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ADLOC: An Aptamer-Displacement Assay Based on Luminescent Oxygen Channeling

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Abstract: Functional nucleic acids, such as aptamers and allosteric ribozymes, can sense their ligands specifically, thereby undergoing structural alterations that can be converted into a detectable signal. The direct coupling of molecular recognition to signal generation enables the production of versatile reporters that can be applied as molecular probes for various purposes, including high-throughput screening. Here we describe an unprecedented type of a nucleic acid-based sensor system and show that it is amenable to high-throughput screening (HTS) applications. The approach detects the displacement of an aptamer from its bound protein partner by means of luminescent oxygen channeling. In a proof-of-principle study we demonstrate that the format is feasible for efficient identification of small drug-like molecules that bind to a protein target, in this case to the Sec7 domain of cytohesin. We extended the approach to a new cytohesin-specific single chain DNA aptamer, C10.41, which exhibits a similar binding behavior to cytohesins but has the advantage of being more stable and easier to synthesize and to modify than the RNA-aptamer M69. The results obtained with both aptamers indicate the general suitability of the aptamer-displacement assay based on luminescent oxygen channelling (ADLOC) for HTS. We also analyzed the potential for false positive hits and identified from a library of 18000 drug-like small molecules two compounds as strong singlet-oxygen quenchers. With full automation and the use of commercially available plate readers, we estimate that the ADLOC-based assay described here could be used to screen at least 100000 compounds per day.

Keywords: aptamers · biosensors · high-throughput screening · luminescence · small-molecule inhibitors

Introduction

High-throughput screening (HTS) of compound libraries to identify small molecules that interact with target proteins and modulate their biological activity is a major task in drug development. Therefore, broadly applicable sensor methods that are amenable to HTS conditions are highly desirable. Functional nucleic acid sensors, such as aptamers, molecular beacons, allosteric ribozymes, and riboswitches, have proven to be valuable tools for obtaining and storing information about a target. Some of these sensors have successfully been manipulated in a way that enables translation of this information into a variety of read-out signals with the potential of applications in HTS formats. In most cases, the applied read-out systems rely on introducing one or two fluorescent groups at appropriate sites within a nucleic acid to generate a fluorescent signal either in the presence or absence of the respective ligand. Consequently, various functional artificial and natural nucleic acids have been adapted to make them suitable for biosensing approaches,[1,2] but the majority of examples applied artificial nucleic acid sensors comprised of aptamers or aptamer-regulated allosteric ribozymes.

Aptamers are in vitro generated single-stranded oligonucleotides that have become invaluable tools for a variety of applications in basic and applied research.[3] Similar to antibodies, aptamers are able to bind different biological targets that can range from small organic compounds to protein domains, and from multimeric proteins to complex targets, such as cells, viruses, or tissues.[4] These functional nucleic

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acids are routinely selected from large combinatorial nucleic acid libraries (~10^{14}–10^{15}) against a variety of targets ranging from small molecules to proteins using a process called SELEX. Moreover, they can be readily adapted in vitro to meet certain criteria, such as increasing stability, affinity and specificity, or traceability. In addition, aptamers can be chemically synthesized in fairly large quantities. These properties make this class of nucleic-acid-based binding elements suitable for a variety of applications that require the reliable, specific and sensitive detection of target compounds.

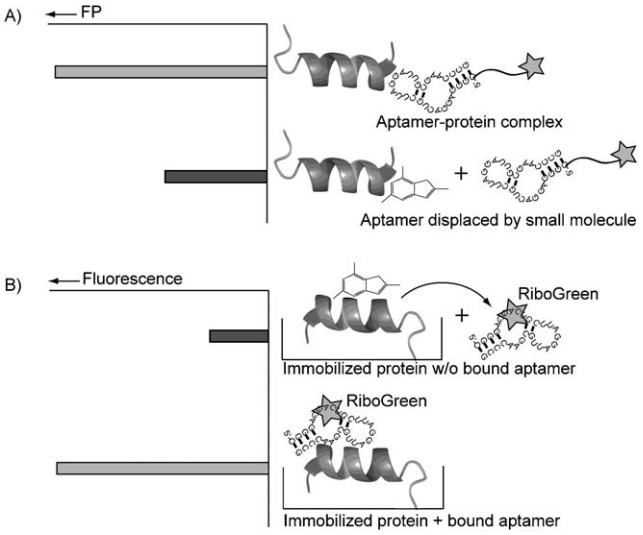
It has been shown that aptamers can be applied as tools for the discovery of small organic compounds which compete with the aptamer for protein binding, thus exhibiting similar inhibitory properties on the same target proteins.[5] The majority of newly identified small molecule modulators for biological targets is normally accomplished by functional assays that have been adapted to a high-throughput readout format. This adaptation of known assays is often hampered by instable reaction partners, slow kinetics, tedious and time- or resource-consuming washing procedures, and other drawbacks.[6] A promising alternative to tailoring a screening method to an individual drug target is the use of aptamers. This is due to the ease by which aptameric inhibitors can be identified and synthesized and advantageously aptamers can be also applied to validate a target protein as "drugable". Screening assays based on aptamer displacement have the advantage of providing access to target-independent assays for the identification of small molecules—particularly in those cases where assay development is a limiting step.

Here, we describe a new and highly modular principle based on aptamer displacement sensed by luminescent oxygen channeling that we designate with the acronym ADLOC (Aptamer Displacement detected by Luminescent Oxygen Channeling). We show that ADLOC is, in principle, compatible with high-throughput screening for the detection of small molecules that displace aptamers bound to their protein targets in solution. In this proof-of-principle study, we demonstrate that ADLOC provides a format that can be used to identify small molecule ligands for binding to a model protein, the Sec7 domain of the ARF-GEF class of cytohesins.

Results and Discussion

The aptamer-displacement assay systems that have been described in detail by our laboratory until now were based on fluorescence polarization (FP; Scheme 1 a).[7]

In practice, however, for many aptamer–protein pairs the difference in size between the unbound aptamer and the aptamer–protein complex is not sufficient for achieving a substantial fluorescence polarization difference suitable for HTS, since many fluorescence labels show lifetimes of only 4 ns or less, thereby restricting investigations of interacting molecules to sizes below 20 kDa. Another obstacle is the occurrence of the so-called "propeller effect" resulting from flexible movements or oscillations of the linked fluorophor,



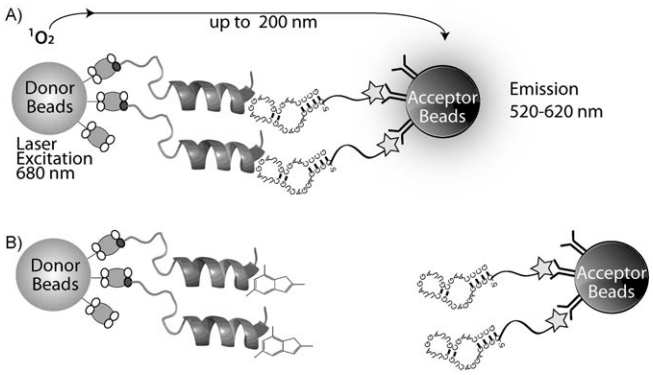
Scheme 1. Aptamer-displacement assays based on fluorescence polarisation (FP) and RiboGreen-fluorescence. A) FP is high in the protein/aptamer complex (top) and low in the displaced aptamer (bottom). B) Fluorescence is low when the displaced aptamer is washed away from the immobilized protein (top) and high in the aptamer/protein complex in presence of RiboGreen (star; bottom).

which can make the correlation between polarization and molecular mass of the complex difficult.[8]

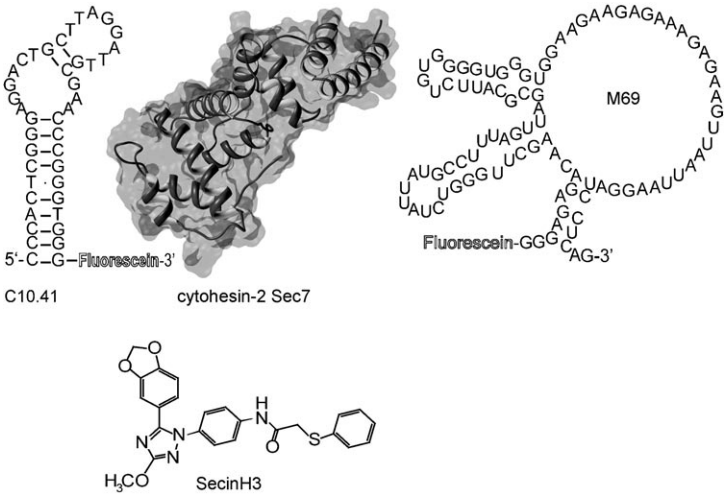
Another strategy that has been described for aptamer-displacement screening involves the use of specific nucleic-acid-binding dyes such as RiboGreen that relies on the detection of aptamers bound to the immobilized protein target by RiboGreen fluorescence. This assay has the advantage that it neither requires labeling of the protein nor of the aptamer, but a severe disadvantage is the necessity of several washing steps that counteract high-throughput compatibility (Scheme 1 b).[5e]

Principles of luminescent oxygen channeling (LOC) and ADLOC: In search for an alternative strategy, we aimed for a straightforward and more size-independent aptamer-displacement screening that circumvents the obstacles of these approaches. We thus established an aptamer-displacement assay based on the luminescent oxygen channeling immunoassay (LOCI)[9] that allows the study of biomolecular interactions. Its mode of action rests upon the proximity-dependent energy transfer between two types of microbeads illustrated in Scheme 2.

The donor beads contain the photosensitizer phthalocyanine, which upon excitation at 680 nm converts ambient triplet oxygen to singlet oxygen. These singlet-oxygen molecules have a half-life of 4 μs, within which they are able to diffuse over a distance of approximately 200 nm in solution (Scheme 2 A). Thus, the detectable distance of the two interaction partners exceeds by more than one magnitude the limiting distance in FRET-based assays. In the presence of the second type of beads, the acceptor beads, within this required proximity, energy is transferred from the singlet



Scheme 2. A) Streptavidin (smaller circles in grey) coated donor beads bind biotinylated Sec7 domain, acceptor beads are functionalized with anti-fluoresceinisothiocyanate–IgG and interact with the fluorescein-label (star) on the aptamer. Complex formation of protein and aptamer brings donor and acceptor beads in close proximity. After laser excitation of the donor beads, singlet oxygen ($^1O_2$) diffuses to the acceptor beads which can be detected by light emission between 520–620 nm. B) Small molecule displaces the aptamer from the protein, the complex disintegrates and emission of light is reduced.



Scheme 3. RNA-aptamer M69 (right) and DNA-aptamer C10.41 (left) have previously been selected for specific binding to the Sec7 domain of cytohesins. The displacement of aptamers with small organic compounds often results in hit compounds that show similar properties as the parent aptamer. SecinH3 was discovered in a fluorescence polarization based aptamer-displacement assay of M69 and Sec7 domain of cytohesin-2.[7]

oxygen to the thioxene derivatives contained in these beads leading to a luminescent signal that can be detected at wavelengths from 520 to 620 nm. Because both the donor and acceptor beads can be derivatized with multiple attachment sites, the specific formation of the complex between the aptamer and the protein attached to each bead occurs cooperatively: As soon as the quaternary complex consisting of donor-bead-protein/aptamer-acceptor-bead has formed, chelate effects by additional protein–aptamer interactions on the same beads will occur. This facilitates and stabilizes the attraction of the beads and brings their surfaces into closer proximity by increasing the local concentration of the binding partners (Scheme 2A). Conversely, if a small molecule interferes with aptamer binding to the target protein, the interacting beads rapidly disassemble, resulting in the disappearance of the signal (Scheme 2B).

LOC assay based on the *anti*-Sec7 RNA aptamer M69: For assay development we used the previously characterized RNA aptamer M69, which specifically binds the Sec7 domain of the cytohesin family of small guanine nucleotide exchange factors and inhibits its biological function.[10] The M69–Sec7 pair is particularly suitable as a model for establishing an ADLOC-based screening system because it has previously been used in the screening assays described in Scheme 1 and because specific aptamer displacing small molecules exist for this pair. The ADLOC assay can therefore be directly compared with the two previous approaches regarding its suitability for identifying small molecules specifically targeting the Sec7 domain of cytohesins. For this proof-of-principle-study we used the small molecule SecinH3 as a well characterized inhibitor for the Sec7 domain of cytohesin-2,[7a] which is also known to be capable of aptamer displacement (Scheme 3).

To establish the ADLOC assay we used streptavidin-coated donor beads and anti-fluorescein isothiocyanate-IgG-coated acceptor beads. In order to be recognized by the beads, the interaction partners had to be modified first. The M69 aptamer was labeled by in vitro transcription using T7 RNA polymerase in the presence of 5′-guanosine monophosphothioate (GMPS, **1**) as the initiator nucleotide,[11] followed by alkylation using the electrophilic 5-iodoacetamido fluorescein **2** to attach fluorescein to the 5′-end of the aptamer (Scheme S1, Supporting Information). Biotin labeling of the Sec7 domain was performed using sulfo-NHS-LC biotin. The labeling efficiency was 20% as determined by UV spectroscopy (data not shown).

We next determined the optimum concentrations of the M69 aptamer and the Sec7 domain required for optimizing the detection limits, that is, to achieve maximal response at lowest possible concentrations of the labeled binding partners (Figure 1). As a negative control we used an aptamer sequence similar in length to M69 which does not bind the Sec7 domain. Although M69 binds the Sec7 domain with a dissociation constant in the order of a two-digit nanomolar range (~16–60 nM),[10] as low as 5 nM M69 aptamer concentration was observed to be sufficient for achieving maximal signals; at 10 nM M69 no significant further increase of the signal was detectable (Figure 1A). This increased sensitivity presumably reflects the fact that the local concentration of reaction partners on the bead surface is considerably higher than in solution, likely due to chelate effects. Indeed, an unambiguous signal as compared to the negative control was already detected at 100 pM M69, indicating that the sensor system on which the ADLOC assay is based is highly specific and sensitive.

Similarly, when 5′-fluorescein–M69 was kept at a constant concentration of 5 nM, a maximum signal was obtained at

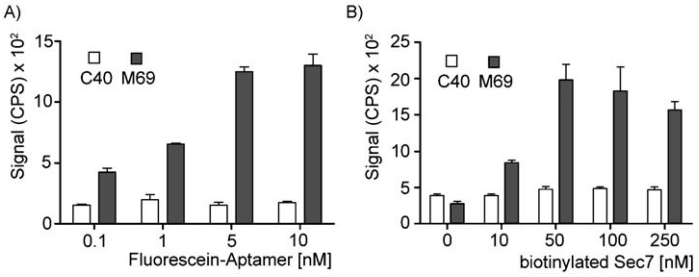


Figure 1. Cross-titration of RNA aptamer M69 and non-binding aptamer sequence C40 with biotinylated Sec7 domain to determine optimal concentration of RNA and protein, respectively. A) Increasing concentrations of 5′-fluorescein-labeled aptamer M69 at 50 nM of biotinylated Sec7 domain. B) Increasing concentrations of biotinylated Sec7 domain at 5 nM 5′-fluorescein-labeled aptamer M69. CPS: counts per second.

50 nM of biotinylated Sec7 domain; again, increasing the protein concentration further to 100 nM had no effect on the magnitude of the detection signal, whereas at 250 nM protein the signal began to slightly decrease (Figure 1B).

ADLOC-assay based on the anti-Sec7 DNA aptamer C10.41: Performing the ADLOC assay system under high-throughput conditions increases the risk of contamination with ubiquitous ribonucleases if an RNA aptamer is used as the binding partner, which is far more prone to degradation than a DNA aptamer, particularly at the low concentrations applied here. We therefore sought to expand the assay to DNA aptamers. For that purpose we used DNA aptamer C10.41,[12] which also targets the cytohesin family of Sec7 domains and thus can be used in direct comparison to its RNA-counterpart M69 (Figure 2). The fluorescence labeling

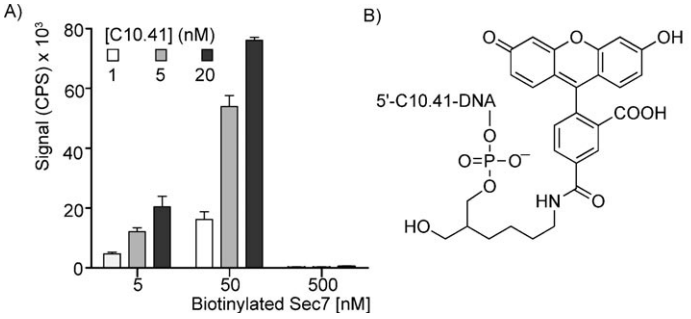


Figure 2. A) Cross-titration of DNA aptamer C10.41 with the biotinylated Sec7 domain to determine optimal concentration of DNA and protein, respectively. B) 3′-FAM labeled DNA aptamer C10.41.

of the DNA aptamer C10.41 was done synthetically at the 3′-end using CPG-beads derivatized with a fluorescein phosphoramidite, to yield the product shown in Figure 2B. As evident from Figure 2A, cross-titration of C10.41 and biotinylated Sec7 domain revealed maximum signals at similar combinations of concentrations as seen in the M69/Sec7 pair, that is, 5 nM C10.41 and 50 nM Sec7. At 500 nM Sec7 domain, a dramatic drop in the signal was observed. This decrease in the signal intensity reflects a concentration of binding partners that is higher than the concentration required to reach complete saturation of all binding sites available on the beads. Beyond this concentration, a further increase in ligand concentration will merely increase the amount of soluble ligand, which will compete with bead-bound ligand thus resulting in a decrease of the detection signal.

Interestingly, the absolute signal intensity obtained for the DNA-aptamer C10.41 was roughly 30-fold higher as compared to the M69/Sec7 pair. This may be due to more quantitative fluorescein labeling of C10.41 during solid-phase synthesis than the labeling of M69 during in vitro transcription (Figure S1, Supporting Information). It may also reflect a preference of the fluorescein-binding IgG for the fluorescein moiety used for labeling C10.41, which also contained a short spacer, over the iodoacetamidofluorescein label that was used for M69.

ADLOC-based assay system: Having demonstrated that both RNA- and DNA-aptamer/protein complexes are well suited for high-sensitivity detection in a sensor system based on luminescent oxygen channeling, we next sought to apply the sensor approach in a small molecule displacement assay that aims for the identification of low molecular weight compounds which disrupt aptamer–protein interactions. To evaluate the potential of this approach we applied the known small molecule inhibitor SecinH3, a *pan*-selective inhibitor of cytohesin Sec7 domains.

We first examined the ability of SecinH3 to disrupt the quarternary complex of the donor and acceptor beads bridged by the M69–Sec7 complex (Figure 3A). Increasing concentrations of SecinH3 led to a continuous drop in the signal until about 15 μM compound. A further increase did not lead to a further reduction of the signal, presumably due to solubility of SecinH3 which is limited to about 20 μM in aqueous solutions (data not shown). Already at 1.5 μM SecinH3 a significant signal reduction was obtained which corresponds to a substantially higher sensitivity of displacement detection than other assay setups such as fluorescence polarization.[7b]

When the experiment was performed with the Sec7-specific aptamer C10.41 the overall signal intensity again was more than 20-fold higher than with the M69–Sec7 complex. The margins of signal reduction after SecinH3 addition were also increased, namely from 54000 counts per second (CPS) in absence of SecinH3 to 45000 CPS at 15 μM of the compound with a clearly detectable signal reduction already at concentrations of 1 μM of the compound. As a negative control we used a point mutant of the aptamer C10.41, C10.41 (C18T) that does not bind to the Sec7 domain at all. This mutant did not show any response to SecinH3 treatment, indicating that the signals obtained specifically measure the SecinH3-induced displacement of C10.41 from the complex with the cytohesin Sec7 domain (Figure 3B). As a further control, we tested SecinH3 in the same concentration range

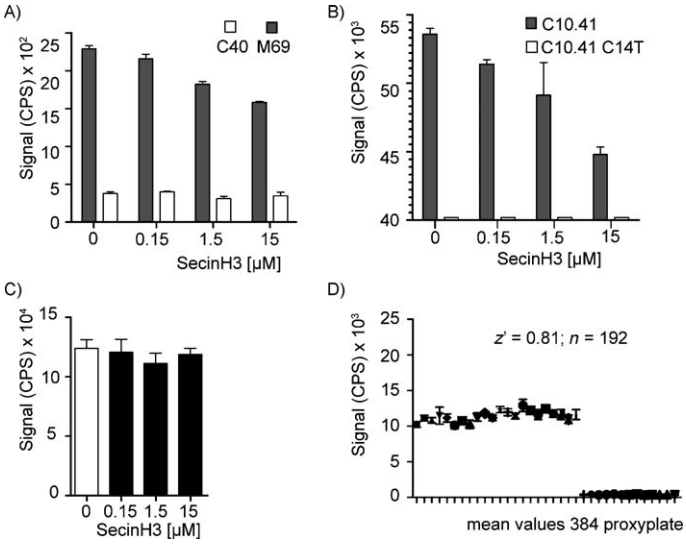


Figure 3. A) 5 nM M69/C40 and 50 nM Sec7 was incubated with increasing concentrations of SecinH3. B) 5 nM C10.41 and 50 nM Sec7 was incubated with increasing concentrations of SecinH3. C) 1 nM biotinylated FITC was incubated with increasing concentrations of SecinH3 in the presence of streptavidin- and anti-FITC mAb-coated donor and acceptor beads, respectively. D) Adaptation of the assay setup C10.41/Sec7 to an automated liquid handling device. Mean values of each column are plotted, z' value was calculated from 192 replicates.

for assay interference using streptavidin-coated donor beads, biotinylated fluorescein (Bio-FITC) and acceptor beads conjugated with the anti-FITC antibody. No effect by SecinH3 could be detected (Figure 3C), indicating that the SecinH3-dependent signal reduction observed in Figure 3A and B, respectively, is not a result of non-specific interference artefacts of the compound with the interacting beads.

Adaptation of ADLOC to high-throughput screening conditions: We next examined the performance of the assay under high-throughput conditions by using an automated liquid handling platform and by obtaining z' factors under these conditions. The z' factor measures the strength of the relationship between variables in the statistical population of samples and is used to quantify the suitability of a particular assay for high-throughput screening.[13] This analysis was done in 384-well plates in final volumes of 16 μL. First, we compared the signal distribution of the C10.41 DNA aptamer and the non-binding mutant C10.41 (C14T) by pipetting 192 replicates (Figure 3D). We noted that direct light exposure led to significant variations in signal intensities, resulting in suboptimal z' factors. A dramatic improvement was achieved when the automated pipetting was performed under subdued light conditions. The z' factors[13] obtained under these conditions were between 0.7 and 0.8, rendering this assay setup suitable for HTS.

To explore the same assay format under genuine screening conditions, we applied a model library of 85 randomly picked drug-like small molecules that do not interact with cytohesin. SecinH3 was included as a positive control in plate rows 1, 3, and 5 (Figure 4). Each compound was applied at a final concentration of 10 μM in a 1% DMSO containing screening buffer. As negative controls we included 1% DMSO in screening buffer in row 9, and the non-binding mutant C10.41 (C18T) in row 10. The z' value we obtained using the standard deviations of the data in rows 9 and 10, respectively, (Figure 4) was 0.78 which matches very well with the more rigorously determined z' value in Figure 3D. The average of the values obtained in the DMSO control in row 9 were set as 100% with a standard deviation σ of ±6%. The three-fold ±σ value is generally considered as a suitable threshold value for hit-definition.[13] If a compound gives a signal that exceeds the ±3σ value (i.e., ±18% in the present screen; see grey shadow in Figure 4) it can be considered as a potential hit compound. As evident from Figure 4, all non-binding compounds, except compounds **3** and **4**, lie well within this margin, whereas SecinH3 is identified as a hit in all three cases. This result indicates the principal suitability of the ADLOC assay for high-throughput screening.

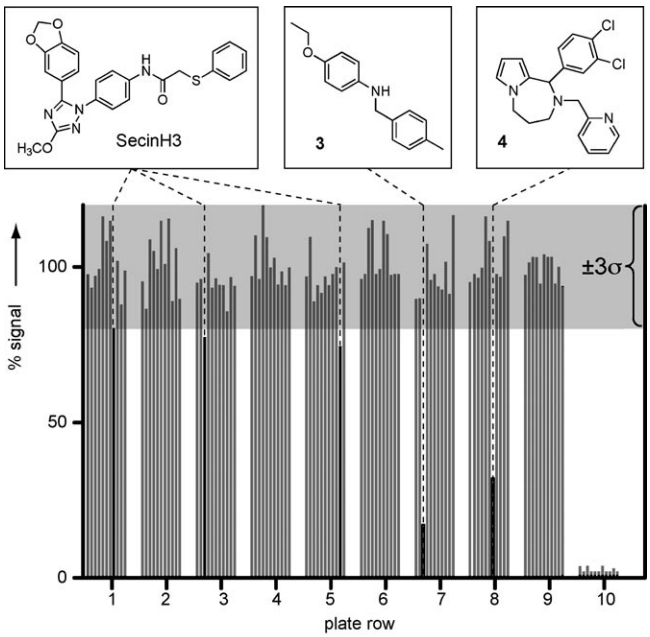
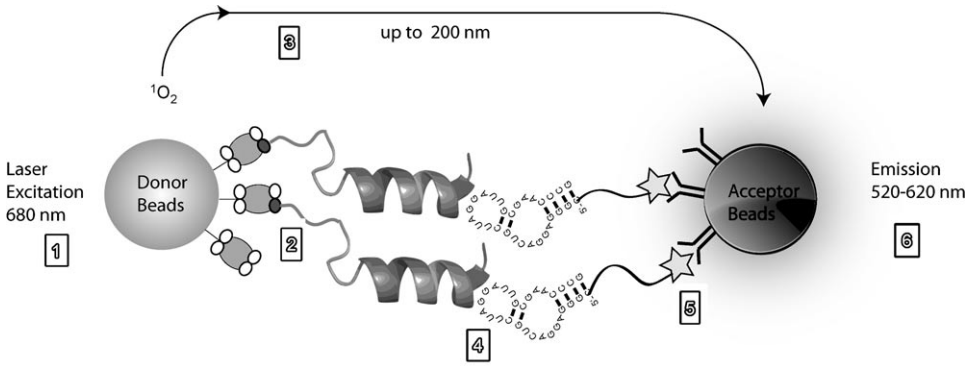


Figure 4. Pilot screen using the DNA-aptamer C10.41 (5 nM) and Sec7 domain (50 nM) with 88 model compounds, including the Sec7-specific inhibitor SecinH3 and two unrelated compounds 4-ethoxy-*N*-[(4-methylphenyl)methyl]aniline (**3**), and 1-(3,4-dichlorophenyl)-2-[(pyridin-2-yl)methyl]-1,3,4,5-tetrahydropyrrolo[1,2-*a*,1,4]diazepine (**4**) that interfere strongly with the assay. The z' value was calculated to be 0.78. Plate row 9 and 10 represent the positive (without compound) and negative (C10.41 C18T) controls.

Potential false-positive compounds: Because the assay exhibits a certain complexity it is crucial to rule out possible false positives that may appear during the screening of large compound libraries. Several basic scenarios for interference with the assay are conceivable (Scheme 4).

First, the compounds might interfere with the assay by absorbing light at either the excitation or emission wave-



Scheme 4. Potential scenarios for mechanisms that might result in false positive hits in an ADLOC screening assay.

lengths (see Scheme 4, boxes 1 and 6). Second, the compounds might compete with the binding of the biotinylated interaction partner to either the streptavidin residues on the donor beads by mimicking the biotin structure, or to the anti-FITC monoclonal antibody by mimicking the FITC structure (see Scheme 4, boxes 2 and 5). Third, compounds that displace the aptamer from the complex with the protein target by binding to the nucleic acid rather than to the protein (Scheme 4, box 4) also represent potential false positives. Probably the most common reason for a false positive are compounds that act as quenchers for singlet oxygen (see Scheme 4, box 3).

To estimate the false positives we performed a pre-screen of a library of about 18000 different drug-like small molecules in the absence of binding partners by using biotinylated acceptor beads and streptavidin-coated donor beads (data not shown). From this pre-screen, two compounds **3** and **4** (Figure 4) emerged that turned out to be strong quenchers of singlet oxygen. No interfering compounds that acted by the mechanisms indicated by box 1 or 2 in Scheme 4 were present in this library. Compounds **3** and **4** were included in our pilot screen to illustrate the importance of a post-screening validation of obtained hit compounds.

To exclude the possibility that the positive control compound SecinH3 might act not by an aptamer-displacement mechanism, we used biotin-labeled acceptor beads and streptavidin-coated donor beads in a post screening set-up (Figure 5). First, we applied 10 μM SecinH3, **3**, and **4** under the standard assay conditions to reconfirm the results obtained in the screening, by triplicate measurements (Figure 5A). The decrease in the signals was virtually identical to those in the pilot screen with very low standard deviation. However, using biotin-labeled acceptor beads and streptavidin-coated donor beads showed no effect of SecinH3 but the same signal decrease in case of **3** and **4**, respectively. This clearly confirms **3** and **4** to reduce the signal by interfering with the assay itself rather than representing a true hit compound.

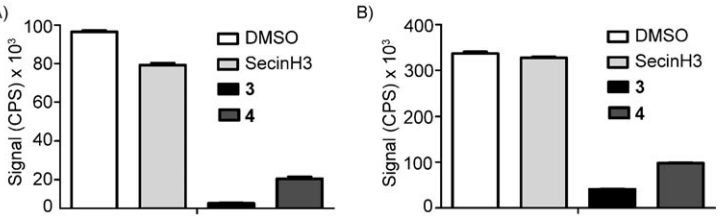


Figure 5. Triplicate measurement of 10 μM hit compounds identified in the pilot screen. A) C10.41/Sec7 concentrations identical to pilot screen: the Sec7 domain inhibitor SecinH3 shows a stable signal decrease of approximately 20%, **3** and **4** decrease the AlphaScreen signal by 90 and 70%, respectively. To determine potential interfering compounds biotinylated acceptor beads (5 $\mu g\,mL^{-1}$) and streptavidin coated Donor beads (5 $\mu g\,mL^{-1}$) are incubated with the identified compounds. B) Compounds **3** and **4** show a substantial interference with the signal detection and thus are clearly identified as false positives. 10 μM of SecinH3 does not exhibit interference with the detection.

Conclusion

In summary, we have developed a luminescence-based aptamer displacement (ADLOC) assay that has the potential to become the method of choice for the screening of large compound libraries by aptamer displacement. The assay is basically independent from the size of the aptamer–protein complex and is therefore superior to FRET or fluorescence polarization techniques. We could show the quick adaptability of the basic setup for different sequences, no matter whether RNA or DNA aptamers were used. The coupling of fluorescein-labeled aptamers to the acceptor bead surface appears to occur with high efficiency, since the theoretical binding capacity of the anti-fluorescein isothiocyanate-IgG-coated acceptor beads was around 5 nM which is also the aptamer concentration showing the strongest signal. In contrast, coupling of a His6-tagged Sec7 domain to Ni–NTA functionalized acceptor beads did not work at all (data not shown), probably due to the positively charged surface which could easily interact electrostatically with the negatively charged nucleic acid phosphate backbone. Thus, using the fluoresceinisothiocyanate–IgG functionalized acceptor beads will likely be superior to other functionalizations when establishing aptamer displacement assays for different protein–aptamer pairs.

In addition, the ADLOC assay is highly cost-effective (currently <0.20 Euro per well), due to the requirement of very low aptamer and protein concentrations and the ease of assay miniaturization and is therefore particularly suitable for the screening of large compound libraries. The aptamer-displacement screenings by this highly modular system

promises to be highly versatile as many aptamers that can easily be functionalized at their 5′/3′ termini will be suitable for this system irrespective of their shape or size. It is reasonable to expect that the ADLOC system will easily be adaptable to fully automated HTS platforms and that it will be feasible for screening 100000 compounds per day or more.

The ADLOC assay complements the tool box of nucleic-acid- and aptamer-based screening methodologies. Particularly the use of aptamers as molecular probes during HTS will benefit from this assay development. The ADLOC assay should be compatible with the use of aptamers in various sophisticated screening set-ups. Covering previously described FRET-, FP-, and fluorescence intensity-based assays together with the luminescence-based format described within this study, aptamers represent versatile, ready-to-use reagents for HTS approaches.

Experimental Section

Labeling of reagents: C10.41 and C10.41(C18T) DNA aptamers were generated and 3′-FAM-labeled by solid-phase synthesis (Metabion, Martinsried, Germany). M69 and C40 RNA aptamers were obtained by standard in vitro transcription using T7 RNA polymerase as described previously,[14] supplemented with 20 mM GMPS (guanosine 5′-monophosphothioate) and successive coupling to 5-iodoacetamidofluorescein (Pierce Protein Science) as previously described.[11] Biotin-labeling of the proteins was done with 5-(2-oxo-hexahydro-thieno[3,4-D]imidazol-4-yl)-pentanoic acid 2,5-dioxo-3-sulfo-pyrrolidin-1-yl ester Na salt (NHS-biotin, Pierce) at primary amine residues. The efficiency of the biotinylation was determined spectroscopically at 354 nm. SecinH3 {(*N*-[4-[5-(1,3-benzodioxol-5-yl)-3-methoxy-1*H*-1,2,4-triaz-ol-1-yl]phenyl]-2-(phenylthio)acetamide)} was synthesized as described previously.[7d]

Adaptation to HTS conditions: The assay was performed in 384-well plates (Perkin–Elmer Proxyplate 384) in a final volume of 16 μL which ensures that only minimal amounts of reagents are needed. The assay setup and conditions which gave a stable signal and that were then subsequently used for all experiments were as follows: First a four-fold buffer premix was given into the wells to obtain final concentrations of 3% DMSO and 0.1 mg mL^{-1} BSA. After the successive addition of fluorescein-labeled aptamer and the biotinylated protein, the assay plates were incubated for 30 min at room temperature to allow the binding reaction to reach equilibrium, before a mixture of donor and acceptor beads was added to obtain a final concentration of 40 μg mL^{-1} per bead type. After an incubation time of 2 h the plates were measured on the Plate reader Berthold Mithras LB 940 in the AlphaScreen Mode. The excitation and measuring time was both 0.5 s. All pipetting steps by the automatic liquid handling device (Tecan Evolution) were done under subdued light conditions, in a total volume of 16 μL under the same conditions.

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