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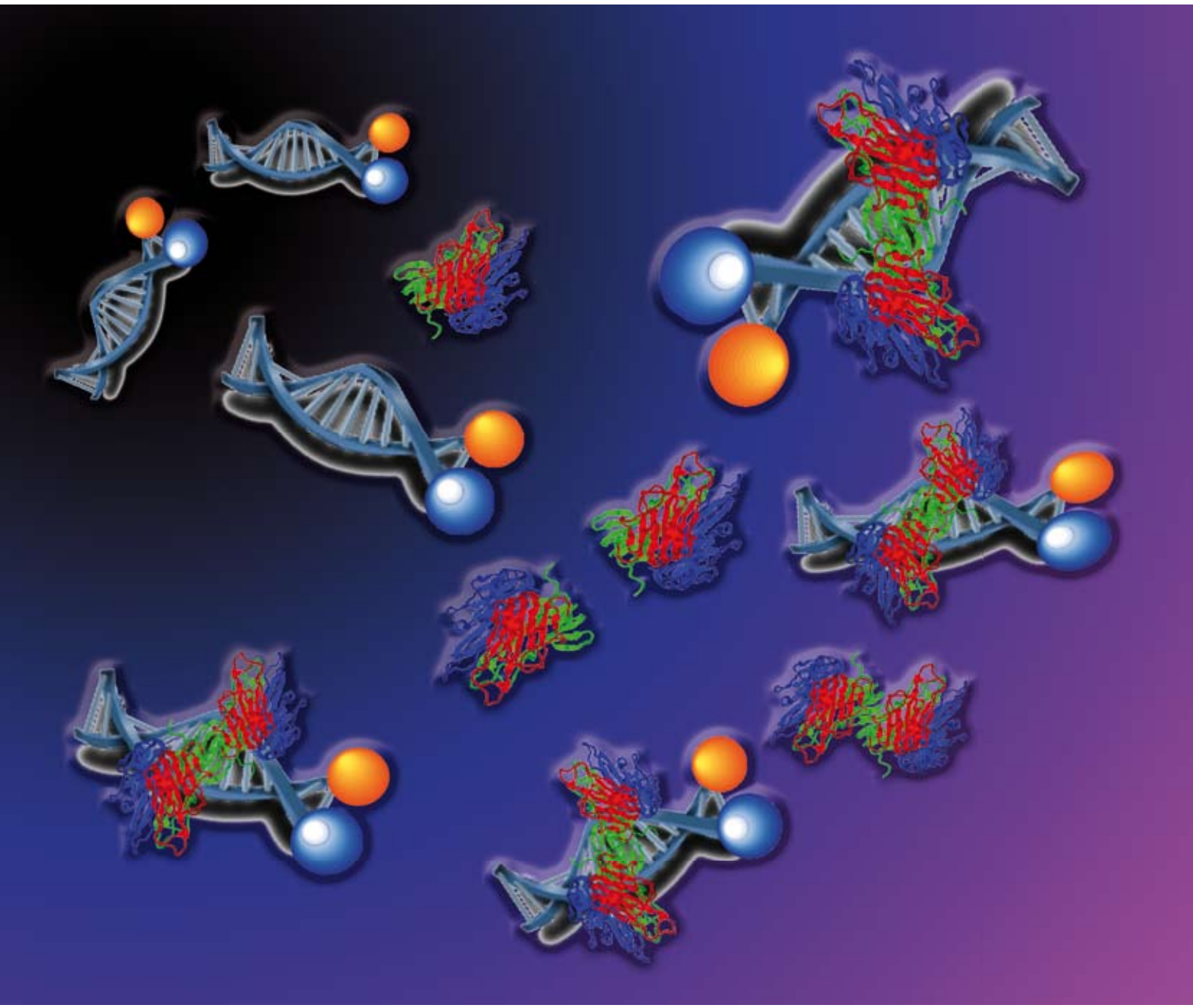

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Pak Kin Wong *et al*.
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Separation-free detection of nuclear factor kappa B with double-stranded molecular probes

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This paper reports a simple, rapid molecular binding scheme for the detection and quantification of the transcription factor nuclear factor kappa B and other DNA binding proteins without any separation or immobilization step.

The DNA binding proteins, such as transcription activators and repressors, are the key elements in the regulation of gene expression. Rapid quantification of the activities of these DNA binding proteins is of great importance in high-throughput drug screening and in the interrogation of complex cellular regulatory circuitries.[1,2] Currently, Electrophoretic Mobility Shift Assays (EMSA) and reporter genes are employed for the measurement of the activities of DNA binding proteins. The time- and labor-intensive nature of these techniques, however, often presents a bottleneck in large-scale biomedical and pharmacological studies. Furthermore, these techniques are typically not quantitative due to the perturbation of the equilibrium dynamics during gel electrophoresis for EMSA and the variation in the transcriptional–translational efficiency for reporter genes. Therefore, a transcription factor assay that is rapid, quantitative, and has a readout that is amenable to automated analysis is highly desirable.

Several molecular approaches have been developed for homogeneous detection of protein–DNA binding. For instance, fluorescence correlation spectroscopy (FCS) using a single molecule detection system has been demonstrated for studying DNA–protein interaction.[3] Fluorescence resonance energy transfer (FRET)-based techniques can also be applied to study protein–DNA interaction.[4] However, FRET-based techniques often require labeling of the target protein. Alternatively, molecular beacons, which consist of two fluorophore-labeled DNA fragments each containing half of the DNA binding site, have been developed for label-free detection of DNA binding proteins.[5] Endonuclease or exonuclease protection strategies can also be coupled with fluorescence measurement for rapid detection of DNA binding proteins.[6]

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Herein, we report a generic molecular binding scheme for homogeneous detection of DNA binding proteins, specifically the transcription factor nuclear factor kappa B (NF-κB). NF-κB regulates numerous genes that mediate several important cellular functions and is one of the major drug targets for cancer and chronic inflammatory diseases.[7–10] NF-κB consists of a family of structurally related subunits including p50, p52, p65, c-Rel, and Rel-B. The p50/p65 heterodimer is present in most cells and is usually the most abundant dimeric complex.[11] NF-κB can be activated by various stimuli and tumor necrosis factor α (TNFα) is one of the most potent activators in a wide variety of cell types.[12] To detect active NF-κB, we develop an homogeneous binding scheme that takes advantage of the molecular interaction between NF-κB and the corresponding binding sequence. In this binding scheme, a double-stranded DNA probe is designed based on the consensus κB DNA sequence.[13] The DNA probes are labeled with a fluorophore and a quencher, which are brought into close proximity. A similar probe design has been developed for homogeneous nucleic acid analysis.[14] To detect a DNA binding protein, a single-stranded DNA competitor, which is complementary to one of the probe strands, is also designed. In the absence of the NF-κB proteins, the competitor hybridizes with the fluorophore probe and separates the fluorophore and the quencher. The existence of the NF-κB proteins stabilizes the probe and impedes the competitor from separating the fluorophore–quencher complex (Fig. 1). Therefore, the fluorescence intensity decreases with an increase of the concentration of NF-κB if a fluorophore–quencher pair is applied. Alternatively, a donor–acceptor pair can also be applied for measuring a FRET signal.[15]

Table 1 summarizes the probe sequences used in this study. In this assay, the probe sequence is primarily determined by the consensus binding sequence. In order to prevent unnecessary interaction between the fluorophore and the protein, two bases were introduced on the 5′ end of the fluorophore probe and on the 3′ end of the quencher probe. The introduction of the bases also served to avoid guanine-induced quenching of the fluorophore.[16,17] The concentrations of the fluorophore probe and the quencher probe were 20 and 60 nM, respectively. The quencher to fluorophore ratio was maintained at 3 : 1 to minimize the background level.[14] In the experiment, the fluorophore and quencher probes were pre-hybridized at 90 °C for 5 min and cooled to room temperature slowly in a dry bath incubator. Recombinant proteins and cell lysates were incubated with the probes in a 96-well plate at room temperature. The binding buffer consisted of 10 mM Tris-HCl, 50 mM NaCl, 3 mM $MgCl_2$, 10% glycerol, 0.5 mM dithiothreitol, and

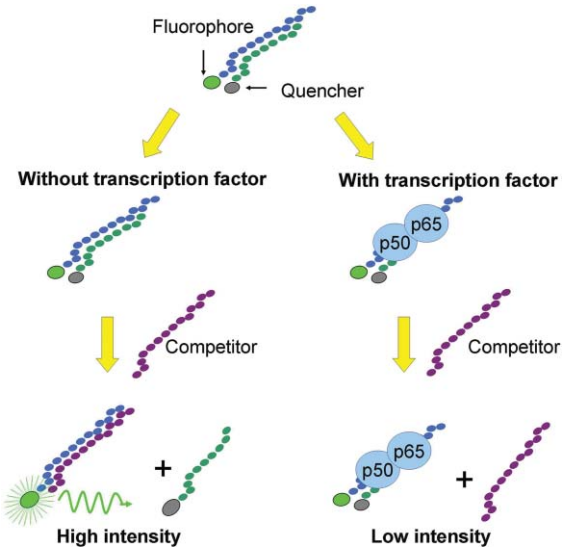

Fig. 1 Schematic of the molecular binding scheme for homogeneous detection of DNA binding proteins. Without the transcription factor, the competitor switches with the probe and separates the fluorophore from the quencher. With the transcription factor, the probe is stabilized by the protein–DNA interaction. In this binding scheme, the fluorescence intensity decreases with the target protein concentration.

0.5 mg/ml bovine serum albumin (BSA). The competitor probe (30 nM) was then introduced into the well. The fluorescence intensities were monitored using an automated fluorescence microplate reader.

To evaluate the performance of the molecular binding scheme, we first characterized the probes with recombinant proteins. Fig. 2a shows the fluorescence responses of different concentrations of p50. Upon the addition of the competitor DNA, the fluorescence intensity increased rapidly, which indicated separation of the fluorophore and quencher pair. We observed that this increase in fluorescence decreased with the concentration

Table 1 Sequences of the nucleic acid probes in this study. The κB DNA sequence is underlined

Probe
5′ Fluorophore- TT**GGGACTTTCC**CAAGATAGTAAG 3′
3′ Iowa black- AA**CCCTGAAAGG**GTTC 5′
Competitor
3′ AACCCTGAAAGGGTTCTATCATTC 5′

of p50. The concentration dependence can be distinguished within one hour after the introduction of the competitor. Fig. 2b shows the effect of the concentration of p50 on the fluorescence intensity at steady state. Since the steady state fluorescence intensity depends on the concentration of p50, the concentration of p50 can be quantified based on the fluorescence intensity. The limit of detection is determined to be in the nanomolar concentration range, which is in agreement with the reported affinity of NF-κB with its binding sequence.

To further characterize the assay, we performed the experiment with p50, p65, p50/p65, and BSA (Fig. 2c). We observed a lower fluorescence at steady state for the p50/p65 heterodimer compared to p65–p65 and p50–p50 homodimers. This indicates that the p50/p65 heterodimer has a higher affinity compared with the p50–p50 and p65–p65 homodimers, which is consistent with previously reported values.[18] In addition, the fluorescence signal for 5000 nM BSA (100-fold higher) was indistinguishable from the control. We have also tested the applicability of the assay with HeLa nuclear extracts treated with and without TNFα, a well-know inducer of the NF-κB signaling pathway.[13] As shown in Fig. 2d, the assay was able to distinguish between the samples with and without TNFα treatment.

In summary, we have developed a homogeneous molecular binding scheme for the rapid detection of NF-κB without any protein labeling or separation step. The scheme is demonstrated using recombinant proteins and nuclear extracts. The limit of detection is on the order of nanomolar concentration,

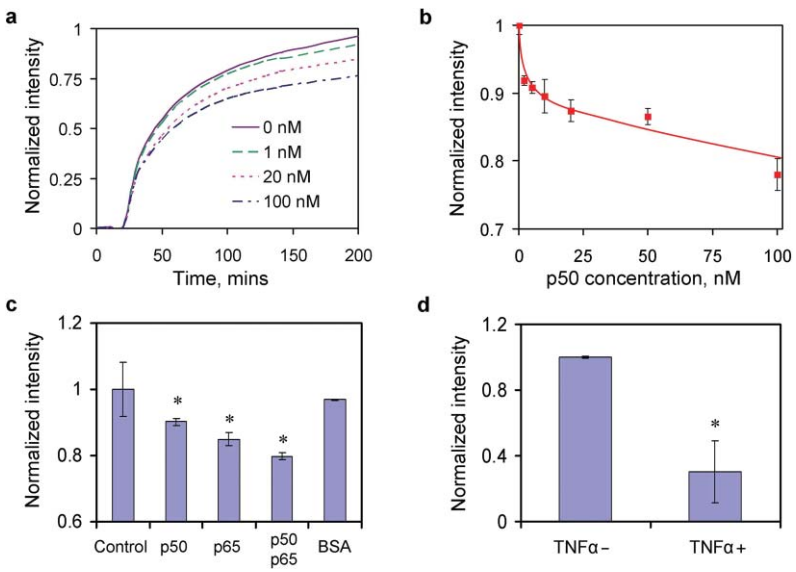

Fig. 2 (a) Kinetics of the binding assay. Competitors were introduced at time = 20 min. (b) Quantification of the p50 protein using the homogeneous molecular assay. (c) Specificity of the NF-κB probes. The concentrations are: p50 (50 nM), p65 (50 nM), p50/p65 (25 nM each), and BSA (5000 nM). (d) Detection of TNFα-induced NF-κB activity in nuclear extracts. * $p < 0.05$.

which is compatible with other NF-κB assays. Compared to the molecular beacon approach,[5] our binding scheme prevents potential interferences between the protein and the fluorophore, as the fluorophore and quencher are labeled at the end of the probe molecule. The probe can be easily designed based on the consensus binding sequence. We believe that the molecular binding scheme can be generically extended to other DNA binding proteins. The molecular binding scheme can easily be incorporated into an automated system for high-throughput drug screening and large-scale interrogation of signaling networks in the future.

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