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Programmable binary chimera aptamer probes for intelligent fluorescence imaging of cell membrane receptors

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ABSTRACT

Fluorescence imaging is a powerful tool to characterize cell membrane receptors which involve many important intracellular activities. However, the study of the membrane receptors' senior behaviors is hindered by the static model characteristic of current fluorescence imaging strategies. To address this issue, we adapted a novel type of programmable binary chimera aptamer probes for intelligent fluorescence imaging of cell membrane receptors. Firstly, one binary chimera aptamer probe was successfully engineered to reiteratively image target receptor PTK7 on living cells. Besides, it also showed a distinct advantage of selectivity for signal regulation over conventional method such as trypsin treatment. Secondly, another probe was developed by employing a layered DNA nanostructure as the programmable signal regulating moiety. It allowed *in situ* visualizing the membrane receptor PTK7 in an AND logic manner using two DNA strands as inputs. Interestingly, it was among the first cell imaging probes which could differentiate the order-dependent interaction in a keypad-lock logic manner. Furthermore, an internal control was also successfully employed in these probes to improve imaging reliability by accounting for cell-to-cell variations and background.

1. Introduction

As well known, cell membrane receptors participate in the precise regulation of intracellular biological activities and also are important biomarkers of cancer cells [1–4]. Due to extreme sensitivity, high selectivity, and microscopic detection, fluorescence imaging has become one of the most popular and powerful tools for the study of cell membrane receptors [5,6]. Selected using a SELEX approach (Systematic Evolution of Ligands by EXponential enrichment), aptamers are known as single-stranded DNA/RNA oligonucleotides with high affinity and specificity to their targets [7,8]. Compared with antibodies, they possess kinds of advantages, including reproducible production, high stability, programmable structures, low molecular weight, and little immune response [9]. Therefore, aptamers serve as the ideal recognition moiety for the design of excellent cell imaging probes with high specificity even under complex biologic environments [10].

However, most of the current aptamer-based fluorescent probes functioned only in a static model, in which the fluorescence imaging signal couldn't be altered after the recognition of the aptamer probes to their target membrane receptors [10]. This characteristic of static imaging model is unfavorable for aptamers' further application. On one hand, selective fluorescence imaging of different membrane receptors is desirable for the study of the membrane receptors. But it is usually hindered by the spectral overlap of fluorophores in different probes [11,12]. Therefore, it's necessary to cage the fluorescent signal for one membrane receptor, so that another signal for the other membrane receptor could be more accurately imaged. But most of fluorophores are covalently linked to probes, and the signal's caging process requires the employment of acid wash or trypsin digestion which will disrupt cell morphology and compromise further research [13,14]. On the other hand, membrane receptors often interact with other signaling molecules in advanced logic relationships to precisely regulate intracellular biological activities [1,2]. Besides, cell is also not a homogeneous medium but non-uniform "stratified" media, where different regions contain different biomolecules, and various signal transductions function in an order-dependent manner, such as cell migration [15,16]. In this way, the current fluorescent probes with single-input diagnostics are not suitable to monitor the advanced biological activities in living cells. To overcome these limitations, it is ideal to design a novel type of aptamer probes, in which the fluorescent imaging signals could be gently adjusted in a temporal and spatial model compatible for intelligent imaging.

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The specificity and predictability of the Watson-Crick complementarity principle make DNA an excellent material for bio-nanotechnology, enabling the development of a dreamy set of programmable dynamic DNA nanodevices. In particular, toehold-mediated DNA strand displacement is a simple but powerful approach to form advanced DNA devices for various applications [17,18]. Different from purely classical hybridization process, it can facilitate the exchange reaction of oligonucleotides with different thermodynamic stabilities. The principle of toehold-mediated DNA strand displacement has inspired the development of various dynamic DNA devices, including programmable logic gates, chemical amplifiers, DNA machines, and even high-order DNA circuits [19–22]. The resulting DNA devices have also been successfully involved in kinds of intracellular sensing applications, such as immunofluorescence labeling *in situ*, autonomous cancer targeting and therapy, and even biomedical diagnostics *in vivo* [12,23–25].

In this study, we developed a novel type of programmable binary chimera aptamer probes (**BcAPs**) for intelligent imaging of cell membrane receptors. The **BcAPs** employed binary chimera structure, where one part is a recognition moiety using aptamer, the other one is a programmable signal moiety. The imaging signal of the BcAPs could be gently adjusted in a temporal and spatial model *via* the toehold-mediated strand displacement strategy. Based on the results of both flow cytometric analysis and confocal microscopy imaging, the proposed **BcAPs** realized intelligent fluorescent imaging: **BcAP 1** for reiterative and selective label, while **BcAP 2** for keypad-lock logic gate controlled imaging.

2. Experimental section

2.1. Materials and instrumentation

All DNA oligonucleotides were synthesized and HPLC-purified by Sangon Biotechnology Co., Ltd (Shanghai, China). The sequences of the oligonucleotides are listed in Table S1 (Supporting information). Water used in all experiments was doubly distilled from a Milli-Q system (Millipore, USA). Fluorescence measurements were carried out at 25 °C on a FluoroMax-4 spectrofluorometer (Horiba Jobin Yvon, France). All the detail procedures for probe formation, fluorescence kinetics measurements, gel electrophoresis analysis and cell culture can be found in the Text S1 (Supporting information).

2.2. Flow cytometric analysis and confocal microscopy imaging

In Eppendorf tubes, 100 nM probes was incubated with 3×10^5 CCRF-CEM cells at 4 °C in 200 μL of binding buffer for 30 min. The mixture was washed twice with 200 μL washing buffer and then suspended in binding buffer (200 μL). The resultant cell solutions were subjected to flow cytometric analysis on a BD FACSVerse flow cytometer by counting 10,000 events.

For the **BcAP 1**: After the first flow cytometric analysis, 10 μL F_{in} (5 μM, binding buffer) was added into the resultant cell solutions and incubated at 4 °C for 5 min. The mixture was washed with 200 μL washing buffer and suspended in binding buffer (200 μL). After the second flow cytometric analysis, 10 μL F (5 μM, binding buffer) was added into the resultant cell solutions and incubated at 4 °C for 5 min. The mixture was washed with 200 μL washing buffer and suspended in binding buffer (200 μL) for the next flow cytometric analysis. These procedures were repeated for the reiterative fluorescence labeling.

For the **BcAP 2**: Prior to the flow cytometric analysis, 5 μL G_{in} or F_{in} (5 μM, binding buffer) was added into the resultant cell solutions and incubated at 4 °C for 5 min.

Cell samples were prepared for confocal microscopy imaging in the same way that they were prepared for flow cytometry analysis, and the details can also be found in the Text S1 (Supporting information).

3. Results and discussion

3.1. Reiteratively imaging

As indicted in Fig. 1A, the **BcAP 1** was firstly developed *via* specific DNA hybridization process for reiteratively imaging, which comprised of a binary chimera structure: one recognition moiety and one programmable signal moiety. To demonstrate the applicability of this probe, the recognition moiety employed sgc8, an aptamer for selective recognition of Human protein tyrosine kinase-7 protein (PTK7) on CCRF-CEM cells [26]. And the programmable signal moiety was based on a toehold-mediated strand displacement process: the binding of F_{in} strand to a 6-base toehold domain at one end of F strand initiates the strand displacement reaction, which releases an "output" double strand F·F_{in}. Alongside of this process, a black hole quencher 1 (BHQ-1) on F strand will be separated from the aptamer moiety, and its quenching effect on FITC's green fluorescence signal was inhibited (Fig. 1A). To reiteratively imaging of the target protein PTK7, F strand with a BHQ-1 can be further added to quench the FITC's green fluorescence signal, *via* a simple hybridization process with B strand on the **BcAP 1**. In this way, a reiteratively imaging probe was successfully developed *via* alternation of strand displacement reaction and DNA hybridization process. Furthermore, Cy5's red fluorescence signal was employed as an internal standard (Fig. 1A), which can avoid possible errors resulting from fluorescent bleaching or other cell sample treatments. This two color fluorescence imaging strategy offers the opportunity to study a single molecule event by different fluorescence channels, and thus can increase imaging reliability. In the presence of 300 nM F_{in}, a dramatic signal enhancement (>5 fold) was observed (green line, Fig. S1(A), Supporting information), and further addition of F strand resulted in a rapid decrease of green fluorescence intensity to its initial level, demonstrating the effective activation and quenching processes for FITC's green fluorescence signal. Meanwhile, Cy5's fluorescence signal remained almost unaffected during all these processes, indicating its potential role as an internal standard for the **BcAP 1**. Based on a signal-to-noise ratio >3, the detection limit for F_{in} was estimated to be 1 nM, with a linear range of 2–20 nM (Fig. S1(B), Supporting information).

After *in vitro* characterization of probe's fluorescence kinetics responses, we next performed many experiments for *in situ* fluorescence imaging of the target protein PTK7. Firstly, living CCRF-CEM cells were incubated with the **BcAP 1**. The resulting sample showed only little green fluorescence signal due to the quenched state of the **BcAP 1**'s FITC signal by the BHQ-1 on F strand (a, Fig. 2A). After addition of F_{in}, 10 min incubation and subsequent washing procedure, the resultant cell's green fluorescence signal was successfully activated *via* the strand displacement reaction (b, Fig. 2A). Further incubation with a fresh solution of F strand efficiently turned off the cells' green fluorescent signal to its initial level (c, Fig. 2A). Especially, the green fluorescent signal of CCRF-CEM cells could be activated once again by F_{in} (d, Fig. 2A). In contrast, Cy5's fluorescence intensity, a predesigned internal standard signal (Fig. 2B), was unchanged during all the above-mentioned treatments. In addition, FITC and Cy5 signals remained generally correlated for all the cell samples, indicating the excellent stability of this binary chimera aptamer probe (Fig. S2, Supporting information).

To precisely characterize the **BcAP 1**, flow cytometry was further used as a high throughput method to analysis the fluorescent response signals from the cell samples. In consistence with the