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# Control of Immobilization and Hybridization on DNA Chips by Fluorescence Spectroscopy

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A new methodology for the analysis of DNA polymorphisms has been developed using specific oligonucleotide strand arrays bound to a solid silicon support recovered by a thin layer of silica. Arrays of directly synthesized oligonucleotides covalently fixed on $Si/SiO_2$ wafers have been designed at the macroscopic scale. Using suitable nucleotide-labeled units, the fluorescence emission technique has been used as an experimental control of the molecular network bound to the support and as a method for analyzing the hybridizing abilities of the corresponding oligonucleotide array. Fluorescein has allowed us to control the molecular density of the DNA strand resulting from a complete synthetic growing process. A specific protocol using both complementary and noncomplementary units labeled with two probes, Cy3 and Cy5, was used to distinguish clearly nucleotide units fixed on the array either as hybridized sequences or by the unavoidable adsorption process. The present performance of this fluorescence detection procedure will now be used with a scanning fluorescence device to perform the analysis at the microscopic scale.

**KEY WORDS:** DNA chip; oligonucleotide; hybridization; fluorescence detection.

## INTRODUCTION

Sequence analysis, genotyping, and gene expression monitoring find applications in different areas such as biomedical research, genomic and clinical diagnostics, pharmacology, environment, and food industry. Nucleic acid probes show great potential for the rapid identification of human, animal, or plant pathogens [1,2], for the detection of specific genes in animal and plants breeding [3], and for the diagnosis of human genetic disorders.

Access to genetic information is ultimately limited by the ability to screen DNA sequence. In this domain, DNA chips provide a simple and rapid method for simultaneous analysis of several thousands of nucleic acids, and currently, DNA detection techniques use radiochemical, enzymatic, fluorescent, or electrochemiluminescent methods [4–7]. The fluorescence spectroscopy [8–12] is the most widely used detection technique, as it allows both the control of DNA targets immobilized on chips and the detection of hybridization patterns.

Two types of technology concurring to the elaboration of DNA chips are widely described in the literature. In the case of a low-density array of hybridization units, immobilization of presynthesized oligonucleotides seems to be a pertinent way. This process uses defined oligonucleotide moieties previously purified. In contrast, the implementation of several thousands of hybridization units requires the development of a direct synthesis of oligonucleotide targets arrayed on a solid support [13]. In that case, drawbacks come from failure of the coupling reaction yield.

In this work, we introduced a specific protocol of direct synthesis of oligonucleotide targets arrayed on a $Si/SiO_2$ wafer. This substrate was chosen with the view to

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develop an alternative detection method of hybridization without any label [14]. The validation of this new direct detection technique requires beforehand a well-controlled elaboration of samples. So the synthesis protocol is associated to a control procedure of the molecular density of the directly synthesized and covalently fixed oligonucleotides on the solid support. As the detection method we have used, in a first step at the macroscopic scale ($1 \times 1$ cm$^2$), the conventional fluorescence emission measurements on labeled arrays. Then using suitable fluorescently tagged sequences, we have explored the ability of these oligonucleotides arrayed on the support to be hybridized.

## EXPERIMENTAL

### Materials

In oligonucleotide synthesis, phosphoramidite reagents from Perkin–Elmer were used as received. 6-FAM phosphoramidite was involved as the fluorescein-labeling agent of oligonucleotide strands.

For fluorescence detection, two other probes, Cy3 and Cy5, associated to an oligonucleotide sequence, respectively, U185 (Cy3U185: 5′Cy3 A A G G A T T G G C C C G C G T T G G A T T A G C 3′) and L226 (Cy5L226: 5′Cy5 C T A T G G A T C G T C C G C A T G G T A G G A G 3′) were used and purchased from Eurogentech. The $T_m$ for both sequences was determined at 47°C. Cy3 and Cy5 are the usual abbreviated names for 3-1-*O*-(2-cyanoethyl)-(*N*,*N*-diisopropyl)indocarbocyanine and 5-1-*O*-(2-cyanoethyl)-(*N*,*N*-diisopropyl)indocarbocyanine. Excitation and emission wavelengths are located, respectively, at 520 and 570 nm for Cy3, 620 and 665 nm for Cy5.

Tris buffer was prepared using 10 m*M* Tris–HCl, 10 m*M* Tris base, and 50 m*M* NaCl as the final concentrations. The other rinsing reagents were obtained from the commercial 5X SSC (sodium citrate and NaCl) buffer by successive dilutions. All buffer components and usual reagents were purchased from Aldrich and used without further purification.

### Substrates and Surface Treatments

The substrates used were $1 \times 1$-cm$^2$ diced Si wafers covered with a thin $SiO_2$ layer (15 nm) on the front side and purchased from Microsens (Switzerland). The initial derivatisation process of these supports corresponds to the fixation of alkylsilane molecules on the cleaned samples bearing silanol groups at the surface with the view of obtaining an appropriate activated surface for further oligonucleotide fixation.

### Automated Synthesis of Oligonucleotides

The DNA sequence 656 (CTT GCG GGG CGT TCG) was synthesized with a Perceptive Biosystems automated nucleic acid synthesizer using Perkin–Elmer reagents (phosphoramidite derivatives protected by tertiobutylphenoxyacetic groups). The system was connected to a special home-made cell to perform the oligonucleotide synthesis directly on solid substrates.

This process involved four steps: (i) a deprotection of the last nucleotide unit, (ii) coupling of the activated phosphoramidite reagent on this last unit of the growing strand, (iii) capping of the ending nucleotides which do not react in the previous step, and (iiii) oxidation of the orthophosphorous in an orthophosphoric function on the last nucleotide unit. During the final cycle, a phosphoramidite labeled with a suitable fluorescent probe, such as fluorescein, was introduced.

Then the oligonucleotide strands were wholly deprotected by immersing the chip in 30% aqueous ammoniac during 2 h.

The surface density of the synthesized oligonucleotide strands and their molecular structure depend on the efficacy of the different steps of the synthesis. To avoid mismatch in the sequence, the uncoupled nucleotide heads have to be isolated from the synthesis sequence by a capping process.

### Hybridization and Fluorescence Detection

Hybridization was performed at 42°C during 12 h in a 5X SSC solution containing 0.005 μg/μl DNA. The denaturation of the duplex structure is obtained by using 0.1 *M* NaOH during 1 min.

The stationary emission fluorescence spectra were recorded using the front-face mode of a SPEX Fluorolog 2 spectrofluorimeter and corrected from the background arising from scattered light and nonspecific fluorescence.

## RESULTS AND DISCUSSION

### Elaboration and Control of the Support-Bound Oligonucleotides

For the validation of DNA covalently bound to the solid support during the sequence synthesis we have a

DNA strand labeled with fluorescein and examined the fluorescence emission of the sample before and after strong rinsing.

In the synthesis protocol, silicon wafers were previously functionalized with an organic layer activated with an alcohol function. Then the 656 sequences of 15-mer oligonucleotides were bound to the alcohol group according to the phosphoramidite process presented in Fig. 1. A fluorescein-labeled phosphoramidite was included in the last cycle of the synthesis of the 15-mer oligonucleotides to be added at the 5′ end of the nucleic acid sequence. The deprotection process was followed by several rinsing steps with Tris buffer leading to the elimination of undesired reagents of the synthesis lying on the substrate surface in an adsorbed or unbound state, above all the fluorescein-labeled moieties. To characterize the efficiency of the rinsing procedure, the evolution of fluorescence emission was estimated after each rinsing step both at the level of the substrate surface to identify the final signal of bound labeled strands (minimum of the light emission) and at the level of the rinsing liquid to control the amount of labeled nucleotides desorbed from the chip surface. An important signal, corresponding to a signal/noise ratio of 20, is detected (Fig. 2a), indicating that a large amount of labeled oligonucleotides remain linked to the surface of the substrate.

At this step, we needed to identify the origin of the fluorescein emission either coming from adsorbed fluorescein labeling reagent or resulting from fluorescein labeled oligonucleotides covalently bound to the surface of the substrate. Figure 2b gives the reference emission spectra recorded on sample being unreactive with the 6-FAM phosphoramidite. On this sample, the synthesis of the nucleic acid sequence was performed as described previously except at the level of the last cycle, where the nucleotide moiety was capped in a first step to be unreactive. Then the reactivity of the fluorescein labeling reagent with the wafer recovered with the 656 sequences is limited to the adsorption process. At this step, we observed the corresponding fluorescein emission signal which disappeared completely as shown in Fig. 2b after the usual rinsing procedure. The value of the signal/noise ratio was near 1, so 20 times less intense than in the former experiment.

This experiment shows clearly the fluorescence emission coming from oligonucleotides labeled with fluorescein and covalently bound to the solid support. This is also an appropriate indication that, during the direct

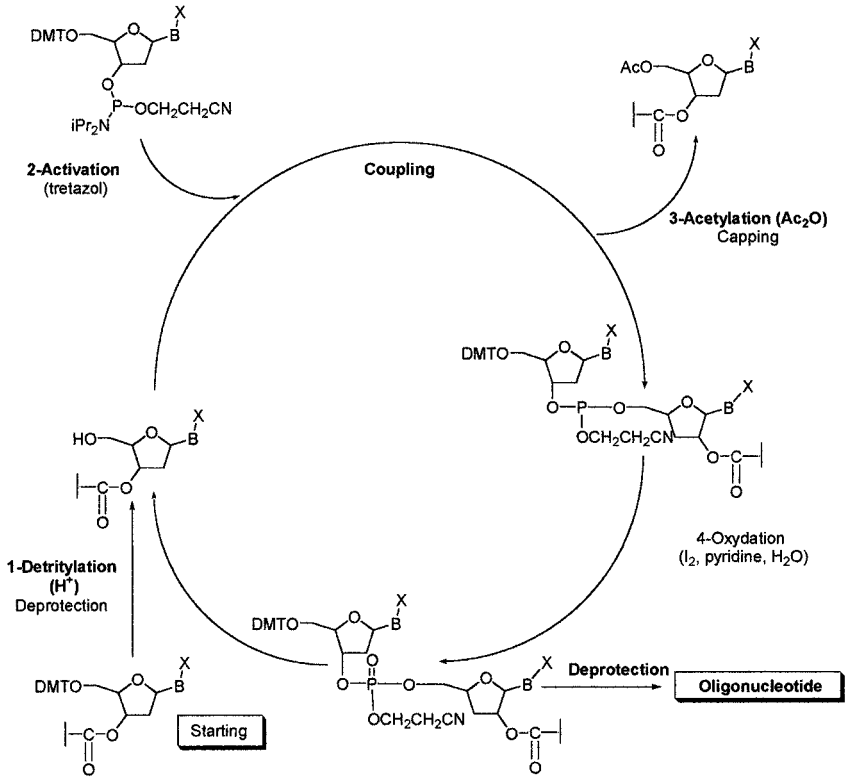



**Fig. 1.** Direct oligonucletide synthesis with the phosphoramidite process.

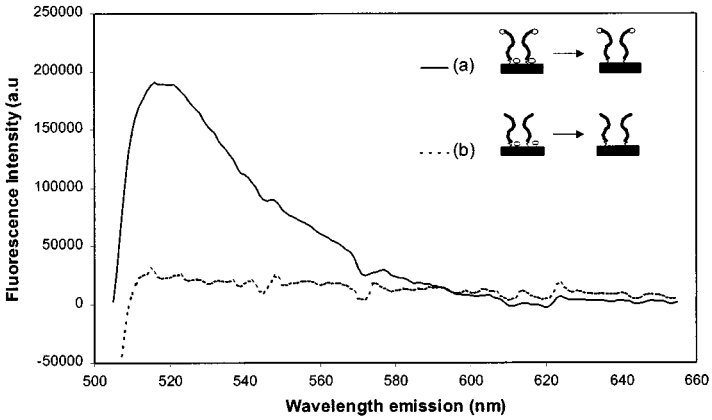



**Fig. 2.** Control of the direct synthesis of 656 DNA single strands. Fluorescence emission spectra of directly synthesized 656 DNA sequences: (a) 656 sequences labeled with fluorescein; (b) no fluorescence with capped 656 sequences after elimination of the adsorbed 6-FAM phosphoramidite reagent.

synthesis procedure, a complete oligonucleotide sequence is realized as a certain amount of DNA strand remain reactive until the last cycle being capable to fix the fluorescent probe.

However, we noted that it was difficult to obtain reproducible emission with fluorescein fixed on the surface, as its fluorescence yield depends on pH and is very sensitive to self-quenching process. So a good correlation between fluorescein emission and the density of labeled oligonucleotide cannot be obtained with unstructured layers of biological material.

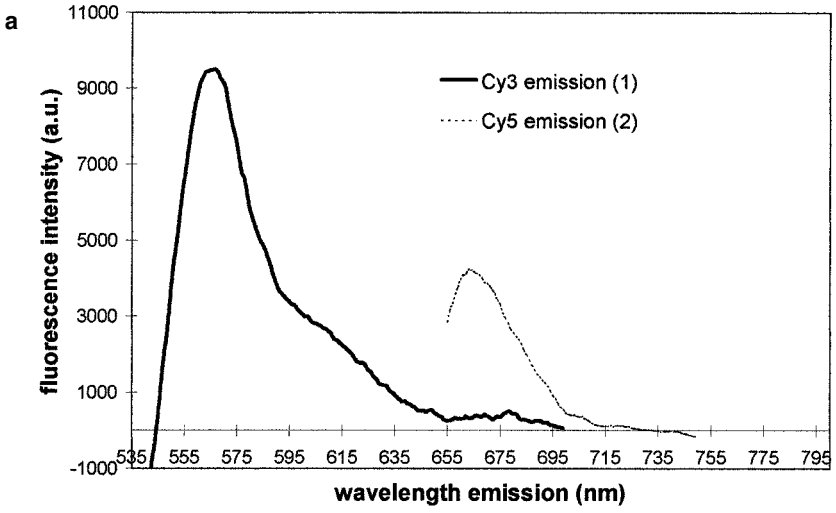
## Hybridization Ability of the Support-Bound Oligonucleotide Array

In fact, fluorescein presents a higher fluorescence yield in basic medium (pH $\geq$ 8) but unfortunately DNA strands cannot be hybridized under these conditions [15]. As we have, in the hybridization reading process, to differentiate specific hybridization from nonspecific one or adsorbed molecules, we introduced the Cy3 and Cy5 probes in our detection methods. Their fluorescent yield did not depend on the pH, and the fluorescence emission can be detected on dry surface with minor self-quenching effects. The fluorescence emission spectra of these probes are located in two wavelength zones to be distinctly distinguished [9,12].

The 656 sequence did not appear as the most suitable DNA strand to finalize the hybridization reading procedure, so we associated the new probes with 25-mer sequences (L or U185 and L or U226). The U185 and L226 sequences were tested with classical biological methods before use. The two new labeled oligonucleotide targets were [Cy3U185] and [Cy5L226], which correspond, respectively, to the noncomplementary (U185) and complementary (L226) sequences of the immobilized strand U226 and labeled with two fluorescent probes, Cy3 and Cy5. With an analogue composition on nucleic bases (36% G, 20% C, 20% A, 24% T), the U185 and L226 sequences have been chosen to present identical molecular interactions with the functionalized surface of the $Si/SiO_2$ wafer.

The DNA sequence U226 was directly synthesized on the support and then hybridized with a mixture of both the noncomplementary DNA strands U185 labeled with the Cy3 group and the complementary sequence L226 labeled with the Cy5 group. As expected, emission spectra of both probe Cy5 and probe Cy3 appeared in preliminary fluorescence measurements before rinsing, due to the duplex sequences [U226–Cy5L226] and

**Fig. 3.** (Opposite) Fluorescence analysis of the direct synthesis of U226 DNA sequences on functionalized $Si/SiO_2$ surface. (a) Hybridization and adsorption of [Cy3U185] and [Cy5L226]. Curve (1), Cy3 emission from adsorbed [Cy3U185] units; curve (2), Cy5 emission from hybridized and adsorbed [Cy5L226] units. (b) Elimination of nonspecific hybridization and adsorption. Remaining of the Cy5 fluorescence coming from labeled duplex sequences [U226–Cy5L226]. Curve (1), Cy3 signal after rinsing; curve (2), Cy5 emission from hybridized [Cy5L226] units after rinsing. (c) Loss of the Cy5 emission signal of duplex sequences [U226–Cy5L226] during the denaturation process. Curve (1), Cy3 signal, and curve (2), Cy5 signal, after denaturation.


a
11000
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fluorescence intensity (a.u.)
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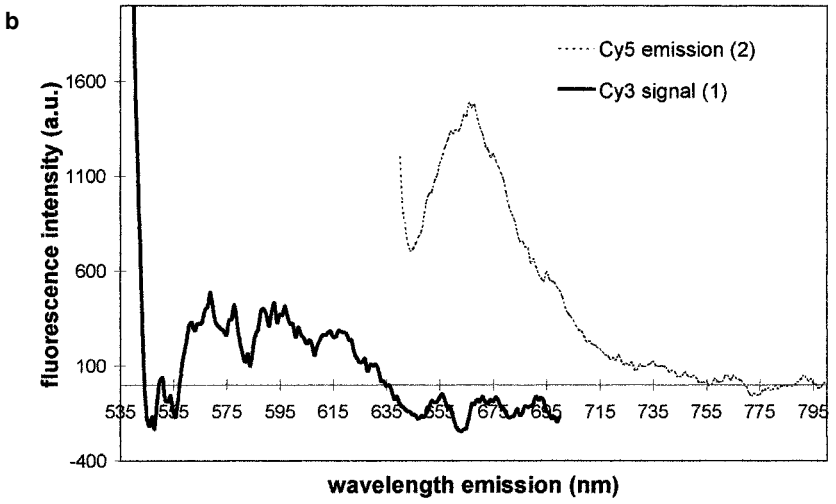
wavelength emission (nm)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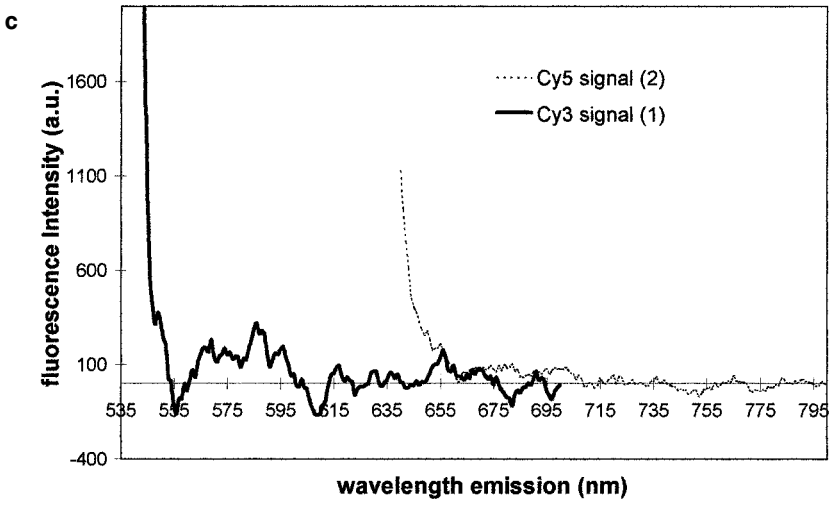
wavelength emission (nm)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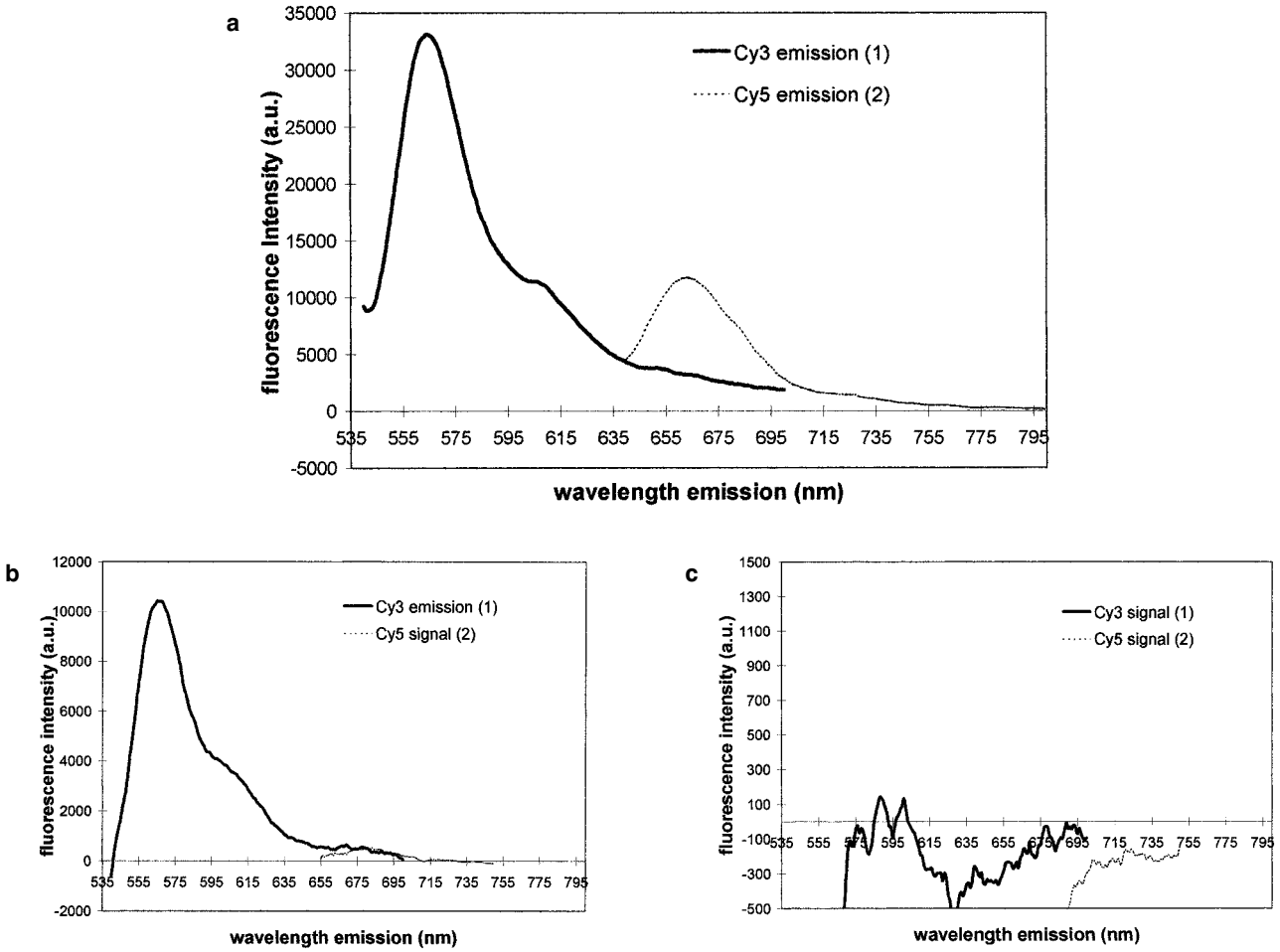


**Fig. 4.** Fluorescence analysis of the direct synthesis of L185 DNA sequence on functionalized $Si/SiO_2$ surface. (a) Hybridization and adsorption of [Cy3U185] and [Cy5L226]. Curve (1), Cy3 emission from hybridized and adsorbed [Cy3U185] units; curve (2), Cy5 emission from adsorbed [Cy5L226] units. (b) Elimination of nonspecific hybridization and adsorption. Remaining of the Cy3 fluorescence coming from labeled duplex sequences [L185–Cy3U185]. Curve (1), Cy3 emission from hybridized [Cy3U185] units after rinsing; curve (2), Cy5 signal after rinsing. (c) Loss of the Cy3 emission signal of duplex sequences [L185–Cy3U185] during the denaturation process. Curve (1), Cy3 signal, and curve (2), Cy5 signal, after denaturation.

adsorbed [Cy5L226] moieties labeled with Cy5 (Fig. 3a, curve 2) and the adsorbed noncomplementary [Cy3U185] strand labeled with Cy3 (Fig. 3a, curve 1).

As previously presented the efficacy of the rinsing procedure was characterized by the evolution of the respective emission of both probes after each rinsing step. Only the rinsing solvent, 1X SSC, was able to isolate the complementary Cy5 fluorescence (Fig. 3b, curve 2) with elimination of the noncomplementary Cy3 emission (Fig. 3b, curve 1). The rinsing powers of the 5X and 2X SSC solvents were inefficient to obtain this result.

A constant fluorescence emission signal, at the end of the rinsing process, suggests the presence of duplex labeled sequences formed on linked DNA strands with the complementary moiety [Cy5L226] during the hybridization process. The verification of this hypothesis was obtained with the complete disappearance of the Cy5 signal when the molecular hybridized system was submitted to the denaturation process (Fig. 3c, curve 2). The succession of hybridization and denaturation processes have been repeated three times on the same wafer, indicating that DNA strands are strongly linked to the surface and remain active at each experimental step. The support-bound molecular array presents the real functionality of a DNA chip.

We have verified that the behavior of labeled oligonucleotide moieties, such as [Cy5L226] and [Cy3U185], at the level of the hybridization or the adsorption processes is not depending on the nature of the fluorescent probe. We should observe any effect coming from the

sequence units, L226 and U185, as they present an equivalent nucleic base composition. On a new silicon wafer, a 25-mer oligonucleotide array, [L185], was synthesized. Then this new molecular system was submitted to the hybridization process of the complementary unit [Cy3U185]. This hybridization was performed in the presence of the noncomplementary sequence [Cy5L226] to test the ability of the molecular system arrayed on the wafer to adsorb the labeled reagents.

A similar experimental protocol as described previously was applied to this new sample: (i) preliminary identification of the emission of both pigments (Fig. 4a, curves 1 and 2) and (ii) control of the different rinsing steps. As expected, only the fluorescence signal of Cy3 moieties was stabilized at the end of the rinsing process (Fig. 4b, curve 1) and a complete loss of the Cy3 group signal was obtained after the denaturation process (Fig. 4c, curve 1). This new experiment attests, once more, that, with the synthesis and the analysis procedures presented here, we are able to detect hybridized DNA units and to identify clearly oligonucleotides fixed either with the hybridization process or by the competitive adsorption on the functionalized wafer. The Cy3 and Cy5 probes can be indifferently used to characterize both fixation modes of DNA sequences on solid wafers.

## CONCLUSION

We have shown in this work the opportunity to use direct oligonucleotide synthesis on activated $Si/SiO_2$ wafers to elaborate support-bound DNA arrays. By using suitable probes, we have characterized with fluorescence emission measurements either the formation or the functionality of the directly synthesized sequences. The elaboration procedure leads to the complete synthesis of defined sequences covalently linked to the solid support. The corresponding arrays have been found to be active for several cycles of hybridization–denaturation. A fluorescent system composed of two probes, such as Cy3 and Cy5, gives us the possibility of reading, at the macroscopic scale, hybridized DNA array within the interference of the nonspecific hybridization process of labeled DNA targets.

Direct synthesis of support-bound oligonucleotide arrays associated with fluorescence detection offer an attractive and promising solution to the problem of large-scale detection and analysis of DNA polymorphism. Taking into account the necessary high spatial resolution of this technical analysis and the performance of the fluorescence technique for hybridization detection, we are presently building up a scanning fluorescence device to perform the analysis, at the microscopic scale, of the hybridized area mapped on DNA chips.

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