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ARTICLE TYPE

High-throughput imaging assay of multiple proteins via target-induced DNA assembly and cleavage†

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This work integrates the target-induced DNA assembly and cleavage on a DNA chip to design a versatile imaging strategy for assay of multiple proteins. The DNA assembly is achieved via immunological recognition to trigger the proximity hybridization for releasing a DNA sequence, which then hybridizes with FITC-DNA1 immobilized on chip to induce the enzymatic cleavage of DNA1 and thus decrease the signals. The signal readout is performed with both fluorescent imaging of the left FITC and chemiluminescent (CL) imaging by adding peroxidase labelled anti-FITC in assembly solution and CL substrates to produce CL emission. This one-step incubation can be completed in 30 min. The imaging method shows wide detection ranges and the detection limits down to pg mL⁻¹ for simultaneous detection of 4 protein biomarkers. This high-throughput strategy with good practicability can be easily extended to other protein analytes, providing powerful protocol for protein analysis and clinic diagnosis.

Introduction

Simple, high throughput, sensitive and accurate detection of multiple proteins is critical in protein analysis and clinic diagnosis.¹ Although a variety of electrochemical,²⁻⁵ electrochemiluminescent,^{6,7} colorimetric,^{8,9} chemiluminescent (CL),^{10,11} fluorescent (FL)¹²⁻¹⁵ and surface-enhanced raman scattering¹⁶ immunoassay methods have been proposed for multiplex detection of proteins, most of them are carried out on a spatial-resolved protein array, which is usually prepared by immobilizing different capture antibodies (Ab) on corresponding sensing sites, and needs two sequential incubation steps. Therefore, the multiplex detection is high-cost and time-consuming, which greatly limits its utility in protein analysis. Convenient and fast detection principle for multiplex analysis of proteins is still maintained in urgent need.

Recently, many affinity ligand-based proximity assay methods have been designed via target-induced DNA assembly for protein detection.¹⁷ They use two DNA-conjugated affinity ligands to simultaneously recognize the target protein and subsequently induce the DNA assemblies such as DNA ligation,¹⁸ hybridization¹⁹⁻²¹ and strand displacement.²²⁻²⁴ As a result, the protein analysis can be translated to DNA detection, and the sensitivity can be conveniently improved with different DNA

amplification strategies, including polymerase chain reaction,^{25,26} rolling circle amplification,^{27,28} catalytic DNA circuit,²³ and enzymatic cleavage recycling.²⁹ However, these methods can only be used for detection of single protein due to the lack of resolution technique.

To avoid the drawbacks of protein array, this work integrated the target-induced DNA assembly and cleavage on a DNA chip to present a high-throughput imaging strategy for simultaneous detection of multiple proteins. The assembly and cleavage processes were achieved by using two DNA-antibody affinity probes to recognize the target protein, which formed a sandwich immunocomplex and led to the proximity of two DNA labels, thus induced the DNA displacement and hybridization to trigger the enzymatic cleavage recycling for amplify the decrease of FL or CL signal.

The assay strategy was performed with both FL imaging and indirect CL imaging by adding peroxidase labelled anti-FITC in the assembly solution to trigger the CL emission of substrate. Benefiting from the one-step target-induced signal change, the designed imaging strategy showed the advantages of easy operation, short assay time and high throughput. The DNA chip and assembly led to good practicability and convenient extensibility. The enzymatic cleavage recycling improved the detection sensitivity. Thus the proposed high-throughput strategy provided an avenue for multiple protein analysis.

The DNA chip was prepared by immobilizing DNA1-FITC on different cells of home-made array, and a DNA3/DNA2 duplex was formed for the preparation of affinity probe Ab-DNA3/2 (ESI†). The purity of the obtained affinity probes was identified to be acceptable with PAGE and mass spectroscopic analysis (Fig. S1 and S2). The FL imaging assay (FIA) was performed by mixing two affinity probes (Ab-DNA3/2 and Ab-DNA4), nicking endonuclease (Nt.BbvCI) and target protein on the chip to achieve the target-induced DNA assembly and release of DNA2, which then hybridized with DNA1 to trigger the enzymatic

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cleavage recycling (Fig. 1a). The FL signals of FITC left on the chip were recorded with a microarray imaging system. By adding horseradish peroxidase labelled anti-FITC (HRP-Ab_{FITC}) in the reaction mixture and CL substrates on the left chip, the CL imaging assay (CLIA) could be carried out (Fig. 1b). Due to the target-induced cleavage of DNA1-FITC, the presence of target protein decreased the amount of FITC or HRP-Ab_{FITC} captured on the chip, leading to a “signal-off” FIA or CLIA method for multiplex protein detection.

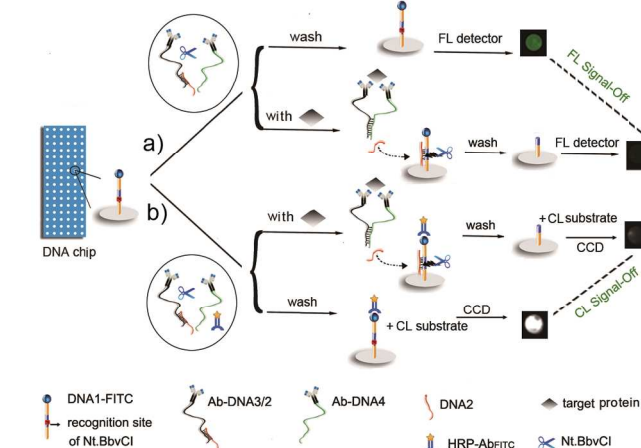


Fig. 1 General principle of a) FIA and b) CLIA of multiple proteins with a target-induced DNA cleavage strategy on DNA chip.

Results and discussion

Characterization of target-induced DNA assembly and cleavage

The target-induced DNA assembly and enzymatic cleavage recycling were firstly verified by using DNA1 and DNA2-Cy5 as the substitutes of DNA1-FITC and DNA2 to record the fluorescent signals (Fig. 2a). The absence of target protein showed negligible FL of Cy5 (I and II), indicating that no DNA2 was captured by DNA1, and the cleavage of Nt.BbvCI did not happen. Thus the proximity hybridization between DNA3 and DNA4 could be excluded. Upon addition of the target protein in mixture (I), a strong FL of Cy5 was observed (III), which could be extinguished by adding Nt.BbvCI in the mixture (IV), suggesting that the target triggered the proximity hybridization to release DNA2-Cy5, and the released DNA2-Cy5 hybridized with DNA1 on the chip to further trigger the cleavage of DNA1, which unlinked the DNA2-Cy5 from the sensing site. It was noted that the target protein led to the FL observation of Cy5 on the chip. However, the absence of enzymatic cleavage recycling in the Cy5-based FL detection led to low sensitivity.

The cleavage was further examined on a chip prepared with DNA1-Cy3 (Fig. 2b). After incubation with Nt.BbvCI, the chip showed strong FL of Cy3 similar to that with blank buffer (I and II), indicating that Nt.BbvCI could not cleave single DNA1 strand. In the absence of target protein, the cleavage also did not happen (III). The presence of target protein in the incubation mixture of Ab-DNA3/2, Ab-DNA4 and Nt.BbvCI led to the disappearance of the FL signal (IV), confirming the target-induced FL “signal off”. The FL of DNA1-Cy3 could be

maintained in the absence of Nt.BbvCI (V), indicating the FL “signal off” depended on the presence of Nt.BbvCI for enzymatic cleavage recycling, which amplified the FL decrease of Cy3 for obtaining high sensitivity.

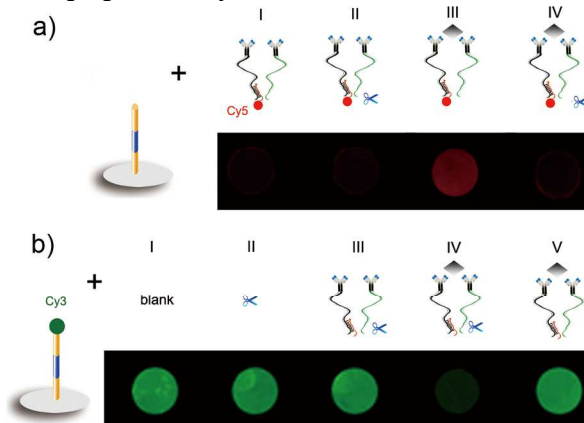


Fig. 2 Fluorescent characterization of target-induced DNA assembly and enzymatic cleavage recycling process. a) Ab-DNA3/2-Cy5 and Ab-DNA4 on chip prepared with DNA1. b) controls, and Ab-DNA3/2 and Ab-DNA4 on chip prepared with DNA1-Cy3. CEA: 670 ng mL⁻¹.

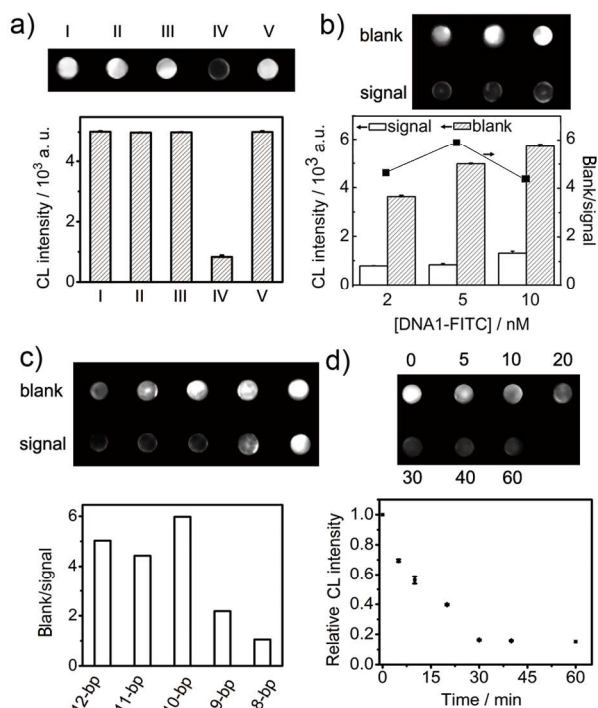


Fig. 3 a) CL imaging of DNA1-FITC chip incubated with (I) HRP-Ab_{FITC}, and (II-V) the mixtures of (II) Ab-DNA3/2, Ab-DNA4, Nt.BbvCI and HRP-Ab_{FITC}, (III) Ab-DNA3/2, CEA, Nt.BbvCI and HRP-Ab_{FITC}, (IV) Ab-DNA3/2, Ab-DNA4, CEA, Nt.BbvCI and HRP-Ab_{FITC} and (V) Nt.BbvCI and HRP-Ab_{FITC}. b) Effect of DNA1-FITC concentration for chip preparation on CL intensity (left Y) and ratio of blank to signal (right Y). c) Optimization of complementary base number between DNA3 and DNA4. d) optimization of incubation time for CLIA. CEA: 670 ng mL⁻¹.

The target-induced CL “signal off” could be confirmed on the DNA1-FITC chip (Fig. 3a). After immunoreaction of the FITC with HRP-Ab_{FITC}, the HRP was captured on the chip to catalyze the CL reaction of luminol-*p*-iodophenol-H₂O₂ system, thus the chip showed strong CL intensity (I). Similar spot was observed after the chip was incubated with the mixture of Ab-DNA3/2, Ab-DNA4, Nt.BbvCI and HRP-Ab_{FITC} (II). Thus the cleavage did not happen in the absence of target protein. The CL intensity was the same as both (I) and (II) in the absence of Ab-DNA4 even if the mixture contained target protein (III). The addition of target protein in mixture (II) led to an obvious CL “signal off” (IV), suggesting the cleavage of DNA1-FITC to decrease the amount of capture HRP-Ab_{FITC}. The cleavage could not be triggered without the presence of proximity hybridization products (V).

Kinetic of CL reaction

Kinetic behavior of the CL reaction catalyzed by the captured HRP on the chip was studied with a static method. Upon addition of CL substrates the CL emission occurred immediately and slightly increased during the first 30 min and then decreased quickly due to the consumption of CL substrates (Fig. 4), indicating the feasibility for CCD imaging. Considering the detection sensitivity, a total exposure time of 3 min was used to collect the CL images, at which the CL “signal off” was used to optimize the detection conditions.

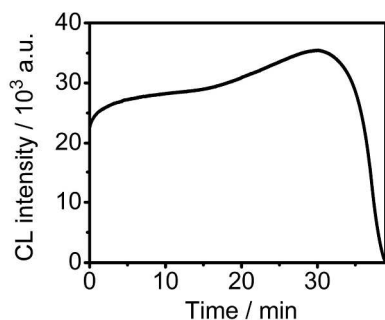


Fig. 4 Kinetic curve of luminol-*p*-iodophenol-H₂O₂ CL reaction catalyzed by HRP-Ab_{FITC} captured on the DNA chip.

Optimization of assay conditions

The complementary bases between DNA1-FITC and DNA2 were chosen to be 11, which ensured the successful enzymatic cleavage recycling. As shown in Fig. 3b, the low concentration of DNA1-FITC could not offer sufficient FITC to capture HRP-Ab_{FITC} and sufficient DNA1 to capture DNA2 released in proximity hybridization, while high DNA1-FITC concentration caused a high density of DNA1-FITC on the chip, which affected the capture of DNA2 and the following cleavage efficiency. Both of them led to low “signal off” and detection sensitivity, thus 5.0 nM of DNA1-FITC was used for the preparation of DNA chip.

The DNA3/DNA2 duplex contained 13 complementary bases. The melting temperature between DNA3 and DNA2 was estimated to be 51.4 °C, which indicated that the DNA3/DNA2 was very stable and could not be affected by DNA1-FITC on the array under the experiment conditions. To efficiently displace

DNA2 from the duplex through the target-induced proximity hybridization between DNA3 and DNA4, the number of their complementary bases was optimized (Fig. 3c). At low number of complementary bases DNA4 could not hybridize with DNA3 to displace DNA2 even with the help of proximate effect, thus the subsequent enzymatic cleavage happened hardly and the strong CL signal did not change. However, when the complementary number was higher than 10 bases, the hybridization between DNA3 and DNA4 could happen in the absence of the proximity effect, which also led to the release of DNA2 to trigger the cleavage of DNA1 and “signal off”. At 10 complementary bases (10-bp), the CL “signal off” reached the maximum value, which was chosen for this work.

Both the FIA and CLIA performances depended on the time for proximity hybridization and enzymatic cleavage processes. The CL intensity decreased with the increasing incubation time (Fig. 3d). The relative CL intensity reached the minimum value at 30 min, and then trended toward a stable value, indicating the target-induced assembly and enzymatic cleavage could be completed within 30 min. This result was the same as that in FIA (Fig. 5). Thus 30 min was selected for incubation in whole work.

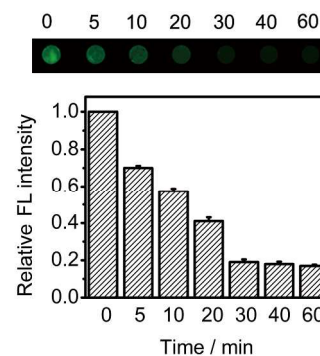


Fig. 5 Fluorescent imaging and relative percentage of fluorescent intensity of FITC on DNA chip incubated with the mixture of Ab-DNA3/2, Ab-DNA4, 670 ng mL⁻¹ target CEA, Nt.BbvCI and HRP-Ab_{FITC} for different times.

Imaging assay of multiple proteins

The practicability of the designed FIA or CLIA strategy was demonstrated using α -fetoprotein (AFP), carcinoma antigen 125 (CA 125), carcinoma antigen 199 (CA 199) and carcinoembryonic antigen (CEA) for simultaneous detection of multiple protein biomarkers. By incubating the DNA chip with the mixtures containing affinity probe pairs, corresponding proteins and Nt.BbvCI in different cells, the FL images were simultaneously collected for the different concentrations of target proteins. The FL brightness of the spot was inversely proportional to the logarithm value of analyte concentration over the ranges of 0.033–330 ng mL⁻¹ for AFP, 0.017–170 U mL⁻¹ for CA 125, 0.017–170 U mL⁻¹ for CA 199, and 0.067–670 ng mL⁻¹ for CEA, respectively (Fig. 6a and 6b). The limits of detection corresponding to the FL signals of 3SD were 0.029 ng mL⁻¹ for AFP, 0.016 U mL⁻¹ for CA 125, 0.011 U mL⁻¹ for CA 199, and 0.060 ng mL⁻¹ for CEA, respectively. When the mixtures containing additional HRP-Ab_{FITC}, the CLIA with CL substrates showed the limits of detection of 0.022 ng mL⁻¹ for AFP, 0.012 U

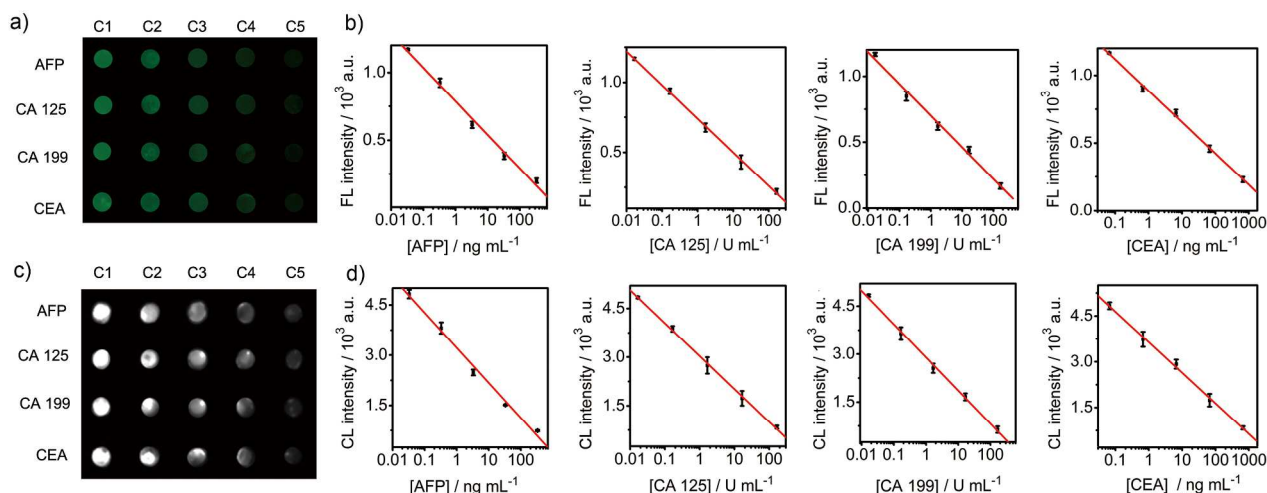


Fig. 6 a,b) FL and c,d) CL images and calibration curves for detection of 4 protein biomarkers. Protein concentrations from C1 to C5 are 0.033, 0.33, 3.3, 33 and 330 ng mL⁻¹ for AFP, 0.017, 0.17, 1.7, 17 and 170 U mL⁻¹ for CA 125 and CA 199, and 0.067, 0.67, 6.7, 67 and 670 ng mL⁻¹ for CEA.

mL⁻¹ for CA 125, 0.010 U mL⁻¹ for CA 199 and 0.048 ng mL⁻¹ for CEA along with the detectable concentration ranges as those in the FIA (Fig. 6c and 6d). Although CLIA required additional reagents and an operation step, it benefited from no need of external optical equipments, which avoids the interference of scattered light caused by incident light and thus possesses weaker background and higher sensitivity than FIA. As a result, the limits of detection obtained by CLIA for four proteins were a little lower than those with FIA strategy. The pg mL⁻¹-level detection limits were lower than those of other immunoassays with different amplification strategies,³⁰⁻³³ and were comparable to the assays based on proximity effect³⁴⁻³⁵ and binding-induced DNA strand displacement strategies.^{23,24}

Evaluation of cross-reactivity and clinical application

In the proposed assay, the cross-reactivity and nonspecific binding among analytes and nonspecific antibodies were demonstrated to be negligible (Fig. 7). To evaluate the accuracy of the proposed detection strategy, the levels of four cancer biomarkers in 5 human serum samples from cancer patients were tested using the designed CLIA and compared with the reference values obtained by commercial electrochemiluminescent single-analyte testing.

Due to the wide detectable ranges and good specificity, the serum samples were detected without any dilution treatment. The results with relative errors less than 8.7% for the detection of all the four biomarkers (Table 1) were acceptable.

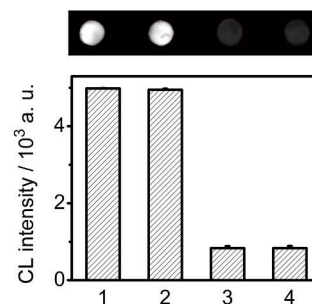


Fig. 7 CL imaging and CL intensity on DNA chip incubated with the mixture of (1) CEA Ab-DNA3/2, CEA Ab-DNA4, Nt.BbvCI and HRP-Ab_{FITC}, (2) (1)+170 U mL⁻¹ CA 125+170 U mL⁻¹ CA 199+330 ng mL⁻¹ AFP, (3) (1)+670 ng mL⁻¹ CEA, (4) (1)+670 ng mL⁻¹ CEA+170 U mL⁻¹ CA 125+170 U mL⁻¹ CA 199+330 ng mL⁻¹ AFP.

Table 1. CLIA results of AFP, CA 125, CA 199, and CEA in clinical serum samples using the proposed and reference methods.

Biomarkers	1	2	3	4	5	
AFP (ng mL ⁻¹)	2.48	1.63	9.32	99.7	22.4	This work
	2.54	1.63	9.28	101.8	20.6	Reference method
	-2.4	0	0.4	-2.1	8.7	Relative error (%)
CA 125 (U mL ⁻¹)	21.9	19.9	20.5	76.9	6.9	This work
	21.3	19.5	21.0	80.3	6.8	Reference method
	2.8	2.1	-2.4	-4.2	1.5	Relative error (%)
CA 199 (U mL ⁻¹)	8.51	4.14	27.3	12.4	13.0	This work
	8.35	4.31	27.6	12.8	13.0	Reference method
	1.9	-3.9	-1.1	-3.1	0	Relative error (%)
CEA (ng mL ⁻¹)	224.2	2.15	52.1	5.64	10.6	This work
	231.6	2.03	49.9	5.75	10.3	Reference method
	-3.2	5.9	4.4	-1.9	2.9	Relative error (%)

Table 2. Performance comparison of this work with previous multiplex immunoassay methods.

Analytical methods	Assay time	Operation steps	Substrate array	References
Imaging assay	35-40 min	1	An array chip prepared with the same DNA sequence for various proteins	this work
Electrochemical assay	~84 min	2	Protein array prepared with different antibodies for corresponding proteins	2
Electrochemical assay	~1 h	2		5
ECL assay	~2.5 h	3		6
Colorimetric assay	~5.5 h	4		8
CL assay	45 min	2		11
Fluorescent assay	~2.5 h	3		13
Surface-enhanced Raman scattering imaging assay	~3 h	3		16

Sample throughput

The whole FIA including the target-induced DNA assembly and enzymatic cleavage recycling on one 6×16 DNA chip could be completed within 35 min, leading to a throughput of 164 tests per hour for single analyte measurement and 41 samples per hour for the simultaneous detection of 4 tumor markers. The CLIA could be completed within 40 min including a 3-min exposure for the CCD-based signal collection, which led to a throughput of 144 tests per hour. By performing parallel imaging assay on several chips, the detection throughput could be enhanced conveniently. The assay time, operation step and sensing chip were further compared with those in previous multiplex protein assays (Table 2). Benefiting from the one-step target-induced DNA assembly and enzymatic cleavage recycling strategy, the imaging assay could carry out the highly sensitive multiplex detection of various analytes using the most universal sensing chip with the shortest assay time and the least operation steps, showing good applicability in clinical diagnosis.

Conclusions

This work reports an imaging assay protocol of multiple protein biomarkers on a normal DNA chip via a target-induced DNA assembly and cleavage strategy. The target protein induces the formation of sandwich immunocomplex and subsequently the DNA assembly via proximity hybridization to release a DNA sequence for triggering the *in situ* enzymatic cleavage. The DNA-chip based imaging assay can carry out the multiplex detection with one 35-min incubation step, leading to a high detection throughput. By coupled flexibly with FL and CL readout, this protocol has been used to detect 4 protein biomarkers simultaneously with wide concentration ranges and pg mL^{-1} -level detection limits. It can also be easily extended to visual detection by using colorimetric substrates for point-of-care testing. In addition, the designed protocol can be extended to the detection of other protein analytes by use of corresponding affinity probes. The excellent analytical performance of high sensitivity and throughput, acceptable selectivity and accuracy, convenient operation and good extensibility for multiple protein markers demonstrates its practicability.

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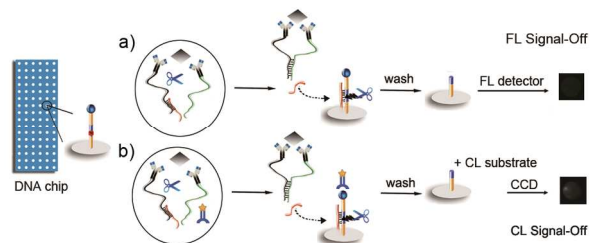
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ARTICLE TYPE

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5 A versatile imaging strategy integrated with the target-induced DNA assembly and cleavage was designed for assay of multiple proteins.