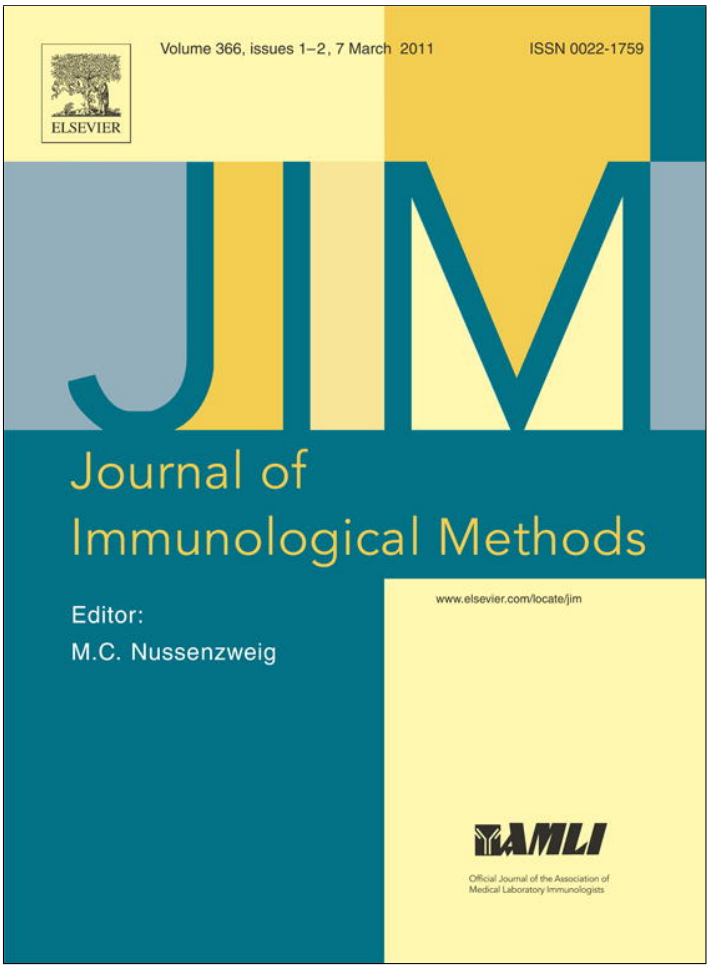

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Research paper

Two-color, 30 second microwave-accelerated Metal-Enhanced Fluorescence DNA assays: A new Rapid Catch and Signal (RCS) technology

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ABSTRACT

For analyses of DNA fragment sequences in solution we introduce a 2-color DNA assay, utilizing a combination of the Metal-Enhanced Fluorescence (MEF) effect and microwave-accelerated DNA hybridization. The assay is based on a new "Catch and Signal" technology, i.e. the simultaneous specific recognition of two target DNA sequences in one well by complementary anchor-ssDNAs, attached to silver island films (SiFs). It is shown that fluorescent labels (Alexa 488 and Alexa 594), covalently attached to ssDNA fragments, play the role of biosensor recognition probes, demonstrating strong response upon DNA hybridization, locating fluorophores in close proximity to silver NPs, which is ideal for MEF. Subsequently the emission dramatically increases, while the excited state lifetime decreases. It is also shown that 30 s microwave irradiation of wells, containing DNA molecules, considerably (~1000-fold) speeds up the highly selective hybridization of DNA fragments at ambient temperature. The 2-color "Catch and Signal" DNA assay platform can radically expedite quantitative analysis of genome DNA sequences, creating a simple and fast bio-medical platform for nucleic acid analysis.

1. Introduction

Identification of living organisms (eukaryotes, bacteria, viruses etc.) by means of quantitative analysis of their specific DNA sequences, which represent different genomes, is a challenging aim, which faces many scientists today. It also concerns the search and detection of different microorganism mutations and strains of pathogenic bacteria and causes of severe diseases in humans. Significant progress in the last few decades was achieved by the discovery and implementation of the PCR approach for genetic material analysis (Mullis, 2003; Saiki et al., 1985). This method is exceptionally sensitive to a small amount of DNA in solution, due to a huge amplification of the selected DNA sequence by numerous cycles of replication. In other words, the PCR method is based on an *artificial increase of the amount of DNA, containing the specific target sequence*, which then can be easily detected by common analytical methods. Despite the obvious advantage of PCR in DNA detection this approach has several disadvantages (Bae and Sohn, 2010; Chiminqgi et al., 2007; Tonooka and Fujishima, 2009), e.g. sensitivity to DNA material contaminants, misreading, quite high cost of analysis, reagents and time to fulfill experiments, and, most importantly, limits in its application as a general fast and easy Point-of-Care method of specific DNA sequence quantification.

Another approach for DNA quantitation is based on the *direct detection* of a small amount of DNA in solution, i.e. *without amplification of the DNA material*. It is based on detection of the

Abbreviations: MEF, Metal-Enhanced Fluorescence;NP, Nanoparticle;SiF, Silver island film;ssDNA, Single stranded DNA;PCR, Polymerase chain reaction;RCS, Rapid Catch and Signal technology.

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bright emission of dyes bound to nucleic acids (Dragan et al., 2010a,b; Lakowicz, 2006). Most popular chromophores for this approach are ethidium bromide, PicoGreen and Syber Green I, which bind DNA non-covalently and dramatically increase their fluorescence yield. For example, the last two chromophores increase their brightness almost 1000-fold upon binding to double-stranded DNA (Cosa et al., 2001; Dragan et al., 2010a,b; Singer et al., 1997; Zipper et al., 2004). It makes them extremely sensitive to a small (<ng/ml) amount of DNA in solution. Moreover, recently we have shown that in the presence of silver nanoparticles, due to the Metal-Enhanced Fluorescence (MEF) effect, the sensitivity of PicoGreen and Syber Green I to dsDNA can be significantly further increased and become comparable to the sensitivity of the PCR technique, i.e. to be in the range of ~pg/ml (Dragan et al., 2010). The great benefit of this approach is its fast, DNA-specific and inexpensive method of DNA quantitation. Disadvantages of this approach include a lack of DNA sequence specificity, which makes it unfeasible to employ directly in analysis of genome-specific DNA samples.

A remarkable improvement of this technique has been achieved by the combination of two approaches: microwave-accelerated sequence-specific hybridization of the target DNA with anchor DNA, immobilized on a metal surface, and the Metal-Enhanced Fluorescence (MEF) effect, responsible for the large enhancement of a DNA's fluorescent label. Whereas the interactions of fluorophores with metallic nanoparticles has been known for some years (Drexhage, 1970; Persson, 1978), the precise mechanism for enhanced fluorescence is still of some debate (Geddes, 2010). Some authors describe the interaction as due to a modification in the radiative decay rate of a fluorophore (Lakowicz et al., 2002; Lakowicz, 2006), while Metal-Enhanced Fluorescence (MEF), described by Geddes in 2002 (Geddes and Lakowicz, 2002), gave raison d'être to this effect as due to a near-field coupling between an excited state dipole and induced surface plasmons, the surface plasmons in turn radiating the coupled quanta (Geddes and Lakowicz, 2002). Fig. 1 shows a graphical representation of the general principles of MEF and our "Catch and Signal" technology. The MEF effect, i.e. enhancement of a fluorophore's brightness, exponentially depends on the distance between chromophore and metal nanoparticle, due to a short-range (0–30 nm) coupling of a chromophore's excited state electronic system with nanoparticle (NP) plasmons (Fig. 1a). As a result, only chromophores proximal to NPs increase their emission a hundreth thousand fold. Subsequently, hybridization is not only the event of a specific recognition of a target DNA but also the creation of the MEF pair (fluorophore–NP plasmons), which enhances the fluorescence signal. Duplex annealing puts a fluorescent label on a short (~7 nm) enough leash, relative to a NP, thereby in a perfect condition for intense MEF. A significant addition to this technology is microwave "heating" of the reacting system, which significantly speeds up the process of DNA hybridization (Aslan et al., 2006), which is an important attractive feature of any bio-assay. In our recent publications we have presented a platform 1-color DNA assay, based on the "Catch and Signal" technology, for the detection of genome-specific DNA and demonstrated the power of this technique by the detection of DNA from *Bacillus anthracis* spores and the vegetative cells (Aslan et al., 2008).

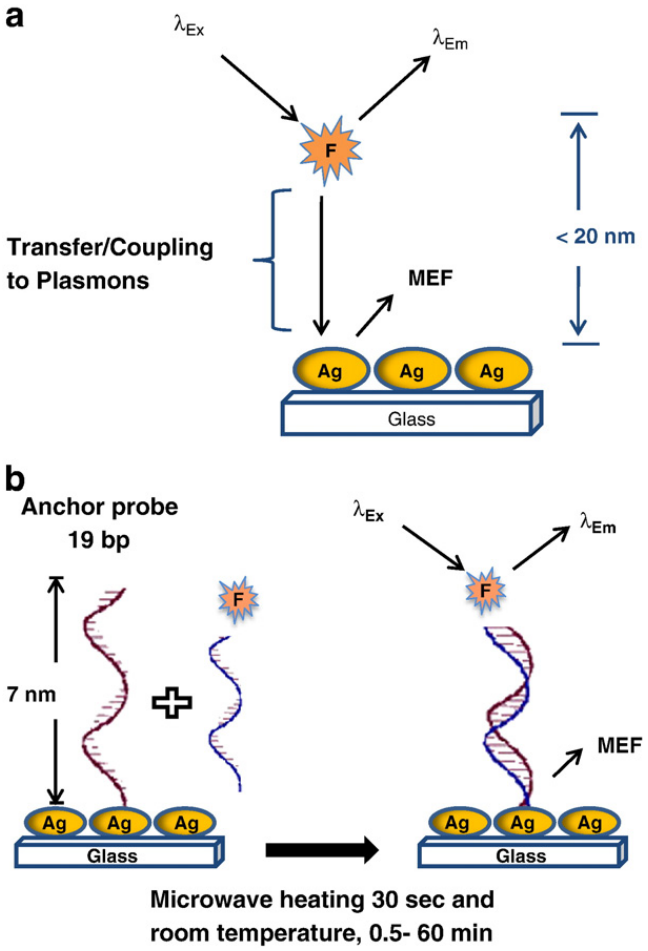

Fig. 1. (a) Graphical representation of the current interpretation of MEF. (b) MAMEF surface DNA capture assay, annealing in microwave cavity and the control assay run at room temperature, "Rapid Catch and Signal" technology.

In this short communication we present further development of the "Catch and Signal" technology—principles of a 2-color DNA assay for the simultaneous detection/quantification of two genome-specific DNAs in one well.

2. Materials and methods

2.1. Materials

Silver nitrate (99.9%), sodium hydroxide (99.996%), ammonium hydroxide (30%), *D*-glucose and premium quality APS-coated glass slides (75×25 mm) were obtained from Sigma-Aldrich.

2.2. The 19 base DNA sequences

The 19 base DNA sequences used in this study are fragments of Chinese hamster ovary (CHO) Alu sequence. Oligonucleotides were purchased from Integrated DNA Technologies, Inc.:

"Target" Green-DNA:

5′-/Alexa488N/TTC TTT TGC TCA TAT CTC T-3′

Complement, "Green-Anchor" thiolated DNAs:

5′-/5ThioMC6-D/ACT TGG AAA GGA GGC TGG A-3′

and

"Target" Red-DNA:

5′-/Alexa594N/TTA TTC TCT GTT CCA TTT C-3′

Complement, "Red-Anchor" thiolated DNAs:

5′-/5ThioMC6-D/GAA ATG GAA CAG AGA ATA A-3′

2.3. Preparation of silver island films (SiFs)

Preparation of silver island films (SiFs) on glass slides was performed as described previously (Aslan et al., 2008). Optical density of SiF-slides measured at the plasmon resonance absorption maximum (~410 nm) was 0.5–0.6 o.u.

2.4. Attachment of thiolated anchor DNA to SiF coated slides

The thiolated DNA, 5′/5ThioMC6-D/-ssDNA, contains a "cap" (5′-Thio-Modifier), which prevents DNA strands from spontaneous dimerization by formation of a S–S linkage. Subsequently, before attachment of the DNA to silver island films, the first step is an activation of DNA, i.e. a cleavage of a disulfide bond between a thiol-modifier group and DNA. DNA was subsequently resuspended in TE buffer, and 100 μl of DNA solution (43 μM) was mixed with 20 μl of DTT (250 μM in TE buffer) and incubated at ambient temperature for 30 min. The deprotected DNA solution was diluted 40-fold with TE buffer and immediately loaded on SiF-slides. Excess of the activated DNA in 6 mM DTT, TE buffer, pH 7.4 was stored at −20 °C. Incubation time of the activated DNA on the SiF surface was 1 h at room temperature.

2.5. Fluorescence measurements and real-color photographs

Measurements of fluorescence of the DNA samples were performed by collecting the emission intensity through an optic fiber, using a Fiber Optic Spectrometer (HD2000) from Ocean Optics, Inc., after excitation using 473 or 594 nm CW laser lines. Long-pass razor-edge filters were used to cut-off excitation light in the registration channel.

The real-color photographs of Alexa 488(594)-fluorophore-labeled DNAs, attached to SiFs, were taken with an Olympus digital camera (C-740, 3.2 Mega Pixel, 106 Optical Zoom) through the same long-pass filters as was used to record the emission spectra.

2.6. Microwave-accelerated and room temperature DNA hybridization

DNA annealing in wells was performed by the incubation of 70 μl of fluorophore-labeled DNA oligos (target DNA) with thiolated oligomers (anchor DNA), immobilized on SiFs, in TE buffer for 30 s in a microwave cavity (GE Compact Microwave Model: JES735BF, frequency 2.45 GHz, power 700 W). Microwave irradiation power was reduced to 20%, which corresponded to 140 W over the entire cavity. Room temperature DNA hybridization was performed using the procedure above, except that the assay was completed at room temperature instead of using microwave irradiation.

2.7. Kinetics of DNAs hybridization in wells

The hybridization kinetics of the target-ssDNAs with the target DNA, immobilized on the SiFs, was studied by measuring the fluorescence intensity from the wells at certain time intervals. For the DNA hybridization in individual wells, the reaction was stopped at different incubation times by washing well with buffer.

2.8. Thermal imaging of the samples upon microwave irradiation

Thermal imaging was undertaken using the procedure described previously (Previte et al., 2007). A sapphire plate 2.54 cm in diameter and 1 mm thick (Swiss Jewel), transparent for the infrared (IR) spectral region, was placed above the cavity opening to allow collection of the thermal image, i.e. the temperature distributions of the samples. The sapphire plate and the SiF-glass slide formed a sandwich with the DNA solution contained inside. The samples were inverted such that the SiF surface faced downward towards the registration channel. Infrared emission from the sample in the microwave cavity was imaged by reflecting the IR-radiation from a gold mirror onto a thermal imaging camera (Silver 420 M; Electrophysics Corp., Fairfield, NJ) that is equipped with a close-up lens, and provides a resolution of approximately 300 μm.

3. Results and discussion

3.1. Characterization of DNA hybridization on SiF surface

Attachment of anchor DNA to a SiF surface forms a DNA scaffold around the NPs, which can effectively catch a complementary fluorophore-labeled DNA, target DNA, and form double-stranded DNA. Upon the process of hybridization, the observed fluorescence intensity is linearly proportional to the amount of the chromophore-labeled target DNA that forms a duplex with an anchor DNA. The amount of hybridized duplex molecules is limited by the quantity of the target DNA, but cannot exceed the amount of the anchor DNA on the SiF-DNA scaffold. Therefore, the observed fluorescence intensity is limited and upon annealing, approaches saturation. Subsequently by measuring the fluorescence of the target DNA one can register kinetics of the annealing and estimate the percentage of hybridized DNA. Fig. 2 shows kinetics of hybridization of target DNA with an anchor DNA scaffold both with and without microwave irradiation. Remarkably, the hybridization of DNA proceeds almost immediately (<30 s) with microwave irradiation (MW) and, in the absence of microwave "heating", it takes almost 1 h to approach the same level of fluorescence signal. Subsequently, MW acceleration dramatically (~1000-fold) speeds up the kinetic process and, thus, shortens the time of the macromolecular recognition, which indeed is a critical factor for creation of fast bio-assays. The slow kinetics of duplex formation at ambient temperature are simply explained by:

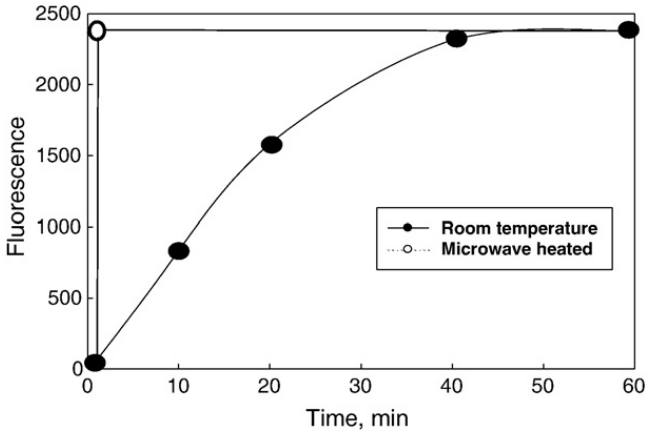

Fig. 2. Room temperature real-time hybridization kinetics for Alexa 594 nm and microwave acceleration for 30 s. The "Rapid Catch and Signal" assay is complete in less than 30 s.

quite slow diffusion of the target DNA from the solution to the DNA scaffold, formed on the silvered surface, and by competition between inter- and intra-molecular interactions (it is well known that single stranded DNA can form intra-molecular structures at room temperature (Jelesarov et al., 1999)). These two processes may significantly increase the time of hybridization (Aslan et al., 2006; Aslan and Geddes, 2006, 2008). The true nature of the MW effect on kinetic parameters of DNA annealing is not fully clear today, and it is assumed that MW irradiation influences both processes, i.e. rate of diffusion and competitive inter-molecular recognition (Aslan and Geddes, 2008). It is well known that MW irradiation, by interacting with a dipole moment of water molecules, increases the rate of this movement and subsequently enhances molecular thermodynamic temperature. Silver NPs, immobilized on a glass surface, cannot move and electrons, trapped in a small (100–300 nm) particle volume, as compared to MW radiation wavelength, are outside of the resonance condition and, consequently, could not absorb MW energy. (It should be noted that silver electrons (plasmons) in nanoparticles of that size can effectively absorb light (Geddes, 2010), with a maximum at ~400 nm, which is far away from the MW wavelength of ~12 cm.) Therefore intuitively, pulses of MW radiation applied to the reacting system, induce a rapid temperature gradient between the "cold" SiF surface and a solvent that would stimulate the rapid movement of molecules, due to mass transport and/or increased molecular diffusion within the wells. Interestingly, the total temperature of the solution in the wells does not sufficiently increase in this condition (see Fig. 1S). Another factor, which MW can provoke, is destabilization of intra-molecular structures in ssDNA, by disordering the hydration of the DNA polymer, which plays a significant role in stabilization of the DNA molecule conformation (Dragan et al., 2009; Privalov et al., 2007), and by increasing internal DNA strand flexibility. This ultimately accelerates specific inter-molecular hybridization.

3.2. Characterization of the 2-color DNA assay

The 2-color DNA assay is based on both the detection and registration of two color emissions, which correspond to two different target DNAs, which ultimately could be any DNA of interest. To reach good discrimination between the two different specific DNA sequences by means of color measurement we have used Alexa 488 and Alexa 594 dyes. Both dyes have large extinction coefficients (71,000 and 90,000 M^{-1} cm^{-1}, respectively), high brightness (quantum yield of the free dyes in aqueous solution—0.92 and 0.66, respectively) and emit light within different spectral regions: Alexa 488 is a green (520 nm) and Alexa 594 is an orange-red (620 nm) fluorescent chromophore, therefore their fluorescence spectral overlap is insignificant (see Fig. 2S, Supplemental material).

Fig. 3a shows emission spectra collected from the SiF bottom well after hybridization of the anchor DNA, attached to NPs, with the green target DNA. It should be noted that in all cases hybridization was accelerated by the 30 s MW irradiation at 20% low power. The real-color photograph shows a green spot of high intensity, corresponding to the emission spectrum, shown in Fig. 3a, insert. A strong fluorescence signal, taken from the mono-layer of the DNA scaffold, demonstrates the high sensitivity of the "Rapid Catch and Signal" (RCS) assay. Taking into account geometrical sizes of dsDNA, one can estimate the maximum density of dsDNA on the SiF surface, which is about (0.02 pmol DNA)/mm^2, i.e. quite a small amount. Meanwhile the fluorescence signal is strong due to the MEF effect and can be easily registered using fiber optic instrumentation. After hybridization with 19 base anchor DNAs, the distance of Alexa 488 dye, attached to the 5′-end of a target DNA, to silver NPs is about 7 nm. At this distance a strong coupling between chromophore and NP plasmons occurs and the expected enhancement of emission can be more than 100-fold (Aslan et al., 2005; Geddes and Lakowicz, 2002; Geddes, 2010). A similar result was obtained with the red target DNA sequence, also hybridized with the corresponding complement of any anchor DNA SiF-wells (Fig. 3b). A real-color photograph (Fig. 3b, insert) shows a bright red fluorescent spot excited by the 594 nm CW laser line.

The Metal-Enhanced Fluorescence effect (Aslan et al., 2005; Geddes and Lakowicz, 2002; Geddes, 2010), which underpins the 2-color ultra-high sensitivity DNA assay, also affords for a decrease in the excited state lifetime and, consequently, an increase in chromophore photostability (Dragan et al., 2010a; Geddes and Lakowicz, 2002; Geddes et al., 2003a,b). The nature of the lifetime decrease upon MEF is thought to be due to energy transfer from an excited state dipole of a chromophore to induce NP plasmons, due to near-field coupling between them (Aslan et al., 2005; Geddes, 2010), followed by surface plasmon emission of the coupled quanta (Geddes, 2010; Aslan et al., 2005; Geddes and Lakowicz, 2002; Geddes et al., 2003a). Subsequently, chromophores with a reduced excited state lifetime are more resistant to photo-oxidation or other excited state processes, which ultimately influence fluorophore stability and the observed intensity over time. We have subsequently measured the intensity decay functions of the chromophores (Alexa 488 and Alexa 594), attached to DNA in solution and in the SiF/DNA complex (Fig. 4a and b, Table 1). The observed decay functions for both chromophores clearly show a drop in the observed lifetime on SiFs, as compared to solution. The results correlate well with the enhanced photostability of the dyes (Fig. 3S). This result suggests a significant advantage of employing short (~20 base) DNA fragments, attached to a

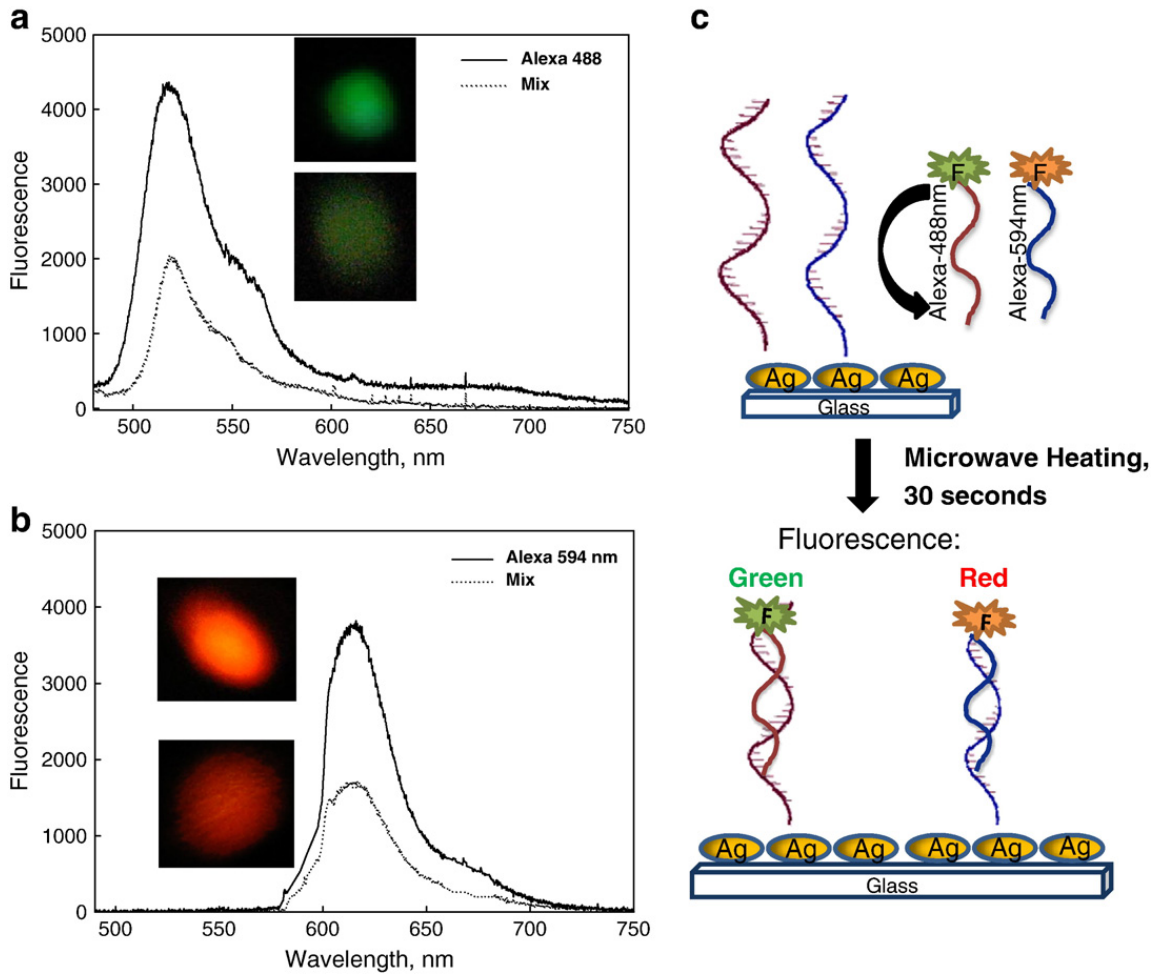

Fig. 3. (a) Emission spectra of Alexa 488 and a 1/1 mixture of both Alexa 488 and 594 dyes attached to a DNA/SiF film. Excitation was undertaken with a 473 nm laser line. (b) Emission spectra of Alexa 594 and a 1/1 mixture of both Alexa 488 and 594 dyes attached to a DNA/SiF film. Excited with a 594 nm laser line. (c) General scheme of 2-color DNA assay.

silver NP surface, for achieving both strong MEF and photostability of fluorescent labels and, ultimately, in establishing an ultra-high sensitive and non-photobleached fluorescence-based DNA quantitation assay.

The 2-color DNA assay employs a mixture of two types of anchor DNA on one surface with the "catching and signaling" of two different target DNAs. This system is quite complex and there are, at least, two conditions at which the 2-color DNA quantitation assay needs to be optimized: a) homogeneous covering of the silver surface with DNA that does not depend on the type of DNA fragments, i.e. the ratio of different DNA fragments in solution and on the surface should be the same; b) the density of DNA on SiFs should be low enough to prevent energy migration (FRET) between labeled DNA molecules, which otherwise would result in selective quenching of one reporting chromophore and enhanced emission of the other. Subsequently, we have studied the system, when both anchor DNAs, complementary to the green and red target DNAs, were mixed at 1/1 mol/mol ratio and incubated on SiF-slides, to get equimolar amounts of 2-anchor DNAs surface. The prepared mixed-scaffold DNA was then hybridized with an equimolar mixture of the two target DNAs and the fluorescence from the wells was analyzed. Fig. 3a and b show the fluorescence spectra collected from the wells. Emission was excited using 473 and 594 nm laser lines, respectively. It is notable and indeed encouraging that the intensity of fluorescence in both cases was 50% of the intensity taken from each well, containing only one kind of anchor DNA, recalling that the DNA was mixed in a 1/1 ratio. This result clearly shows that a) the attachment of two different thiolated anchor DNAs to silver NPs is proportional to the ratio of molecules in the loading solution; b) hybridization of a target DNA to its complement is highly sequence-specific and c) finally that no FRET occurs between the Alexa 488 and Alexa 594 dyes, which is strongly suggested by the measured ratio of fluorescence intensities (i.e. 50% in our case). For the pair of Alexa fluorophores used, the Forster distance is $R_o = 6$ nm, and subsequently we can intuitively suggest that the average distance between labeled DNA on the silver surface is therefore larger than 6 nm.

3.3. Benefits of the Rapid Catch and Signal technology

In this manuscript we have shown the proof of concept for a rapid, <30 s, 2-color DNA hybridization assay platform. The

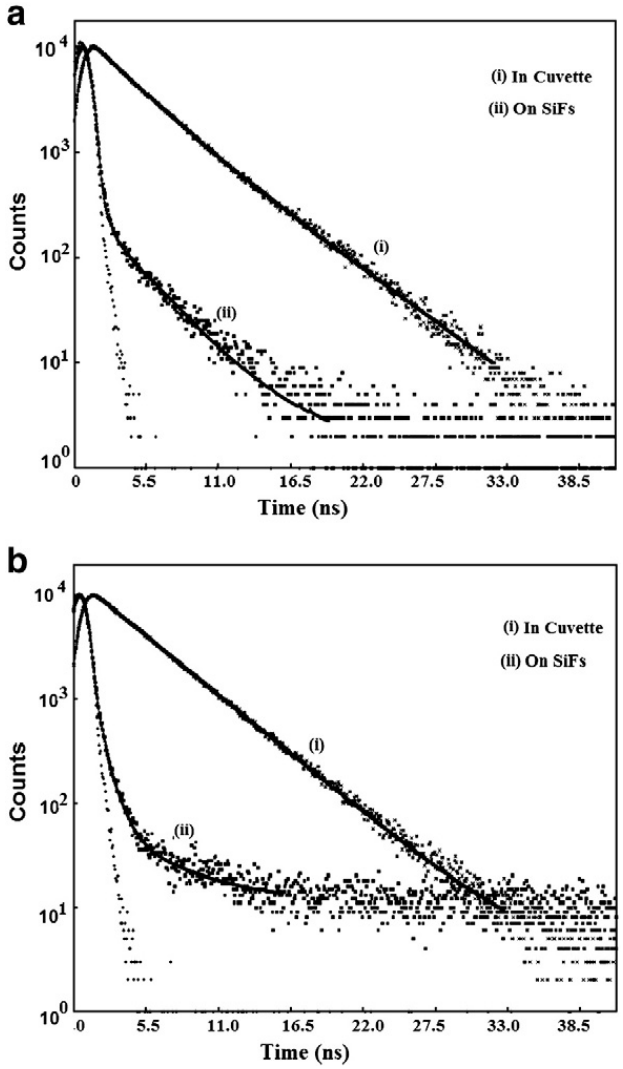

Fig. 4. Intensity decay profile of (a) Alexa 488 and (b) Alexa 594 on glass, a SiFs sandwich and in solution (cuvette) $\lambda_{ex} = 467$ nm.

new platform technology has many significant benefits, including:

- Two DNA targets can be detected within 30 s. The 2-target DNA assay can use any 2-color fluorophores, where the best results will be obtained for the condition where little to no spectral overlap occurs.
- The Rapid Catch and Signal (RCS) technology can target any genome of interest by using specific DNAs as anchor-probes, which encode for the specific genome of interest.
- The assays are highly photostable, considerably more so then in the absence of silver. This ultimately allows for the collection of data over much longer times than is traditionally used.
- While not shown here, we have recently developed an analogous 1-color, 200 genome copy, 20 second assay platform using a 3-piece DNA scaffold construct. Our current RCS technology, described here, could readily be used in this format for the detection of two DNAs. Work is currently underway in this regard and will be reported in due course.
- The new RCS technology is suitable to be used in conjunction with previously reported rapid (<15 s) bacteria lysing strategies (Aslan et al., 2008), suggesting that DNA from organisms can now be lysed, detected and quantified in a 2-plex embodiment, with a total measurement time of less than 1 min.
- The "Rapid Catch and Signal" technology could also significantly improve sensitivity and specificity of chip-based assays, e.g. oligonucleotide microarrays (DNA chip)-based hybridization analysis (Hacia, 1999), which are widely used nowadays for analysis of all possible mutations and sequence variations in genomic DNA.

Table 1
Fluorescence intensity decay parameters for Alexa 488 and Alexa 594 dyes attached to DNA, bound to SiF, and free in solution.

Sample	τ_1, ns	α_1%	τ_2, ns	α_2%	$\langle\tau\rangle$, ns	$\overline{\tau}$, ns	χ^2
(a) Alexa 488 in cuvette	4.80	61	2.63	39	3.95	4.23	1.049
(b) Alexa 488 on vSiFs	3.29	6	0.12	94	0.32	2.13	2.034
(a) Alexa 594 in cuvette	4.45	78	3.20	22	4.17	4.23	1.056
(b) Alexa 594 in SiFs	2.07	7	0.14	93	0.27	1.156	1.811

$\langle\tau\rangle$—amplitude-weighted lifetime; $\overline{\tau}$—mean lifetime.

4. Conclusions

The "Rapid Catch and Signal" RCS technology has been applied to a 2-color DNA assay, which shows high sensitivity, sequence specificity and DNA quantitation ability. The DNA scaffold formed on the SiFs is proportional to anchor DNAs fragment molar concentration ratio in the loading solution. The density of double-stranded DNAs on the silver surface is sufficiently low enough to avoid inter-molecular energy transfer (FRET) between labels (Alexa 488 and Alexa 594), i.e. an average distance between duplexes >6 nm (Förster distance for the donor (Alexa 488)–acceptor (Alexa 594) pair is 6 nm). "Catching" of the complementary target DNAs using the DNA scaffold on a surface is sequence-specific. Our results clearly show that the 2-color DNA assay can effectively be employed as a new "Rapid Catch and Signal" technological platform in the creation of an ultra-sensitive, sequence-specific approach for the fast analysis of genetic material from different organisms, for potential analysis of bacteria and virus pathogens, and search for possible mutations and sequence variations. This technology being fast, ultra-sensitive and inexpensive can effectively compete with the PCR technique, especially for the routine and rapid analysis in Point-of-Care settings and bio-medical laboratories.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jim.2010.12.002.

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