Detection of fluorescent-labeled probes at sub-picomolar concentrations by magnetic modulation

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Abstract: A sensitive and rapid method for detecting fluorescent dyes at low concentrations in homogenous solution is experimentally demonstrated. Fluorescent-labeled DNA probes are detected by attaching magnetic beads and applying alternating magnetic field gradient. This condenses the fluorescent probes into a small detection volume and eliminates the scattering noise from solution by synchronous detection. For DNA probes concentration of $1 \cdot 10^{-13}$ M the detection signal was 3.3 times higher than the noise, thereby implying detection sensitivity of $3 \cdot 10^{-14}$ M.

OCIS codes: (170.6280) Spectroscopy, fluorescence and luminescence; (170.2520) Fluorescence microscopy;

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1. Introduction

Sensitive and rapid detection of fluorescent labels at low concentrations is a well known challenge in many biological applications. Fluorescent-labeled biosensors are widely used in detection of specific DNA sequences and protein-protein interactions. For example, low levels of cell-free fetal DNA are present in the plasma of pregnant women. Analysis of this DNA can provide a non-invasive method of fetal genotyping [1]. Furthermore, diseases such as sickle cell anemia, Alzheimer's, cystic fibrosis and certain cancers are associated with changes in the sequence of particular genes. These changes can serve as biomarkers and may be useful for medical diagnosis at early stages of the disease [2].

In general, current approaches to detect specific DNA sequences require pre-amplification of genomic DNA via polymerase chain reaction (PCR). However, PCR is costly, time consuming and laborious. Alternatively, DNA sequences can be directly detected from unamplified genomic DNA, e.g. by using fluorescent dyes. To improve sensitivity, a long non-PCR pre-amplification phase is generally required (e.g. rolling circle amplification [3]). Another approach also uses fluorescent dyes; however, the detection sensitivity is improved by time-resolved fluorescence detection (e.g. single molecule fluorescence burst detection [2]). A third approach uses nanoparticles and a detection system which is either electrical [4] or optical [5, 6]. While the last two methods usually offer higher sensitivity and faster results, the required analysis time may still be relatively long. Moreover, some of these methods require washing and separation steps which complicate the detection process.

In this paper we present a new approach for rapid and sensitive detection of fluorescent dyes in homogenous solution at low concentration. The main challenge in specific DNA sequence detection while using fluorescent-labeled probes is the fact that the fluorescent signal is very weak and surrounded with background noise, mostly owing to scattering of light from the solution (e.g. Raman scattering [7]). Condensation and separation of fluorescence-labeled probes using magnetic beads is a common procedure in many biological assays. Furthermore, magnetic modulation has been used to rotate magnetic beads and increase signal to noise ratio [8]. In this paper we show that streptavidin-coupled magnetic beads can be used to condense most of the biotinylated fluorescent-labeled probes into the detection area while they are in the solution. Moreover, external periodic maneuver of the fluorescent-labeled probes using an alternating magnetic field gradient can be used to separate the signal from the background noise of the non-magnetized solution and improve measurement sensitivity.

2. Material and methods

2.1 DNA sequence detection method

There are several approaches to recognize the existence of a specific DNA sequence [1-5]. In general, any approach which as a result of the biorecognition event produces fluorescent light; can utilize the magnetic modulation system to detect low concentration of labeled probes. One of the most widely used approaches is based on fluorescence resonance energy transfer - FRET (e.g. Taqman® probes, molecular beacons, etc. [9]). A suggested FRET-based synchronous detection assay is illustrated in Fig. 1. A nucleic acid probe is double-labeled with a fluorescent dye and biotin on the same nucleotide at the 5' prime. A dark quencher is connected at the 3' prime. When detection is made, the discriminating probe attaches to the target DNA. Similar to real time PCR reaction and Taqman® probes [9], after one cycle of PCR, the fluorescent dye molecule, still connected to the biotin, is separated from the dark quencher and fluorescent light is produced. Conversely, if the discriminating probe is not connected to the target DNA, the fluorescent dye and the dark quencher remain connected. Note that in this work, only the last step of detecting low concentrations of fluorescent light from the disconnected probes was experimentally demonstrated.

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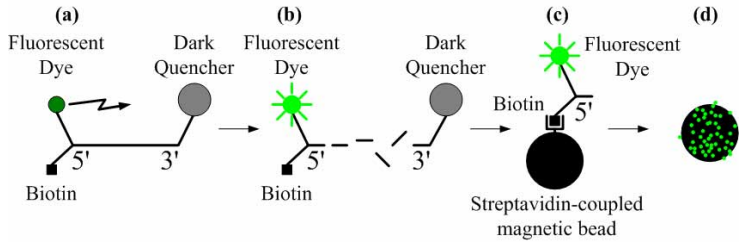

Fig. 1. Illustration of the FRET based synchronous detection assay: (a) a nucleic acid probe is double-labeled with a fluorescent dye and biotin at the 5' prime and a dark quencher at the 3' prime. (b) When detection is made, one cycle of PCR separates the dark quencher from the fluorescent dye and fluorescent light is produced. (c) The fluorescent dyes are connected via the biotin to streptavidin-coupled magnetic beads. (d) Each magnetic bead can be attached to thousands of fluorescent labeled probes and can be set in 1-D periodic motion.

The novel magnetic modulation system utilizes Streptavidin-coupled magnetic beads which are attached to the biotinylated probes. Each bead can be attached to thousands of fluorescent labeled probes. Two external electromagnetic poles condense the magnetic beads to the detection area and set them in a 1-D periodic motion by modulating the magnetic field gradient. This periodic motion, in and out of the orthogonal laser beam, produces a periodic fluorescent light which is collected by a photomultiplier (PMT) and demodulated using a lock-in amplifier. The high sensitivity magnetic modulation system is illustrated in Fig. 2(a).

A 488 nm frequency-doubled diode laser (Sapphire 488-50, Coherent) is used as an excitation light source. The laser beam is directed using a 506 nm long-pass filter (LPF-506-HC, CVI) onto a microscope objective lens (M-10X, 0.25 N.A, 16.5 mm focal length, Newport). The microscope objective lens focuses the laser beam to a 27 μm beam waist in a 500 μm wide borosilicate glass tube (0.5 x 5.0 mm I.D, RT4905, Vitrocom). The emitted light from the sample is collected using the same microscope objective lens, transmitted through the long-pass filter and spatially filtered by a 500 μm diameter slit. The light is then spectrally filtered by three successive band-pass filters (F40-536.0-HC, CVI) and detected by the PMT (H5784-20, Hamamatsu). The detected light is demodulated using a lock-in amplifier (SR760, Stanford research systems).

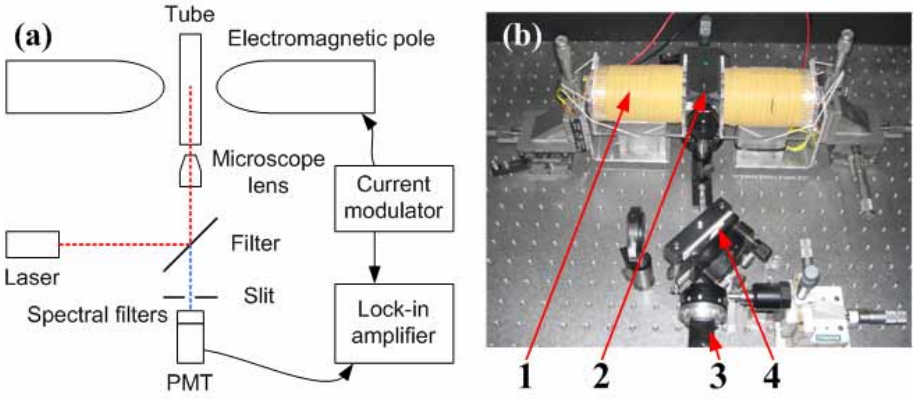

Fig. 2. (a). Schematic diagram of magnetic modulation system, (b) The magnetic modulation detection system is composed of: (1) two electromagnets, (2) rectangle tube, (3) photomultiplier, (4) long-pass filter.

2.2 Magnetic poles design and fabrication

The magnetic force F_m on a magnetic bead with magnetic moment m is proportional to the local field gradient as given in Eq. (1):

$$\vec{F}_m = \vec{m} \cdot \left(\vec{\nabla} B\right) \tag{1}$$

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where B denotes the magnetic field. Thus, the amplitude of the force depends on the magnetization of the bead and the field gradient. For a parabolic shaped pole tip, the field gradient at a given distance *r* from the pole tip is defined as in Eq. (2) [10]:

$$\nabla B(r) = \frac{4 \cdot \mu_0 \cdot M_m \cdot \beta}{(4 \cdot \beta \cdot r + 1)^2} \tag{2}$$

where $\mu_0 = 4\pi \cdot 10^{-7}\ Tm/A$ is the vacuum permeability, M_m – the magnetization saturation of the pole material and 1/β is the pole tip radius. The maximum field gradient for a given distance *r* follows the condition $\partial(\nabla B)/\partial\beta = 0$ and yields $\beta = 1/4r$.

In order to apply high forces on relatively small magnetic beads, very high magnetization saturation material is used to construct the poles (M140-30S, grain oriented silicon steel, 3% Si, Cogent). The 125 mm long poles are designed to have maximum field gradient at $r = 0.55$ mm (i.e. $\beta = 454.54$ 1/m). Each parabolic shaped pole is made of 33 layers of 0.3 mm thick and 10 mm wide strips which are annealed for 2 hours at 800ºC with protective nitrogen atmosphere. After annealing, the strips were vacuum impregnated with epoxy resin, assembled together as pen-shaped units and cured for 6 hours at 160 ºC.

The 101 mm long coils are made of copper wire (0.71 mm diameter) and have 4500 turns; their measured resistance (R) and induction (L) are, respectively, ~26 Ω and ~195 mH (@120 Hz). The coils, with the poles inside, are placed on XYZ translation stages, one at each side of the rectangle tube (see Fig. 2(b)). In order to maneuver the magnetic beads in a periodic 1-D movement we use a homemade current modulator which produces successively 1.4A to each coil at a chosen frequency in the range of 1-10Hz.

The magnetic field of each pole was measured using a Hall probe (AHP-H3Z, Arepoc), which was placed at the parabolic shaped pole tip, at different currents. The magnetic field gradient was measured at magnetization saturation of $\mu_0 \cdot M_m = 0.62$ Tesla (at a DC current of $I = 1$A). The measured field gradient of the poles was compared with the theoretical curve using Eq. 2 (see Fig. 3(a)).

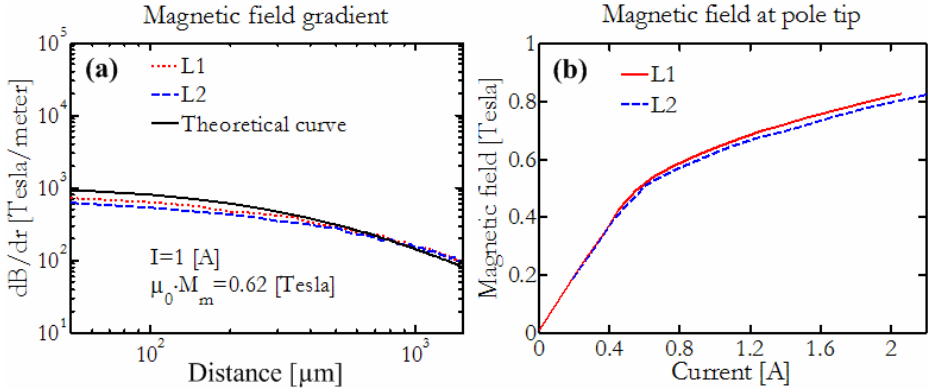

Fig. 3. (a). Magnetic field gradient as a function of the distance from the pole tip, (b) magnetic field at pole tip vs. current.

The magnetic modulation scheme has an important advantage. The magnetic gradient created by the electromagnetic poles attracts most of the magnetic beads in the solution and forms a very narrow path between the two poles. Thus, in a 270 μliter solution containing $7.4 \cdot 10^{-17}$ M magnetic beads, most of the ~12,000 beads will be condensed and maneuvered in a narrow line between the poles (see Fig. 5(c)). This feature enables us to detect very low DNA concentrations.

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2.3 fluorescent-labeled DNA primers preparation

In order to demonstrate our system capability to distinguish fluorescent-labeled DNA probes from the background noise, we used 7.1 nanomol of 17bp DNA probe which was labeled with AlexaFluor488 and biotin on the same nucleotide at the 5' end (5' (A488/biotin-dT)CT GGA AGC TTG GCC AT, Sigma-Proligo). The probe (oligonucleotide) was sequentially diluted in Tris-HCl buffer. Streptavidin-coupled magnetic beads with 2.8 μm diameter (Dynabeads M280, Invitrogen) were used to bind the oligonucleotides (oligos). According to manufacturer datasheet, the binding capacity of the beads is ~1,938,000 of biotinylated oligos per bead. However, much lower binding capacity was utilized in our experiment.

In order to verify the affinity between the fluorescent-labeled oligos and the streptavidin-coupled magnetic beads we mixed 60 μliter of $7.42 \cdot 10^{-14}$ M magnetic beads with 60 μliter at different concentration of oligos ($1 \cdot 10^{-8}$ M - $1 \cdot 10^{-11}$ M). We received different amount of fluorescent-labeled oligos per bead for each solution. The beads were examined using a laser scanning microscope (LSM-510-META, Zeiss), see Fig. 4. The fluorescent signal is clearly seen in Figs. 4(c) to 4(e). It scales up with the number of oligos per bead and it is significantly higher than that of a naked magnetic bead. The fluorescence spectrum exhibited a peak around 520 nm. However, the negative charge of the fluorescent-labeled oligos turns the beads into negative particles. Thus, in order to allow aggregation of the beads, low ratio of fluorescent-labeled oligos per bead should be kept (e.g. ~1348 in our tests).

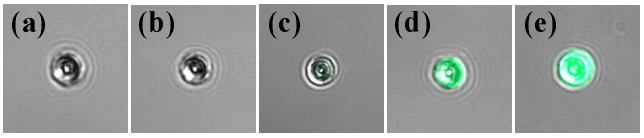

Fig. 4. Magnetic beads attached to different concentrations of fluorescent-labeled oligos: (a) without oligos, (b) ~134 oligos per bead, (c) ~1348 oligos per bead, (d) ~13482 oligos per bead and (e) ~134824 oligos per bead.

We have generated two series of solutions. The "test series" was prepared by mixing 60 μliter of $7.42 \cdot 10^{-14}$ M magnetic beads with 60 μliter of $1 \cdot 10^{-10}$ M fluorescent-labeled oligos. The average fluorescent-labeled oligos per bead was calculated to be ~1348 (as noted above). After 15 minutes we have prepared 7 diluted solutions (1/10, 1/20, 1/40, 1/80, 1/100, 1/200 and 1/500) resulting with final DNA concentrations of: $5 \cdot 10^{-12}$ M, $2.5 \cdot 10^{-12}$ M, $1.25 \cdot 10^{-12}$ M, $6.25 \cdot 10^{-13}$ M, $5 \cdot 10^{-13}$ M, $2.5 \cdot 10^{-13}$ M and $1 \cdot 10^{-13}$ M. The "reference series" was prepared by mixing 60 μliter of $7.42 \cdot 10^{-14}$ M magnetic beads with 60 μliter of Tris-HCl buffer (without any fluorescent dye). The mixture was further diluted to receive similar concentrations of magnetic beads as in the "test series". A CCD camera, placed instead of the PMT, allowed us to accurately position the magnetic poles so the beads were swept in and out the laser beam spot (see Fig. 5).

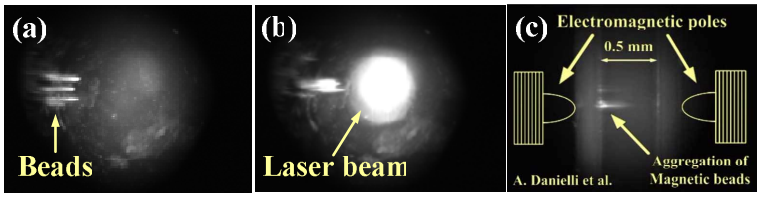

Fig. 5. Magnetic beads moving in and out of the laser beam spot: (a) magnetic beads only, (b) magnetic beads entering the laser beam, (c) (multimedia online) aggregation of the magnetic beads (Media 1).

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3. Results and discussion

The two series of solutions were tested with laser power of 4.6 mW, modulation frequency of 2 Hz and PMT control voltage of 0.5V (which yields a gain of 1.125 V/nW at the emission wavelength, $\lambda = 520$ nm). The results for the 2 types of solutions are summarized in Fig. 6.

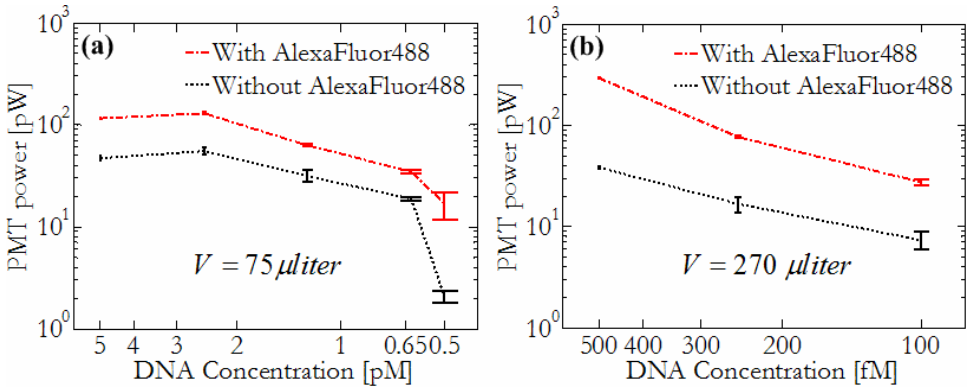

Fig. 6. PMT Power vs. fluorescent-labeled oligos concentration: (a) using a 75 μliter tube, (b) using a 270 μliter tube.

In all concentrations, the signal of the "test solution" (marked in red) was between 2 to 8 times higher than the "reference solution" (marked in black). The main noise factor in the system was caused by autofluorescent light emitted from the beads [11]. At high concentrations, the size of the aggregated beads is larger than the laser beam size. Hence, the signal and the reference remain constant (e.g. at concentrations of $5 \cdot 10^{-12}$ M and $2.5 \cdot 10^{-12}$ M). In order to allow aggregation at lower concentrations we enlarged the solution volume from 75 μliter to 270 μliter. Higher volume results with higher number of beads for the same concentration, therefore the higher noise level and signal level seen in Fig. 6(b). At the lowest oligos concentration of $1 \cdot 10^{-13}$ M, the detection signal was 3.3 times higher than the noise, thereby implying detection sensitivity of $3 \cdot 10^{-14}$ M for a signal to noise ratio of 1. For comparison, the sensitivity of commercial available system is $5 \cdot 10^{-12}$ M [12]

A rough estimation for direct detection limitation of fluorescent dye (e.g. AlexaFluor488) due to Raman scattering is considered in the next calculation. In water, the scattering cross-section of the dominant Raman line (3400 cm^{-1}) for excitation at 488 nm is [13]: $\sigma_W = \Omega \cdot 8.2 \cdot 10^{-34}$ $m^2 molecule^{-1}$ (Ω is the solid angle of observation). Inhomogeneous broadening of Raman linewidth results with less than 0.0001 of peak power at detection bandwidth (515-565 nm). In order to achieve signal to noise ratio of $\sqrt{SNR} = 10$, the total photon emission rate of all the AlexaFluor488 molecules should be at least 100 times higher than the total photon emission rate of water molecules. Assuming fluorescent emission collective efficiency of $\Omega / 4\pi$, the minimal concentration for direct detection is:

$$C = \frac{4\pi \cdot 100 \cdot 0.0001 \cdot \sigma_W \cdot C_W}{QY \cdot \sigma_{AF488} \cdot \Omega} \tag{3}$$

Where σ_{AF488} is AlexaFluor488 cross section, QY is AlexaFluor488 quantum yield and C_W is the water concentration (55.5 M). Using manufacturer's suggested extinction coefficient and quantum yield of 71,000 $cm^{-1}M^{-1}$ and 0.92 respectively, the estimation for minimal concentration for direct detection is: $C \cong 2.3 \cdot 10^{-13}$ M.

The theoretical limit of synchronous detection is based on the following best case assumptions: detector's shot noise limited and 100% modulation of the photocurrent. The shot noise standard deviation is $2 \cdot e \cdot I_{DC} \cdot B$, where e is the electric charge, I_{DC} is the average DC

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current at the PMT and *B* is the lock-in amplifier bandwidth. Assuming a signal to noise ratio of 10 and lock-in amplifier bandwidth of $B=1$ Hz, the minimal signal current required is $I_{DC}^{\min}=3.2\cdot10^{-17}$ A. Divided to the energy of the 520 nm emitted fluorescent photons and to the PMT efficiency ($\eta\cong0.2$), the average number of photons at the PMT input per second is 420 photons/sec. Taking into account the microscope objective numerical aperture and the losses of all the filters and lenses in our setup, one obtains total collective efficiency of 0.84%. Hence, the average number of photons emitted from the solution per second should be at least 50,000 photons/sec. Using manufacturer's suggested extinction coefficient and quantum yield (AlexaFluor488), the emission rate of a single fluorescent molecule is:

$$W_{emission}=W_{absorption}\cdot QY=\frac{\sigma\cdot P}{h\cdot\nu\cdot A}\cdot QY\cong133{,}951\frac{photons}{\sec} \tag{4}$$

Where $W_{absorption}$ is the absorption rate of a single molecule, *P* is the laser power (5 mW), σ is the absorption cross section (as concluded from the extinction coefficient), *h* is plank constant, ν is the absorption frequency and *A* is the laser beam area. Therefore, assuming shot noise limitation, even a single molecule can be detected using synchronous detection. In a 75 μliter tube, it corresponds to a minimum concentration of:

$$C=\frac{N}{Na\cdot V}=\frac{1}{6.022\cdot10^{23}\cdot75\cdot10^{-6}}=2\cdot10^{-20}\ M \tag{5}$$

4. Summary

In conclusion, we have shown that magnetic modulation and synchronous detection can be utilized to rapidly detect small concentrations of fluorescent-labeled DNA probes. The modulated motion of the fluorescent dye in and out of the orthogonal laser beam produces a periodic signal at the PMT. The effect of modulation shifts the signal from DC to the modulation frequency, reduces the 1/*f* noise and separates the signal from the background residual scattering. The magnetic modulation-based detection system provides results within seconds after aggregation and avoids washing steps, typically incorporated in most heterogeneous assays.

The detection system is not limited to the suggested FRET-based biological recognition assay of specific DNA sequences. An alternative to this approach involves a three-component 'sandwich' assay, in which the magnetic beads are coupled to DNA probes designed to be complementary to a portion of the target [14]. Modulation of the fluorescent dye can also be done using an external electric field rather than magnetic field, thereby taking advantage of the nucleotides electric charge. Moreover, as some fluorophores are very sensitive to the ph, modulation of the signal can also be achieved by periodically changing the solution ph. Nevertheless, one of the advantages of magnetic modulation is the condensation of most of magnetic beads in a small area where the beam is focused. Hence, lower concentrations can be detected by simply increasing the solution volume. Further improvements of the fluorescent signal can be achieved by adding anti photo-bleaching reagents.

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