reguleeritakse valkude aktiivsust rakkudes. Valkude fosforüülimine võimaldab suurendada proteoomi mitmekesisust, seeläbi mõjutatakse rakkude normaalset ja patoloogilist toimimist. Valkude fosforüülimistasakaalude nihkumine PKde häiritud aktiivsuse tõttu on paljude raskete haiguste (vähktõve erinevad vormid, diabeet jne) põhjuseks või märguandeks nende tekkimise kohta. Sellest tuleneval on proteiinkinaasid olnud 21. sajandi olulisteks ravimiarenduse sihtmärkideks. Viimase 15 aasta jooksul on 30 proteiinkinaaside inhibiitorit jõudnud vähiravimitena haiglatesse.

Lisaks olulisele rollile ravimiarenduses on proteiinkinaasid tähtsad biomarkerid vähktõve erinevate vormide ning teistele haiguste tuvastamisel. Sellest tuleneb ka suur nõudmine analüüsimeetodite järele, mis võimaldaksid määrata kinaaside kontsentratsiooni ja aktiivsust kliinilistes proovides, näiteks kehavedelikes ja biopsiaproovides.

Viimasel aastakümnel on mõistetud, et just kahe uurimisala, sünteetilise keemia ja valkude konstrueerimise geneetiliste meetodite kooskasutamine on vajalik uudsete omadustega molekulide ja nende komplekside konstrueerimiseks. Need struktuurid lisaksid uudseid võimalusi biomeditsiiniliste uuringute läbiviimiseks, ravimiarenduseks ning haiguste tuvastamiseks.

Käesolevas töös ühitati keemilised ja geneetilised meetodid selleks, et välja arendada uudsed sensorsüsteemid PKde ja nend inhibiitoride uurimiseks. Ühelt poolt toodeti imetajate rakkudes mitmete proteiinkinaaside (PKAc, CK2α, and PIM-kinaasid) liitvalke fluorestseeruvate valkudega. Teisalt konstrueeriti ARC-inhibiitoritest lähtuvalt uudsete luminestsentsomadustega kõrge afiinsusega sondid kinaasidede jaoks [ARC-Lum(Fluo)-sondid].

Seoses PIM-kinaaside (PIM1, PIM2 ja PIM3) olulise tähtsusega nii ravimarenduse sihtmärkidena kui ka vähktõve biomarkeritena, on abivahendite väljatöötamine nende kinaaside uurimiseks suure praktilise väärtusega. PIM2 liitvalke punase fluorestseeruva valguga (TagRFP) ekspresseeriti imetajate rakkudes kahel erineval kujul (TagRFP-PIM2 ja PIM2-TagRFP). Töös näidati, need erineva märgise paigutusega liitvalgud omavad kompleksis ARC-Lum(Fluo)-sondidega erinevaid luminestsentsomadusi, mis tuleneb erinevast Försteri-tüüpi energiaülekandest vastasmõjus olevate luminofooride vahel. Töös näidati esmakordselt, et kompleksis on võimalik energiaülekanne seleeni-sisaldava aromaatse süsteemi ergastatud tripletest olekust kinaasiga seotud fluorestseeruvale valgule, mille tulemusena fluorestsentsvärvilt kiiratav valgus on küll värvile omase kiirgusspektriga, kuid enam kui 10000 korda pikema elueaga.

See tulemus loob eelduse rakusiseste sensorsüsteemide loomiseks raku plasmamembraani läbivate ARC-Lum(Fluo)-sondide ja rakkudes ekspresseeritud proteiinkinaasi ja TagRFP liitvalkude baasil, mis võimaldaks kaardistada proteiinkinaaside aktiivsust elusrakkudes

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Scientific publications

1. **Ganesh babu Manoharan**, Erki Enkvist & Asko Uri (2016). Combining chemical and genetic approaches for development of responsive FRET-based sensor systems for protein kinases. *Biophysical Chemistry,* 211, 39–48.

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Teaduspublikatsioonid

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