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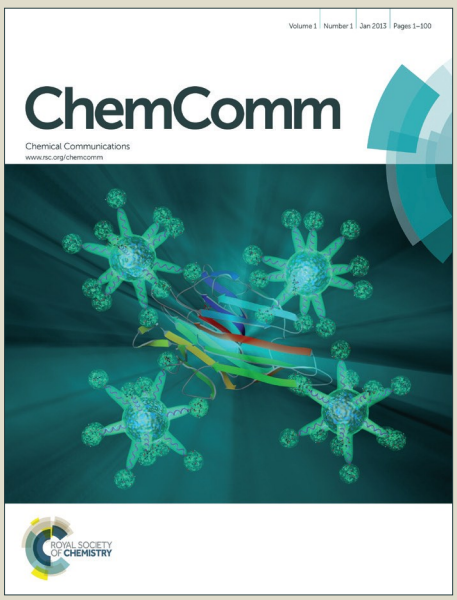





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# A signal-on split aptasensor for highly sensitive and specific detection of tumor cells based on FRET†

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**We present here a signal-on fluorescence biosensor for highly sensitive and specific detection of tumor cells with split aptamers based on fluorescence resonance energy transfer (FRET). This sensor holds considerable potential for simple, rapid, sensitive and specific tumor cells detection in early clinical diagnosis.**

Early diagnosis is essential for effective treatment of malignant tumors which are serious diseases endangering human life and health. However, the number of tumor cells is so few in blood at the early stage that they are difficult to be detected. In addition, complex environment and cell endocytosis can cause high background signal, which greatly limits the sensitivity and specificity for numerous detection methods. According to the current related researches, the improvement of the tumor cells detection strategies is mainly focus on molecular probes[1], signal-readout model[2], and signal amplification[3].

Molecular probes with high sensitivity and specificity for tumor cells have emerged as important tools in the field of cells detection. Aptamers offer several advantages over antibodies since they are stable, non-immunogenic, and can be easily synthesized and modified. They have been widely applied in areas such as tumor classification or early diagnosis.[4] In recent years, aptamers have gained increasing popularity in establishing aptasensors for tumor cells detection coupled with fluorometric[1b], colorimetric[2b], electrochemical[2c,f], Raman signal[2d] or quartz crystal microbalance[2e] sensing methods. Despite the good results of these methods, they still have some problems originated from the complexity of tumor cells, especially high background signal, resulting in difficult detection of complex samples.

In the case of fluorometric methods, some excellent works have been done to improve the sensitivity or specificity in cells detection. For instance, the aptamer-modified fluorescent nanoparticles were applied to detect target cells, which resulting in amplified signal intensity compared to individual dye-labeled probes[5]. In addition, taking advantage of the exceptional quenching capability of several two-dimensional nano-materials to single-strand DNA (ssDNA), such as graphene oxide[6] or single-walled carbon nanotube[7], the background signal decreased thus improved the signal contrast for tumor cells detection. However, these methods can not avoid the background signal induced by non-specific adsorption of ssDNA to cells. In another case, an activatable aptamer probe targeting tumor cells was developed, which achieved contrast-enhanced tumor visualization in living mice[1b]. But, this strategy required complicated design of probes. Therefore, it is in urgent need of developing new methods for tumor cells detection with high sensitivity, great specificity and low background signal.

Split aptamers, which comprised of two or more fragments of the intact aptamer, having great advantages in reducing non-specific signal and improving signal-to-background ratio (SBR).[8] The split aptamers can not fold into the right secondary structure in the absence of the target molecules. Only in the presence of the target molecules will the split aptamers assemble selectively. In this case, it greatly decreases the background signal from non-specific adsorption on cell surface. Based on the excellent features of split aptamers, many aptasensors aimed to detect small molecules[8a] and biomacromolecules[9] have been described. However, using split aptasensor to detect tumor cells is still rarely reported.

FRET is a nonradiative process whereby a donor fluorophore transfers energy to a proximal an acceptor fluorophore through long-range dipole–dipole interactions[10]. It is often used to reduce background noise and therefore improve detection limit effectively, which has been widely applied to detect ions[11], biomolecules[12] and tumor cells[13]. Based on above mentioned case, we ingeniously designed a signal-on fluorescence split aptasensor for highly sensitive and specific detection of tumor cells based on FRET. Because of good performance of the split aptamers and the signal readout mode of FRET, this strategy has advantages regarding time-

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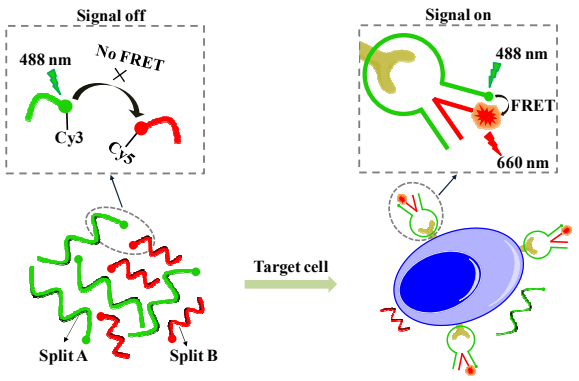


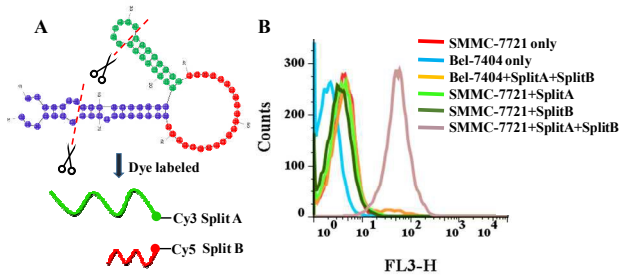

**Scheme 1.** Schematic representation of the split aptasensor for tumor cells detection.

saving, low background signal, high sensitivity and great specificity.

Scheme 1 illustrates the principle of the signal-on split aptasensor for tumor cells detection. 3' terminus of split aptamer A (Split A) and 5' terminus of split aptamer B (Split B) were labeled with Cy3 and Cy5 fluorescent group which serve as excellent FRET pairs respectively. There is equilibrium for the split aptamers disassociation and association, while the disassociation state is much favored in solution, giving very low background signal. Upon binding with the target cells, the equilibrium was significantly driven to the formation of the assembled hairpin-like complex. The probes association results in the proximity between Cy3 and Cy5 and thus the FRET signal.

The key to this strategy is to acquire the split aptamers. Here, we chose the aptamer ZY11 that was screened by our lab using cell-SELEX[14]. The secondary structure of ZY11 simulated by the Mfold software demonstrates that it consists of a loop structure and two stem structures (Figure S1A†). According to this structure, we optimized the sequence of ZY11 using five optimization strategies (Figure S1B†). Interestingly, flow cytometry assays showed that the loop of the aptamer ZY11 is highly sequence-conserved, indicating its importance in interacting with the target molecule, while the two stems of the aptamer ZY11 are structure-conserved that they are tolerant to multiple base pair substitutions as long as they form the stem structures, indicating their important roles in stabilizing the overall structure of the aptamer (Figure S2†). Based on this information, ZY11 was subtly split into two fragments by ripping through the stems (Figure 1A). The experimental observations revealed that the split aptamers retain high affinity to target SMMC-7721 cells (Figure S3†). After labelled with FRET pairs, a split aptasensor was constructed.

Flow cytometry assays was used to test the feasibility of this method. The channel 3 (EX 488 nm, EM 660 nm) of flow cytometry was used to collect FRET signal because Cy3 signal excited by 488 nm light was beyond channel 3 and Cy5 could not be excited by 488 nm light, only FRET signal between Cy3 and Cy5 could be detected in channel 3. As shown in Figure 1B, flow cytometry assays indicated that neither Split A nor Split B




caused the FRET signal when work alone. Only when Split A and Split B coexist would they give efficient signal on target cells, indicating FRET between Cy3 and Cy5. In addition, in the presence of control cells Bel-7404, no obvious signal changes were detected, suggesting the specificity of this method. The confocal imaging results also demonstrated the selective binding of the split aptamers, proven by a strong signal surrounding the target cells after incubation with both split aptamers (Figure S4†).

**Figure 1.** (A) Cutting strategy of ZY11 for the split aptamers. By cutting the terminus of the two stem structures, the split aptamers were obtained. (B)Flow cytometry assays of SMMC-7721 cells, Bel-7404 cells, SMMC-7721 cells incubated with Split A, SMMC-7721 cells incubated with Split B, SMMC-7721 cells incubated with Split A and Split B, and Bel-7404 cells incubated with Split A and Split B. The fluorescence signal was determined by counting 10000 events in channel 3 (EX 488 nm, EM 660 nm long-pass) for FRET signal.

In order to obtain a maximum SBR for detecting target cells, incubation time, incubation temperature, probe ratio, and probe concentration were investigated by using flow cytometry. As shown in Figure S5†, different SBR were displayed with different incubation time (15, 30, and 45 min), incubation temperatures (4, 25, 37, and 40 $^{o}$C), probe ratio (split A to split B, 3:1, 2:1, 1:1, 1:2 and 1:3) and concentration (0.5, 1, 2.5, 5, 25, 50, and 75nM) respectively. The fluorescence signal increasing factor used here was FT/FC, where FT and FC represented the fluorescence intensities of the split aptasensor with target cells and with control cells respectively. From these results, we found that the best experimental condition was 4 $^{o}$C with 5 nM probes in 1:1 probe ratio for 15 min of incubation.

With the optimized conditions, cell samples in 200 µL binding buffer were quantitatively detected by this method with cell number ranging from 0 to 200,000. Target cells quantification was carried out by counting the labeled events appearing in the upright (UR) window. The number of events in the UR region decreased corresponding to the decreased cell number in the sample (Figure 2A). Background signal was measured without any cells in 200 µL binding buffer. Background counts plus three times standard deviation were subtracted from total counts to obtain effective counts. The plotted data presented a good linear response, as seen in Figure 2B. The number of SMMC-7721 cells detected by this method (Y) versus the cell number measured by hemocytometer (X) was used to calculate detection limit. The regression equation was log Y = 0.8857 log X + 0.4124 with the minimum cell number of 20 detected in 200 µL binding buffer. This detection limit was obviously lower than most other

fluorescent aptamer methods with activatable aptamer probe or a turn-on aptamer probe[1b,2a], especially our non-split

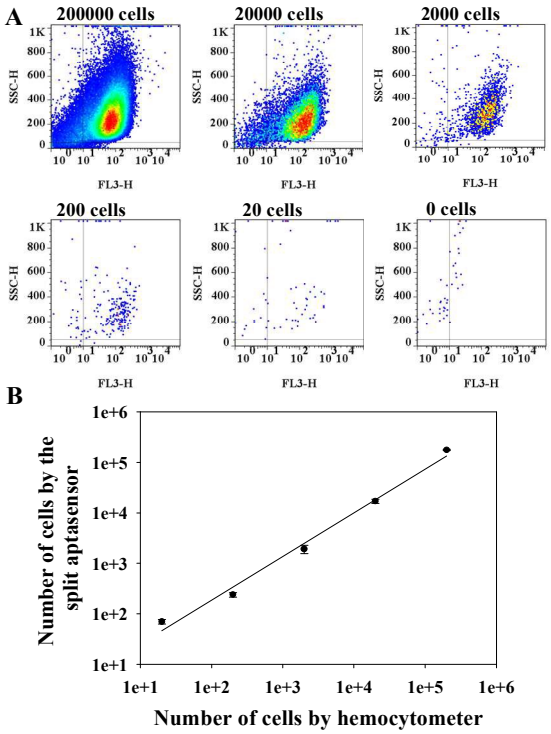



**Figure 2.** (A) Flow cytometry assays of SMMC-7721 cells with decreasing cell counts in 200 μL binding buffer by the split aptasensor. (B) Corresponding calibration curve demonstrating the linear relationship between the number of SMMC-7721 cells counted by the split aptasensor and the number of SMMC-7721 cells counted by hemocytometer. All the error bars represent standard deviations of three repeated measurements.

aptamer ZY11 with the detection limit of 200 cells in 200 μL binding buffer[6]. However, the sensitivity of this assay is lower than some aptamer methods based on electrochemical signal[2c], Raman signal[2d] and Quartz crystal microbalance[2e] (Table S2). Furthermore, this method could specifically detect target cells in complex sample. Figure S6† showed the results of target cells detection in 20% human serum sample. It also showed the same performance with a detection limit of 20 cells in 200 μL sample. This result revealed that this method has great potential in detection of complex samples.

The specificity of this method towards SMMC-7721 cells was evaluated by measuring the fluorescence signal of the split aptasensor for five control cells, including Bel-7404 cells, QBC-939 cells, L02 cells, MCF-7 cells and A549 cells. As shown in Figure 3, the fluorescence signal for SMMC-7721 cells was clearly stronger than the control cells. This result demonstrated the excellent specificity of this method.

We further evaluated the capability of the split aptasensor to detect target SMMC-7721 cells from mixture samples of different cells. As demonstrated in Figure S7†, with the ascending percentage of target SMMC-7721 cells in the cell mixture, increasing number of cells appeared in the high fluorescence (FLH3>$10^1$) population and correspondingly decreasing number of cells remained in the low fluorescence population (FLH3<$10^1$), indicating the prominent specificity in complex samples.

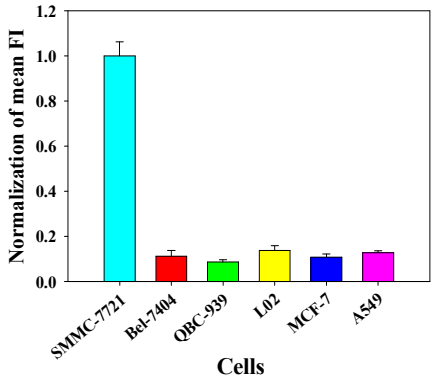



**Figure 3.** The signal comparison between target SMMC-7721 cells with other control cells (Bel-7404, QBC-939, L-02, MCF-7, and A549 cells) based on this split aptasensor by using flow cytometry. The FRET signal of the target detection was collected by counting 10000 events in channel 3 (EX=488 nm, EM=660 nm). While the fluorescence signals of control cells was collected in channel 1 (EX=488 nm, EM=514-545 nm). All the error bars represent standard deviations of three repeated measurements.

In conclusion, we have successfully developed a FRET-based signal-on split aptasensor for highly sensitive and specific detection of SMMC-7721 cells in complex environment. This strategy possesses obvious advantages over existing tumor cells detection methods. Firstly, this method is simple and time-saving that it can be done within half an hour without any cell pretreatment. Secondly, the background signal of this strategy is very low because of the target-induced FRET readout and its excellent selectivity towards target cells. Importantly, this strategy preserves its prominent sensitivity for target cells detection in samples with 20% human serum, indicating its potency in medical diagnostics. Therefore, this simple, rapid, sensitive and specific tumor cells detection method may offer superior performance in tumor research and clinical diagnosis.

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