

Screening unlabeled DNA targets with randomly ordered fiber-optic gene arrays

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We have developed a randomly ordered fiber-optic gene array for rapid, parallel detection of unlabeled DNA targets with surface immobilized molecular beacons (MB) that undergo a conformational change accompanied by a fluorescence change in the presence of a complementary DNA target. Microarrays are prepared by randomly distributing MB-functionalized 3- μm diameter microspheres in an array of wells etched in a 500- μm diameter optical imaging fiber. Using several MBs, each designed to recognize a different target, we demonstrate the selective detection of genomic cystic fibrosis related targets. Positional registration and fluorescence response monitoring of the microspheres was performed using an optical encoding scheme and an imaging fluorescence microscope system.

Keywords: high-density oligonucleotide probe arrays, fluorescence detection, genotyping, DNA diagnosis

An important goal of molecular biology is to identify the mutations that cause genetic diseases and to develop strategies to diagnose them^{1,2}. High-throughput sequence analysis is proving to be a powerful tool for both population-based genetic assessment and rapid and cost-effective diagnostic tests^{3,4,5}. Currently, only DNA chips adapt readily to the parallel format required to screen many samples for many mutations simultaneously. DNA chips are arrays of oligonucleotide probes that are spatially synthesized using either masking techniques or liquid dispersing methods^{6,7,8}. Although this technology is in commercial use and has yielded vast amounts of genetic and cellular information, important challenges remain: (1) reduction of overall substrate and individual feature sizes as the cost and the time for the diagnostic test decreases with sample volume; (2) fabrication using simple and cost-effective protocols; and (3) elimination of target labeling. Target labeling is not only time-consuming but it can change the levels of targets originally present in a sample. In this paper we report the first demonstration of a miniaturized array technology with micrometer-scale feature sizes, capable of detecting unlabeled DNA targets at subnanomolar concentrations in parallel using fluorescence microscopy.

Results

Randomly ordered fiber-optic DNA arrays. The present approach uses a miniaturized array technology in the form of randomly ordered microspheres on an optical fiber⁹, combined with a class of oligonucleotide probes called MBs¹⁰⁻¹⁵. Recently, Kramer et al. developed DNA probes with a hairpin-shaped structure in which the 5' and 3' ends are self-complementary, bringing a fluorophore and a quencher into close proximity. Fluorescence is restored when the MB binds to a complementary target nucleic acid, allowing the detection of unlabeled oligonucleotides. We employ imaging fibers composed of thousands of micrometer-sized optical fibers fused together in a single, hexagonally packed, fiber format. Each individual fiber in the array carries its own isolated optical signal from one end of the fiber to the other. Recently, we have fabricated micro- and nanowell arrays at the distal tip of the imaging fiber using a wet-chemical etching procedure¹⁶. The sensor is prepared by randomly distributing a mixture of microsphere sensors, each containing an immobilized MB, on an optical array containing ~6000 micrometer-scale wells¹⁶⁻¹⁸. The system

has several advantages. First, the DNA sensing chemistry can be attached to microspheres that are dispersed in microwells and held to the optical substrate (see Fig. 1A, B). Second, coupling the imaging fiber to an imaging fluorescence system resolves each fiber independently, while simultaneously viewing the entire array. Third, the feature size of the array (500- μm diameter) and the individual microsphere sensors (3- μm diameter), yield a high packing density (2×10^7 wells/ cm^2). These sizes allow parallel analysis with small volumes of target solutions. Although the individual sensor elements are positioned in a planar format, the microspheres project from the well into the solution, allowing for radial diffusion to occur with faster kinetics.

Microsphere encoding and functionalization. As the microspheres are randomly distributed over the entire array, an optical based registration method was employed. Each sensor in the array is encoded with a particular dye signature, allowing multiple unique DNA sensors to be registered and identified. A family of optically encoded microspheres^{19,20} (Table 1) was created by entrapping two different dyes, Texas red cadaverine (TRC) and europium (III) thenoyltrifluoroacetate-3H₂O (Eu) in the interior of the polystyrene microspheres. By swelling the microspheres in a tetrahydrofuran (or dimethylformamide)/dye solution, followed by rapid shrinking in an aqueous solution (or ethanol), the fluorescent dyes become entrapped in the microsphere's interior. After encoding, the microsphere surfaces were modified with a streptavidin coating for biotin-functionalized DNA attachment according to a previously detailed procedure⁹. Finally, the microspheres were treated with bovine serum albumin to passivate the exposed surface, minimizing unspecific binding.

Molecular beacon design. The MBs were designed with three functional moieties: first, a 5'-terminal biotin serves to immobilize the MB on the microspheres. Second, the loop structures were designed to be complementary with several wild type and mutated genes of the cystic fibrosis transmembrane conductance regulator (CFTR) region. We selected sequences containing the normal and abnormal alleles for cystic fibrosis in which the frequency of occurrence of the mutation is high or moderate (e.g., WT507, DF508 (66%), and G178 (0.2%), Table 2)^{21,22}. F508 was encoded with Eu, G178 with TRC, and WT507 microspheres were not encoded (see Table 1).

As most MB studies indicate that a 15–25 base sequence together with a 5–6 base pair stem yields an appropriate working MB, we

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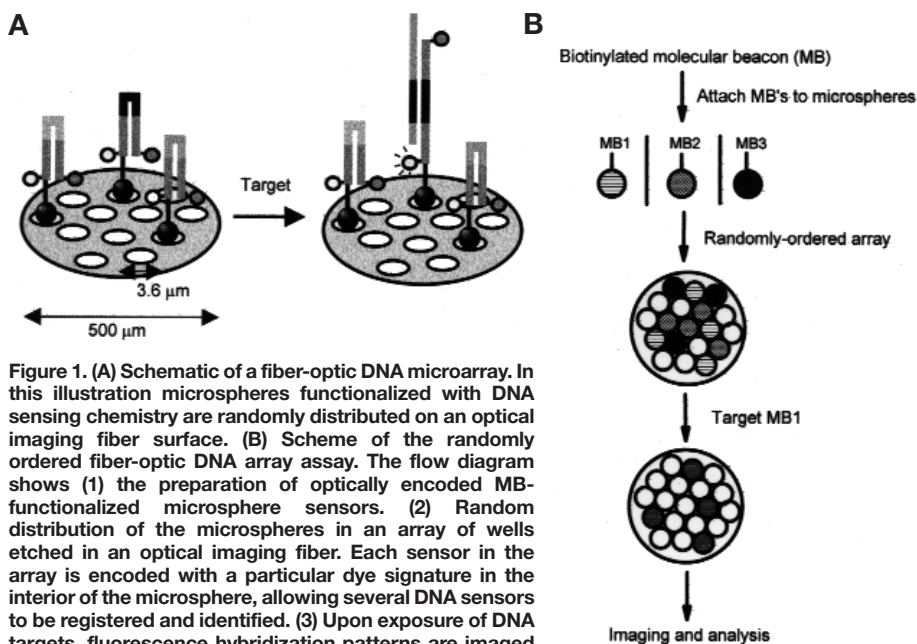


Figure 1. (A) Schematic of a fiber-optic DNA microarray. In this illustration microspheres functionalized with DNA sensing chemistry are randomly distributed on an optical imaging fiber surface. (B) Scheme of the randomly ordered fiber-optic DNA array assay. The flow diagram shows (1) the preparation of optically encoded MB-functionalized microsphere sensors. (2) Random distribution of the microspheres in an array of wells etched in an optical imaging fiber. Each sensor in the array is encoded with a particular dye signature in the interior of the microsphere, allowing several DNA sensors to be registered and identified. (3) Upon exposure of DNA targets, fluorescence hybridization patterns are imaged and analyzed. Individual sensing regions not to scale. Thousands of 3.6 μm sensors are in the array.

incorporated these requirements into our design. The five base pair stem is strong enough to form the hairpin structure for efficient fluorescence quenching, while allowing dissociation when the complementary DNA target hybridizes with the base loop of the MB. For the ΔF508 deletion, we prepared MBs flanking the region of the deletion. Third, we chose fluorescein as the fluorophore and [4-(4-dimethylaminophenylazo)benzoic acid] (DABCYL) as the universal quencher for detecting the opening and closing of the stem-loop structure. The same fluorophore/quencher pair can be used in all the MBs because the fluorescence signals are spatially resolved on the fiber-optic array. The three different MBs were immobilized onto the encoded microspheres (see Table 1) and placed in the array of microwells following a previously detailed procedure⁹.

Sample analysis and microsphere registration. Registration of the microspheres and fluorescence detection upon analyte exposure were performed using a previously described imaging system²³. Images of a representative part of the array containing the three different MBs (WT507, F508, and G178) are shown in Figure 2A-G. Figures 2A-C show fluorescence hybridization patterns upon exposure of DNA targets in 10 mM pH 8.3 TE buffer with 3.5 mM MgCl_2 . Figure 2D depicts the background fluorescence of the MB microspheres in the array in aqueous buffer. When the array is allowed to dry, the fluorescence emission of all MBs significantly increase in intensity as seen in Figure 2E, enabling one to see all the MBs in the array. Figures 2F and 2G depict the fluorescence responses at the Eu and TRC encoding dye wavelengths, respectively, upon excitation. Strikingly, the encoding patterns shown in Figures 2F and 2G are identical to the hybridization results shown in Figures 2B and 2C, respectively. Collectively, all fluorescence signals from individual microspheres were consistent with hybridization of the complementary target containing the corresponding specific MB. The addition of the complementary WT507 oligonucleotide target gave a rapid increase in fluorescence response for the corresponding WT507-

Table 1. Encoding of the microspheres.

	MB	Fluorescein	Eu-dye	TRC
A	WT507	X	-	X
B	F508	X	X	-
C	G178	X	-	X

functionalized microspheres (Fig. 2A). On the other hand, nanomolar concentrations of the F508 and G178 complementary oligonucleotide targets did not have an effect on the fluorescence response of the WT507 functionalized-microspheres (Fig. 2A-C). Complementary results were obtained for the F508 and G178-functionalized microspheres. Quantification of the emitted fluorescence from the MBs indicated a greater than twofold difference in mean intensity between background and target exposed microspheres after 30 min. incubation with 20 nM target concentrations. At higher concentrations (micromolar) and longer exposure times (1 h) a 14-fold fluorescence increase was observed (data not shown).

The immobilized MBs were incubated with the target of interest and the fluorescence response was monitored in real time. Figure 2B shows the kinetic results upon synthetic target exposure with the WT507 MB. After regenerating the sensor with 90% formamide, the response curves were replicated with no significant change. Repetitive

studies showed that the protocol could be repeated at least 50 times, allowing the background signal of the MB to return to its initial value (Fig. 2I). Therefore, the present sensor array can be reused several times for DNA interaction studies. The target incubation times required to obtain a signal-to-noise ratio of two are seconds for micromolar target concentrations and minutes for nanomolar concentrations. As each microsphere is independently addressable through its own light channel, it is possible to combine responses from the same MB-functionalized microspheres randomly distributed throughout the array. The fluorescence responses as depicted in Figures 2H and 2I are the average responses of 33 individual microspheres in a selected area of the array²⁴. Averaging the fluorescence responses of a number of selected microspheres makes possible the reduction of random noise contributions to the measured signal. An additional advantage of having several copies of the same sensor in the array is a reduction of false-positive and/or false-negative signals.

The DNA sensor is able to make quantitative measurements with a detection limit of approximately 100 pM. Although this detection limit is comparable to systems with MBs attached to a solid support^{13,14}, an advantage over other array sensors is the small volume (~10 μl) of target solution used in the experiments. The total volume of target solution times the concentration ultimately determines the amount of DNA necessary for a single experiment. The present sensitivity is approximately a factor of 100–1000 times less than quantitative measurements using other microarrays¹. To increase the sensitivity of the present technique, several factors should be considered. After incubation with their complementary DNA targets the MB show more than a 40–50-fold enhancement in

Table 2. Molecular beacons with complementary targets.

WT507		5' B-FAM-ttgagTATCATCTTTGGTGTTCtcaa-D 3' 3' CCAATAGTAGAAACCACAAAA 5'
F508	66%*	5' B-FAM-ttgcgATATCATTGGTGTTCcga-D 3' 3' CCTATAGTAACCACAAAGGAA 5'
G178MU	0.2%*	5' B-FAM-tagcgAAATAAGTATTAGACAAcgccta-D 3' 3' CCTTTATTCTAATCTGTTGAA 5'

*Frequency of occurrence.

B = Biotin-TEG; D = Dabcyl; and FAM = Fluorescein.

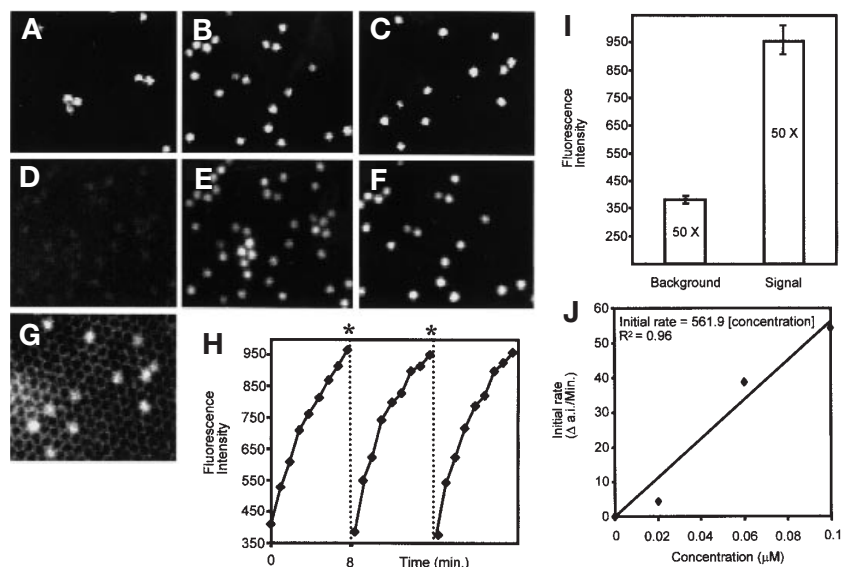


Figure 2. Fluorescence hybridization patterns upon exposure of the array to the synthetic targets. The concentration and the incubation time were 20 nM and 20 min, respectively. Fluorescence response of WT507- (A), F508- (B), and G178-bound (C) microspheres to their corresponding targets. Images were background-subtracted, normalized, and contrast adjusted for clarity. (D) Background fluorescence of the immobilized MBs in a selected area of the array. (E) Fluorescence image of all molecular beacons taken at 530 nm using 490 nm excitation light. Fluorescence image of encoded microspheres taken at (F) 610 nm using 365 nm excitation light (Eu), and (G) 610 nm using 577 nm excitation light (Texas rod). Image 2F was acquired with a 200 ms CCD acquisition time. Image 2G was acquired using a 2 s CCD acquisition time. (H) Kinetic response curves of the WT507 molecular beacon upon exposure of the corresponding synthetic target (average results of 33 individual microspheres). (I) Consecutive studies of hybridization and regeneration show the robust nature of the sensor array. (J) A plot of the concentration of the target versus the initial rate of fluorescence response of the WT507 molecular beacon.

fluorescence signal when measured in homogeneous solution (ABI prism 7700 sequence detection system (PE Biosystems, Foster City, CA). In contrast, when the MBs are attached to microspheres and placed in wells, only a 14-fold enhancement is observed. In principle, the enhancement could be more than 200-fold with optimal design of the sequence, hybridization, and detection conditions¹⁰. Another important factor is the relatively high background fluorescence (see Fig. 2D) of both the MBs and the cladding of the optical fibers. A better quenching efficiency between the fluorophore/quencher pair and optimized optical fiber technology will diminish the background significantly. By employing fluorescent dyes with higher quantum yields than fluorescein (e.g., rhodamine dyes), as well as optimized excitation and detection instrumentation (e.g., a laser system), it should be possible to realize higher sensitivities for the DNA targets.

There was a linear relationship between the initial rate of WT507 fluorescence upon target exposure and the concentration of the corresponding synthetic target (Fig. 2J), enabling the determination of an unknown target concentration. This relationship depends on the sequence of the target DNA employed, and will only hold for target samples without stable secondary structures. Targets with stable secondary structures will seem more dilute than those with weak secondary structures.

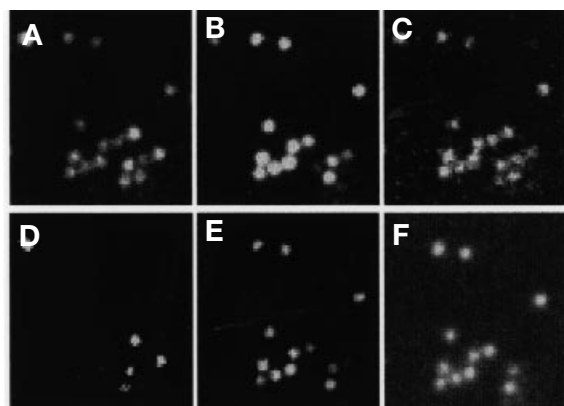


Figure 3. Fluorescence hybridization patterns upon exposure of the array with the PCR amplified samples. (A) Fluorescence response of the array to the WT507 target. (B) Fluorescence response of the array to the F508 target. (C) Background fluorescence of the immobilized MB's in the array. (D) WT507-background. (E) F508-background. (F) F508 microspheres with Eu.

PCR amplified samples from the cystic fibrosis gene in a typical complex background mixture were analyzed. Single-stranded WT507 and F508 hybridization targets were generated using the polymerase chain reaction (PCR), starting from homozygous genomic DNA samples with characterized *CFTR* mutations. To prepare natural single-stranded target DNA by PCR, we used an asymmetric PCR primer (5'-ACC TCT TCT AGT TGG CAT GCT TTG-3')²⁵ to amplify a 491-bp (WT507) and 488-bp (Δ F508) fragment derived from exon 10 in the *CFTR* gene²⁶. The WT507 and F508 PCR products yield a 358-bp and 355-bp single-stranded product, respectively. The synthesis of the PCR products was confirmed by the mobility of the products in 2% agarose gel electrophoresis. Subsequently, the PCR reaction mixtures of the WT507 and Δ F508 were applied to a new sensor array with now only the WT507 and Δ F508 MB sensors present. The optical encoding was performed in the same way as described above (see Fig. 3C, F). F508 microspheres were encoded with Eu, and the WT507 microspheres were not encoded. The results for the fluorescence responses after 5 h incubation with PCR amplified samples are depicted in Figure 3A, B. After background subtraction (Fig. 3C), the net increase in MB fluorescence could be observed (Fig. 3D, E).

As observed for the synthetic targets, all fluorescence signals from individual microspheres were consistent with hybridization of the complementary target to the corresponding specific MB. Cross-hybridization was observed for only a small number of microspheres (<3%) in the array (<1% of the fluorescence signal of the corresponding complementary target). As the magnitude of the complementary fluorescence signal is much higher than the non-complementary signal, and there are multiple copies of each microsphere type in the array, these false-positive signals could easily be excluded. We conclude that the MBs in the array are specific for the corresponding PCR amplified products in a typical PCR complex background mixture. The relatively long hybridization time of 5 h with nanomolar concentrations was necessary to obtain a signal-to-noise ratio of 2. This result is to be expected for the much larger PCR amplified products. With fragmented PCR products (average length=40 bases) similar results could be obtained in 2 h.

Discussion

In this paper we have documented a simple, randomly ordered, fiber-optic, site-addressable detection method for monitoring unlabeled DNA sequences in parallel using surface immobilized MBs. The present array allows high-throughput capabilities and fast

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hybridization times with multiple probes. The number of different probes can be extended easily by entrapping several dyes or combination of dyes in the interior of the microspheres. The high-density of probes on each microsphere and their small size contribute to the short analysis times. Because of their small size, billions of microspheres are prepared at a time with similar binding behavior. In the present form, the randomly ordered array can be used as a specific and effective alternative in screening for 66.2% of the most frequent occurring cystic fibrosis mutations. The combination of self-assembly and selective surface chemistry with microspheres has made it possible to immobilize DNA recognition sites on an optical substrate. Among the greatest strengths of the present array is the incorporation of several copies of each probe, thereby decreasing the chance of both false negatives and false positives. The ability to customize this assay for several genes has advantages over other existing approaches. Additional microsphere-functionalized MB probes can be added to the microsphere probe mixture creating a more diverse array. Finally, it is important to note that arrays produced in, as well as between, manufacturing lots have reproducible hybridization properties, allowing accurate high-throughput mutational analysis.

Experimental protocol

Encoding of the microspheres. Monodisperse $3.1 \pm 0.045 \mu\text{m}$ diameter microspheres (87% methylstyrene/13% divinylbenzene/R-NH₂) were purchased from Bangs Laboratories (Fishers, IN). Three 200- μl aliquots of stock 3.1- μm diameter poly(methylstyrene)divinylbenzene microspheres were prerinced with tetrahydrofuran (THF). Approximately 500- μl aliquots of the Eu (0.1 M, Fisher, Pittsburgh, PA) or TRC (1×10^{-4} M, Molecular Probes, Eugene, OR) solutions made in THF or DMF (TRC) were added to the microspheres, and agitated for 1 h on a shaker. Subsequently, the solutions were filtered (0.5 μm Fluoropore membrane filters, Millipore Co., Bedford, MA) and rinsed with ethanol (Eu) or ultrapure water (TRC).

Molecular beacon synthesis and attachment. MBs were synthesized by Research Genetics, Inc. (Huntsville, AL) The fluorescence ratio between the MB probe-target hybrid (or signal) and the fluorescence of the probe itself in its loop-stem conformation (or background) is at least 25:1 for all the MBs. Three stock solutions of 33 μg MB WT507, F508, and G178 in 10 mM, pH 7.4 PBS (1 ml) containing 0.01% Tween 20 were prepared. From the stock solution, 2 μl was added to each (A, B, or C) corresponding streptavidin-coated microsphere (5 μl ; 10% solids). The microspheres were agitated for 1 h on a shaker. Finally, the microspheres were washed three times using 10 mM pH 7.4 PBS (1 ml) containing 0.01% Tween 20.

Analysis set-up and protocol. The imaging system consists of a light source, inverted microscope, and a modified Olympus epifluorescence microscope/charge coupled device camera (Pentamax ICCD camera; Princeton Instruments, Trenton, NJ) as described previously²³. Fluorescence measurements were acquired and analyzed using commercially available IPLab software (Signal Analytics).

Encoding. Overlay segments were drawn to select the microspheres bearing a hybridization signal using IPLab software (Scanalytics, Fairfax, VA). These overlay segments were copied and pasted onto each of the encoding images and the selected microsphere identity was determined.

Kinetic study. The fiber's distal tip was placed in a 10 μl target solution and images were acquired every minute for 8 min. Subsequently, the fiber was dipped in 90% formamide/TE buffer, and washed with TE buffer to regenerate the sensor. A background fluorescence image was acquired for 200 ms at wavelengths specific to fluorescein (excitation 490 nm and emission 530 nm) with the fiber's distal tip in buffer.

Reproducibility study. The fiber's distal tip was placed in 10 μl WT507 target solution (0.1 μM) for 8 min. and a fluorescence image was acquired for 200 ms. The array was regenerated with 90% formamide/TE buffer removing any hybridized target. This hybridization-regeneration procedure was repeated 50 times.

Hybridization target preparation. A sample of 1 μg of genomic DNA (wild type and F508 PCR products of 491 and 488 base pairs, respectively) was amplified in a 50 μl reaction containing 1 μM of primer (5'-ACC TCT TCT AGT TGG CAT GCT TTG-3'), 50 μM each of dATP, dCTP, dGTP, and

TTP (all dNTP's from Pharmacia, Piscataway, NJ), and 2 U *Taq* polymerase (PE Biosystems, Foster City, CA) in 10 mM Tris HCl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂²⁵. The reactions were cycled 20 times in a thermocycler (MJ Research, Waltham, MA) using the following temperatures and cycle times: 95°C, 10 s, 60°C, 10 s, 72°C, 1.5 min. Fragmented PCR products were prepared as described above with the exception of using 40 μM TTP and 10 μM dUTP instead of 50 μM TTP. The PCR products were fragmented by adding 2U of uracil-N-glycosylase (Gibco, Grand Island, NY) and incubating at 37 °C for 30 min, followed by heating the solution to 95°C for 5 min²⁷. The fragmented PCR product were diluted twofold into 10 mM, pH 8.3 TE buffer with 3.5 mM MgCl₂ and used directly in the hybridization studies.

Acknowledgments

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