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ORIGINAL PAPER

5-Color Multiplexed Microwave-Accelerated Metal-Enhanced Fluorescence: Detection and Analysis of Multiple DNA Sequences from within one Sample Well within a Few Seconds

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Abstract We present a potentially highly sensitive and selective bio-assay for the potential detection of any five different DNA sequences from one sample in one well. The assay is based on a DNA "rapid catch and signal" (DNA-RCS) technology developed for the detection of different DNA sequences from a sample well area. Our signal amplification utilizes the metal-enhanced fluorescence (MEF) of dyes attached to the probe-DNAs, which hybridizes with the preformed mixture of anchor-DNA scaffolds on silver island films (SiFs). Low-power microwave irradiation accelerates both the formation of the anchor-DNA scaffold on the SiF-surface and anchor/probe DNA hybridization, i.e. "rapid catch" of target DNAs from a bulk solution, decreasing the assay run time from hours to only a few seconds. Localization of signaling dye-labels close to the SiFs make them extremely photostable, which allows for collecting/integrating the signal over a long time period. To demonstrate a 5 color DNA assay (5-plex) we have used a range of readily available Alexa™ dyes. Advantages and perspectives of the RCS-technologies ability to detect 5 different DNA sequences from within one plate-well are discussed.

Keywords Metal-Enhanced Fluorescence · Surface Enhanced Fluorescence · Plasmon Enhanced Fluorescence · Plasmon Controlled fluorescence · DNA assays · Point-of Care Diagnostics · 20 s Assays · Pathogen Detection · Bio-agent Detection

Abbreviations

MEF	Metal-Enhanced Fluorescence
NP	Nanoparticle
SiF	Silver Island Film
ssDNAsingle	stranded DNA
dsDNA	double stranded DNA
PCR	Polymerase Chain Reaction
RCS	Rapid Catch and Signal technology
LoD	Limit of Detection
FRET	Fluorescence Resonance Energy Transfer

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Introduction

Detection of small quantities of DNA of a certain sequence in biological fluids is a problem of the up-most importance to the life sciences. DNA sequences contain information about the origin of cells and can serve as markers for pathogens, sources of infection in humans, mutated states of genes, analysis of bacterial diversity and for many other issues concerning living organisms. Success in solving this problem, finding approaches that can register small amounts of DNA sequences will pave the way for the creation of new ways of thinking of how we implement medical care.

Until recently the PCR (polymerase chain reaction) technique was exclusively employed for the purpose of detection of small quantities of specific DNA sequences in solution [1–4]. The power of PCR is in the ability to amplify DNA fragments to the level where simple, usually a fluorescent dye (e.g. PicoGreen, SYBR Green) [2] binding approaches, could easily register the presence of large amounts of amplified DNA.

In the last few years the MEF phenomena has become one of the most promising approaches for lower copy number DNA detection. MEF of fluorophores is a consequence of their coupling with the plasmons of metal nanoparticles (NP), which occurs due to the close proximity of a dye to

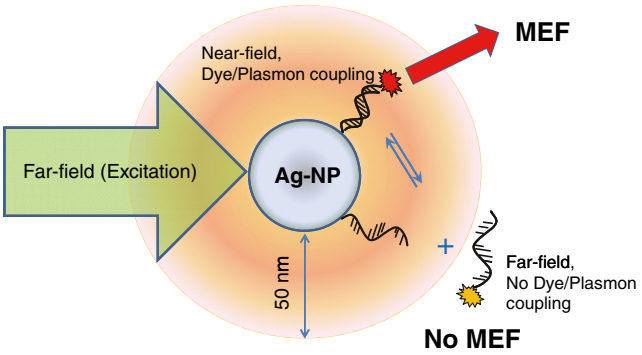

Fig 1 Metal-Enhanced Fluorescence (MEF) principles of the DNA quantitation assay

the NP. Plasmon/dye coupling dramatically increases chromophore brightness, as compared to free dye in the bulk solution [5–6]. To employ the MEF effect in DNA detection techniques, the probe-DNA should be attached (anchored) to NPs and subsequently upon hybridization with labeled DNA, comes into close proximity (~5—10 nm) of the metal NPs. In these conditions the system produces a strong fluorescence detection signal and subsequently senses only the specific near-field recognition event, Fig. 1. In addition to MEF, low power microwave heating speeds up the process of DNA annealing [7]. Combination of both MEF and microwave acceleration, has been termed MAMEF, and was originally developed and successfully utilized in our laboratory for the detection of one bacterial DNA in solution [7–9]

In many ways MAMEF has close similarities to PCR, but with some added features. Both technologies detect small DNA fragments, both have LoDs that are underpinned by sample diversity (blood, serum, Cerebrospinal fluid etc.), and while PCR uses thermal cyclic amplification to replicate the DNA for detection, MAMEF employs direct fluorescence detection, without the need for cyclic amplification. In essence, we remove the need for cyclic amplification, but we use Metal-Enhanced Fluorescence to plasmon amplify the hybridized complimentary DNA probe containing the fluorescent-label. One would therefore expect that PCR and MAMEF would have similar LoDs. In addition, MAMEF does offer a much quicker speed to assay completion / detection, typically less than 30 s due to microwave-acceleration, and can potentially be performed at a much lower cost. Recently our PLOS 1 paper on the 20 s 1 cfu/ml detection of salmonella and our paper on the ultra-fast ultra-sensitive <10 cfu/ml detection of Chlamydia have been published [10–12], which underscores the significant potential of our approach for detecting low levels of disease burden at PCR equivalent levels, the assumption being that we know the target we are looking for. Our MAMEF Chlamydia assays have also been compared to two PCR assays recently in a blinded clinical study using vaginal swabs taken from 270 women. Our CT assay was found to be≈90 % concordant with regard to assay sensitivity and specificity as compared to both PCR tests [12]. In another recent study we have shown the<10 cfu detection of Toxins A and B from the human pathogen Clostridium difficile in 40 seconds using Microwave-Accelerated Metal-Enhanced Fluorescence from stool samples [13].

Fig 2 The sequences of the Anchor and Probe ssDNAs. The DNA sequences used in this study are fragments of Chinese hamster ovary (CHO) Alu sequence

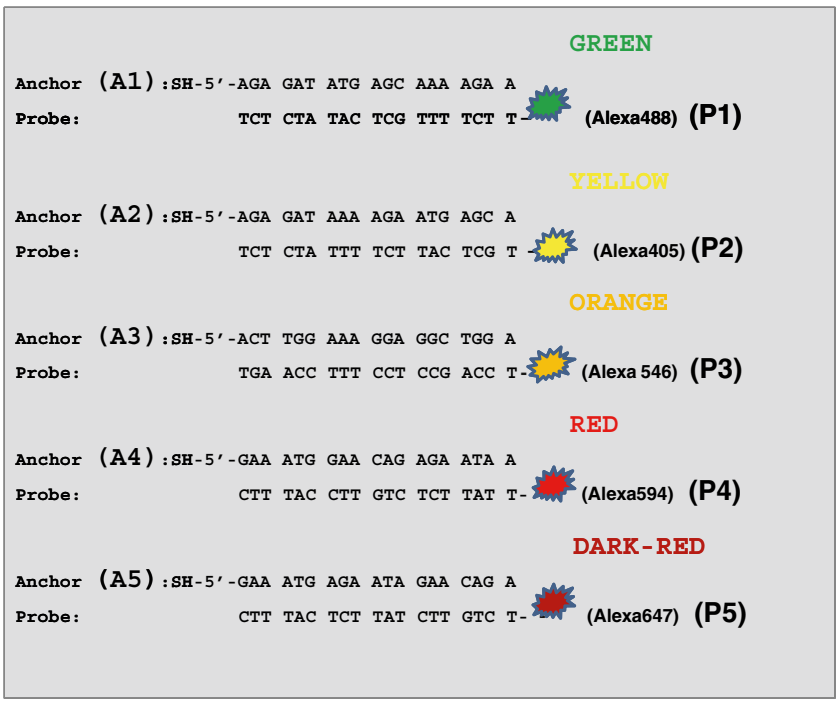

Table 1 Laser lines used for excitation of the Alexa-DNA fluorescence and the chromophore relative brightness

DNA labels	Excitation Laser line (λex), nm	Dye brightness at λex (ε × φ, 10–3 M-1 cm-1)
Alexa 488	473	33
Alexa 514		12
Alexa 546	532	43
Alexa 594		13
Alexa 647	633	44

Dye brightness calculated for different wavelengths, corresponding to the laser excitation wavelength; ε and φ are molar extinction coefficient and quantum yield of the chromophore, respectively

However, as encouraging as our published assays to date have all been, all the assays to date have been monoplex, i.e. detecting only one DNA in a sample. Subsequently, in this paper, we have focused on mulitiplexing the MAMEF technology to detect as many as 5 different DNAs, all from within one sample well, and all within a few seconds. The significance of our approach cannot be overstated with respect to the potential for human health safeguard and diagnosis. It should be noted, that we do not attempt to demonstrate molecular level sensitivity in this paper, moreover we are demonstrating that the MAMEF has the potential to be multiplexed for real world assays, potentially at sensitivity and specificity levels we have published to date for monoplex real world assays in a variety complex matrices, e.g. whole blood, vaginal mucus and feces [10–13].

Materials and Methods

Premium quality Silane-Prep™ glass microscope slides for silver island film (SiFs) preparation were purchased from Sigma-Aldrich. Reagents such as silver nitrate (99.9 %), *d*-glucose and sodium hydroxide were obtained from Sigma-Aldrich.

Five 19 bases long DNA oligos of different sequences were ordered from Integrated DNA Technologies, IDT Inc. (Fig. 2). Since these DNA fragments contain sequences from Chinese hamster ovary (CHO) Alu DNA sequence, they can serve as the markers for clinical diagnostics. The complimentary probe-DNAs were labeled with Alexa^{TM} dyes: 488, 514, 546, 594 and 647. The optical properties of the dyes conjugated to probe-DNAs are shown in Table 1 and Table 2.

Silver deposition on glass slides to make SiFs was undertaken as described below and extensively characterized in [14]. 60 ml (0.83 % w/v) of Silver Nitrate solution is added to a 100 ml beaker with constant stirring. 200 μL of NaOH (5 % w/v) and 2 ml of ammonium hydroxide (28 % w/v) were subsequently added, the beaker covered with aluminum foil and placed on ice for 10 minutes. Subsequently, 13 ml of 4.8 % w/v glucose was added. Silane Prep™ amino-coated glass 1*3 inch microscope slides (Sigma), lightly washed beforehand with deionized water, are subsequently added to the solution, the solution heated rapidly on the hot plate to 40 C. After 3 minutes the slides are removed from the solution and stored in deionized water. The optical density of the silver coated slides is typically 0.4 at λ_{max}, ~ 380–420 nm.

For attachment of anchor-DNA to SiFs we employed thiolated single-stranded DNA activated by the addition 6 mM DTT to 1,000 μL DNA solution. Conjugation of the DNA to the SiFs was performed by incubation of the activated Anchor-DNA on the SiF surface for 1 hour at ambient temperature or *secondly* by 30 sec irradiation of the solution in a microwave cavity (GE Compact Microwave Model: JES735BF, frequency 2.45 GHz, power 700 W).

Fluorescence spectra of the labeled DNA were recorded using a HD2000 Fiber Optic Spectrophotometer from Ocean Optics, Inc. Excitation of the chromophore-labels attached to DNAs was undertaken using 473, 532 or 633 nm CW laser lines, Table 1. To cut-off excitation light from the fluorescence detection channel the same wavelength long-pass filters were used.

DNA hybridization was undertaken in wells by adding 80 μl fluorophore labeled probe-DNA (≈1 nM, TE buffer) to the anchor DNA, already anchored on the SiFs surface, and the corresponding exposure to microwave radiation with 20 % of 140 W power in the cavity. Microwave irradiation was used to promote DNA annealing [7].

3.0. Results and Discussion

In this work we have used five different fluorescent dyes to label five different DNA sequences, (Fig. 2) the optical

Table 2 Optical properties of the dyes conjugated to DNA

Dye	Absorption max, nm	Emission max, nm	*Extinction coefficient, M-1 cm-1	*Quantum Yield (Q)	Lifetime (τ), nsec
Alexa-488	492.6	515.3	71,000	0.92	4.2
Alexa-514	515.6	538.5	80,000	-	4.0
Alexa-546	556.0	570.3	104,000	0.79	4.0
Alexa-594	587.2	609.2	73,000	0.66	4.0
Alexa-647	650.6	664.7	239,000	0.33	1.1

*-data were taken from Invitrogen (www.invitrogen.com)

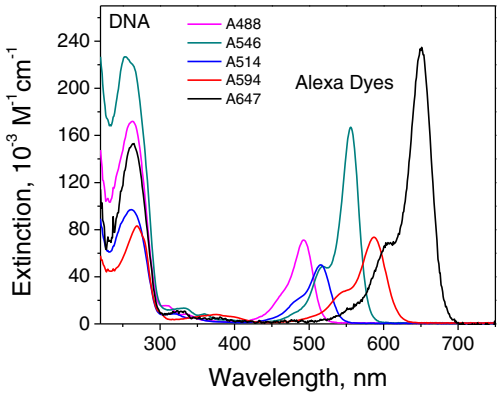

Fig 3 Molar extinction spectra for the set of chromophores used in the 5-Color DNA assay

parameters of which are shown in Table 1, with the absorption and fluorescence spectra presented in Figs. 3 and 4. The brightness of the selected dyes is notable and is in the range 12 – 44 M^{-1} cm^{-1}, which makes them highly suitable for the anchor-DNA/probe-DNA hybridization based-sensing.

A real-color photograph of the anchor-DNA scaffold attached to a SiF-glass slide is shown in Fig. 5. The thin silver layer, consisting of surface-deposited metal nanoparticles, changes color upon conjugation with thiolated DNA. This color effect can be used as an indication of DNA-SiF conjugation. Such DNA/SiF slides, but prepared using different DNA sequences and their different mol/mol mixtures, were used in this study.

For development of the multiplexed multi-color DNA detection assay we have used the principle of microwave-accelerated DNA hybridization, originally developed in our laboratory [7, 8, 15]. In a microwave field, the process of DNA hybridization is significantly accelerated, the time of annealing decreasing from hours to only a few seconds [16].

Another principle that has been utilized in this study is metal-enhanced fluorescence (MEF). The length of DNA fragments employed in the assay was specifically designed to keep the signaling chromophores on a short leash, ~ 6 nm, Fig. 6, from a silver film surface, ensuring the largest

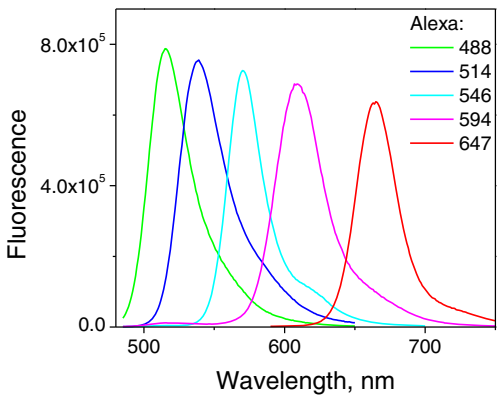

Fig 4 Fluorescence spectra of the chromophores used in the 5-Color DNA assay

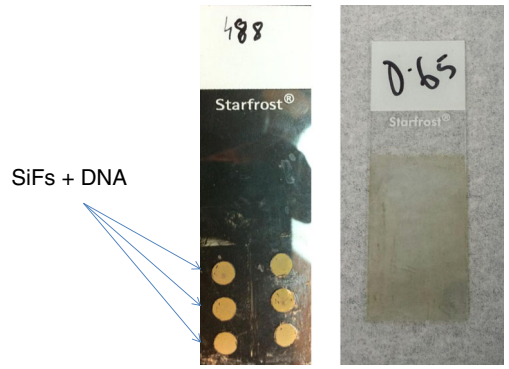

Fig 5 (**Left**) Real-color photograph of the silver surface (SiFs) attached with DNA surface anchor scaffolds. Incubation of DNA solution on SiFs was aided with rubber wells, attached to the slide surface, which formed defined sensing volumes. After incubation, the rubber wells were removed from the slide, as shown in the photograph. (**Right**) – A typical SiFs slide before DNA attachment

enhancement (MEF) of their fluorescence response [7, 16], and, consequently, a high sensitivity of DNA detection, which is extremely important for the development of a highly sensitive multiplexed DNA detection assay.

Figure 7 (a) shows an example of the fluorescence spectra of labeled probe-DNA hybridized with the anchor-DNA/SiF surface. The total amount of anchor-DNA, complementary+ non-complementary DNA, was constant. The fractional amount of the complementary anchor-DNA loaded on SiF was varied from 100 to 20 %. After hybridization of the specific Alexa 647-probe-DNA with anchor-DNA the fluorescence spectra intensity follows the concentration of complementary DNA on SiF. Fig 7 (b) shows a similar result for another probe-DNA sequence, which was labeled with Alexa 514. The obtained linear dependences of hybridized DNA upon the fractional amount of complementary anchor-DNAs on the surface demonstrates that attachment of different DNA sequences to SiF is *independent* and *proportional* to their relative concentrations in the loading solutions and, respectively, on the SiF surface.

Subsequently, as the number of analyzed DNA sequences from within one well increases, the fluorescence signal

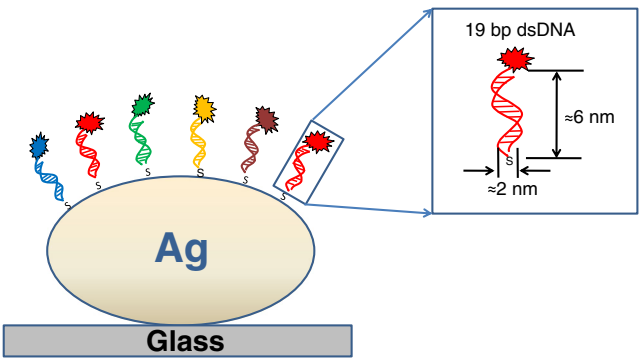

Fig 6 Five different anchor DNAs are attached to SiFs, each of them recognizes and hybridizes with their complementary respective probe-DNA

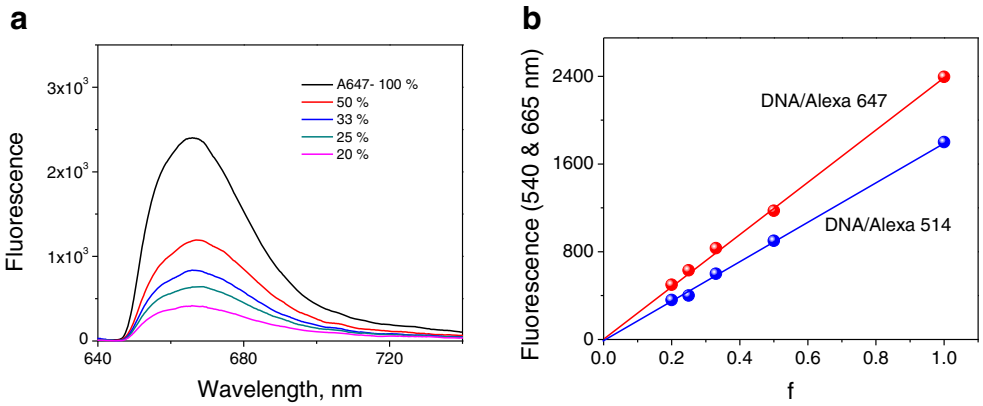

Fig 7 (**a**) Fluorescence spectra of DNA/Alexa 647 recorded for the different loading ratio of DNA/Alexa-647 to the total loaded onto SiFs DNA. (**b**) The dependences of DAN/Alexa647 and DNA/Alexa-514 fluorescence upon the fraction of these labeled DNA on SiFs surface. Attachment of different types (sequences) of DNA to SiFs is *independent* and *proportional* to their relative concentration in the loading solution

exponentially decreases for all analyzed DNAs, Fig. 8, due to the reduced number of complimentary surface capture DNAs. This result also assumes that DNA hybridization ("catch") on a surface is highly specific. Fig 9 shows the results of cross-selectivity in DNA/DNA molecular recognition/hybridization. In this experiment, hybridization was accelerated by using microwave irradiation. For the five different DNA sequences attached to the SiF-surface, the molecular recognition was highly selective. The sequence specific anchor/probe DNA hybridization have shown a high value of the cross-selectivity>98 %. This result is important for the development of highly plexed assays.

The optical properties of the dyes undergo significant change upon probe-DNA attachment to the SiF-surface. In particular, in bound to SiF state, as compared to the bulk solution, their fluorescence lifetime dramatically decreases from typically nanoseconds to picoseconds (Fig. 10), while the emission intensity significantly enhances due to the metal-enhanced fluorescence (MEF) effect [5, 6]. The important consequence of the fluorescence excited state lifetime reduction is an increase in dye photostability, since the fluorophore is less prone to photo-oxidation or other excited state processes, which is demonstrated in Fig. 11 by the profiles of dye photobleaching during constant irradiation, (Alexa-546)-DNA attached to SiFs and free in solution above a glass substrate. Enhanced photostability of dyes plays an important role in maintaining sensitivity, reliability and repeatability of the assay. As shown in Fig. 11, there is a significant enhancement in dye photostability on the SiFs.

Figure 12 a-c shows fluorescence spectra collected from one well containing five different DNA sequences annealed with labeled complementary target probe-DNAs. Five types of DNA were attached to a silver film in equimolar ratios and, subsequently, five different fluorophores were evenly distributed on the SiF well bottom. The fluorescence signal from a well was collected using three excitation wavelengths: 473, 532 and 633 nm, corresponding to the absorption spectra of the dyes, Fig. 3. Fig 12 (a) shows fluorescence spectra of two labeled probe-DNAs (P1 and P2, Fig. 2), DNA/Alexa-488 and DNA/Alexa-514. Fluorescence was excited using a 473 nm laser line. Notably, no other DNA labels contribute to the observed fluorescence. Upon excitation at 532 nm another two labeled probe-DNAs (P3 and P4) signal the hybridization event: DNA/Alexa-546 and DNA/Alexa-594. Hybridization of the fifth DNA sequence (P5), labeled with Alexa-633, can be solely "visualized" using an excitation wavelength of

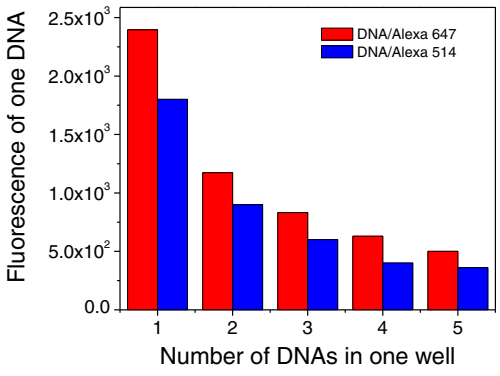

Fig 8 The change in fluorescence signal strength upon the number of DNA molecules in one well. The dependence was measured for two different specific DNA markers labeled with different chromophores (Alexa-647 and −514)

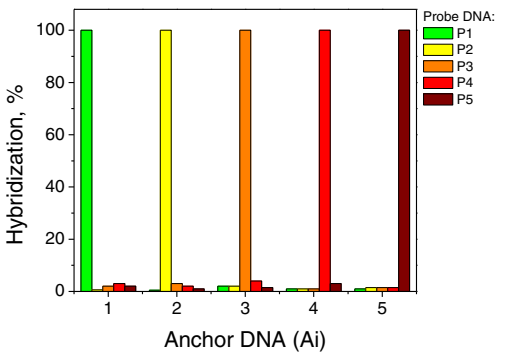

Fig 9 Cross-selectivity of the DNA-DNA molecular recognition (hybridization). Hybridization of a Probe DNA with the Anchor DNA scaffold on SiF was accelerated by microwave irradiation (30 sec). Microwave acceleration induces highly specific hybridization of the probe-DNA with the complementary anchor-DNA attached to SiFs. Cross selectivity is>98 %

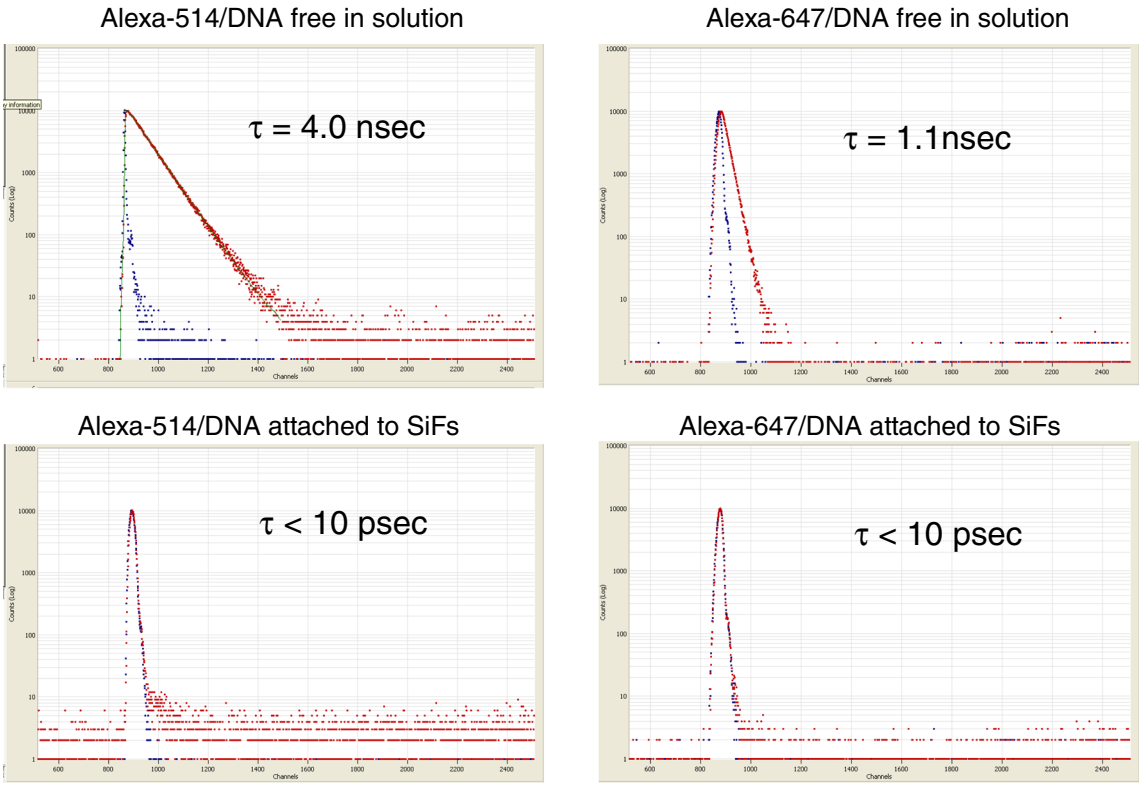

Fig 10 Attachment of the Alexa/DNAs to SiFs dramatically decreases excited state lifetime (τ) of the dyes and, consequently, increases their photostabilty. Time-resolved fluorescence decay profiles were measured by time-domain approach. For the Alexa chromophores attachment of Dye/DNA to SiFs chnages their lifetime from 1 – 4 nsec to<10 psec, i.e. more than 100-fold

633 nm. Consequently, for the first step of using three excitation wavelengths and measuring/observing the visible light signal (color), one can distinguish three sets of DNA sequences: Set I=(P1+P2), Set II=(P3+P4) and Set III=P5. The first and second sets can potentially contain two different DNA sequences.

Further analysis can be applied to separate the contributions of the individual DNA sequences to Sets I and II. For that purpose, the observed fluorescence spectra can be deconvoluted into two components using the following equation:

$$S_{obs}(\lambda) = A_1 \times S_1(\lambda) + A_2 \times S_2(\lambda), \quad (1)$$

where $S_{obs}(\lambda)$, $S_1(\lambda)$ and $S_2(\lambda)$ are the observed fluorescence spectra and standard fluorescence spectra measured for separate components, $S_{obs}(\lambda)$ is normalized to 1.0; A_1 and A_2 are the fractional contributions of the components to the observed spectrum. Fig 12 (a) and (b) show the results of fitting for DNA's Set I and Set II. The best fit gives the following contributions of components to the observed spectra, Set I: $A1/A2=F_{max}(\text{Alexa-488})/F_{max}(\text{Alexa-514})=0.57/0.43$; Set II: $A1/A2=F_{max}(\text{Alexa-546})/F_{max}(\text{Alexa-594})=0.80/0.20$, F_{max} is a maximum fluorescence intensity (Fig. 12). This result was obtained for the case when all DNA sequences are hybridized in an equimolar ratio to the DNA scaffold. The change in ratio (A1/A2) is an indication of the change in amount of analyzed target DNA in solution. For example when one DNA sequence in a Set is absent it will be indicated in A1, A2 values, i.e. A1=1, A2=0 or vise versa, depending on the sequence.

In this study we have used a simplified 2-piece DNA model (Fig. 1) to investigate characteristics and spectral properties of the multiplexed DNA detection assay, which is based on DNA-RCS technology, i.e. "Rapid Catch and Signal". For

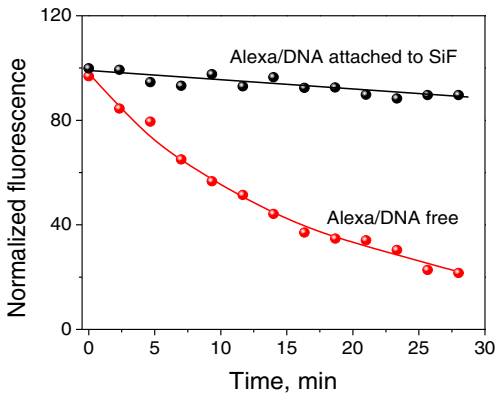

Fig 11 Photobleaching profiles of Alexa546-dsDNA attached to SiFs and free Alexa546-ssDNA on glass. Laser power – 5 mW. Excitation – 532 nm. Photostability of the DNA labels (Alexa dyes) attached to SiFs is greater than that of free dyes in solution

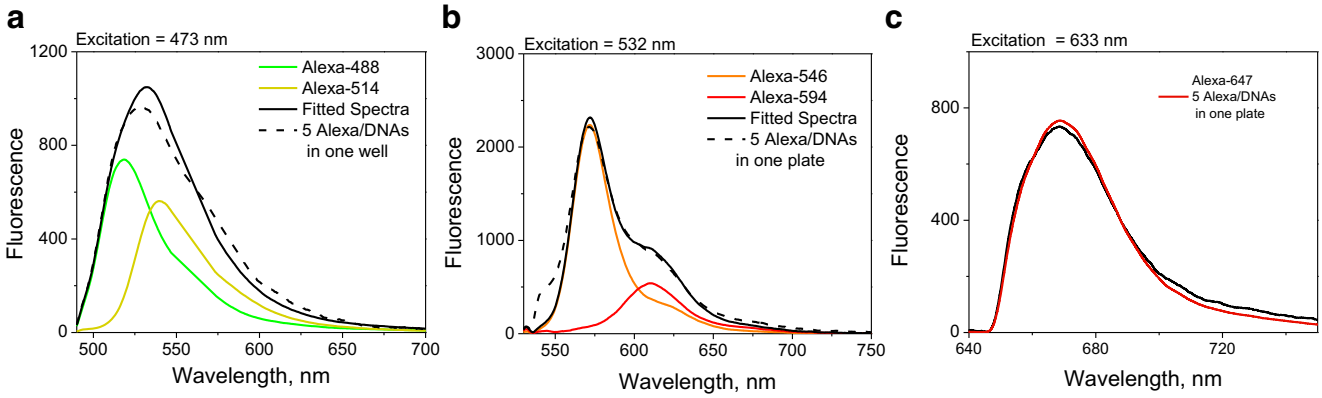

Fig 12 Fluorescence from one wells containing five different labeled DNAs in equimolar ratio. (**a**)-(**c**) Fluorescence spectra of five Alexa-DNAs excited by three different lasers ((**a**) 473 nm, (**b**) 532 nm and (**c**) 633 nm). Recorded spectra were deconvoluted on their components. The best fitting curves (spectra) and their individual components are show in (**a**) – (**c**) graphs

the purpose of DNA detection in real world samples the 3-piece DNA system can be used (Fig. 13). It consists of an anchor-DNA that forms a detection scaffold on the silver film surface, labeled probe-DNA and target DNA sequence, which is a fragment of the DNA of interest, in particular, it can be a fragment of target genomic DNA. Both anchor- and probe-DNAs can readily be designed to be complementary to the specific genome sequence, for the purpose of highly selective specificity of recognition/ hybridization, similar in principle to DNA primers designed for PCR. Previously we have successfully used the 3-piece DNA system in monoplexed DNA assays to detect single DNA sequences at PCR level sensitivities [10–14].

Conclusions

We have developed a multiplexed DNA detection assay which is able to detect five different DNA sequences in one sample from only one well. Our DNA-RCS technology employs the principles of MAMEF, i.e. microwave-accelerated intermolecular recognition and metal-enhanced fluorescence.

In our designed geometry the fluorescent labels are positioned at a close distance to the silver nanoparticles, which facilitates strong MEF. As a consequence, the dyes become extremely photostable and subsequently long-lived, a property which can readily be used to gain enhanced sensitivity of the assay by collecting/integrating the fluorescent signal over a long time period.

Another advantage of our multiplexed five-color DNA assay is the ability to detect five different DNA sequences without a neighboring effect, i.e. showing a high degree of cross-selectivity, > 98 %, and little to no effects of other close-proximity DNA anchors and fluorescent probes., e.g. no FRET.

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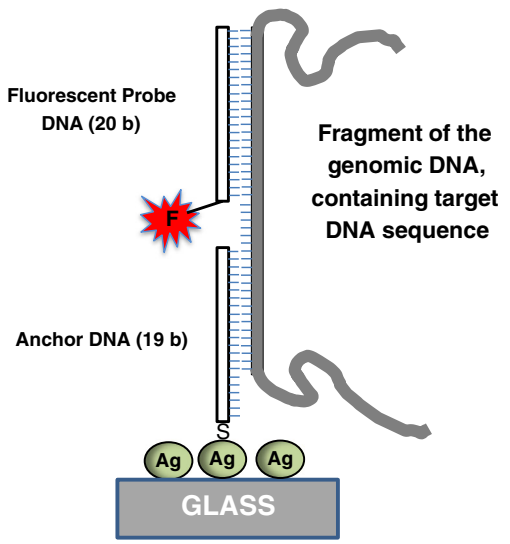

Fig 13 Principles of the 3-piece DNA assay scaffold

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