Article

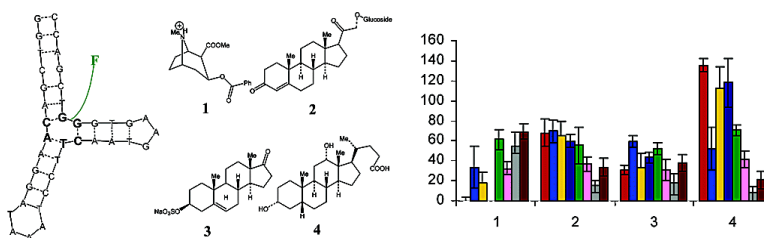# Cross-Reactive Arrays Based on Three-Way Junctions

Milan N. Stojanovi, Eric G. Green, Stanka Semova, Dragan B. Niki, and Donald W. Landry

More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Links to the 1 articles that cite this article, as of the time of this article download
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

Cross-Reactive Arrays Based on Three-Way Junctions

Milan N. Stojanović,* Eric G. Green, Stanka Semova, Dragan B. Nikić, and
Donald W. Landry

*Contribution from the Division of Clinical Pharmacology and Experimental Therapeutics,
Department of Medicine, Columbia University, New York, New York 10032*

Received October 15, 2002; E-mail: mns18@columbia.edu

Abstract: We report herein a novel system for the parallel processing of molecular recognition events utilizing arrays of oligonucleotide-based fluorescent sensors to characterize hydrophobic molecules in solution. The binding domains of the sensors were based on three-way junctions that utilize double helical stems as framework regions to reliably fold regardless of variations in or around the binding domain. A reporting domain was introduced by the specific substitution of a single phosphodiester group with a phosphorothioate, followed by selective functionalization with a fluorophore. The sensors were organized into cross-reactive arrays to yield characteristic fingerprints for samples containing hydrophobic molecules. The fingerprints can be used to characterize steroids in solution, including complex biologically important fluids. Arrays have the potential for clinical applications such as the detection of gross errors in steroidogenesis.

Introduction

The mammalian olfactory system consists of approximately one thousand unique receptors.[1] The distinctive characteristic of this system is cross-reactivity, that is, one receptor may react with many odorants, and one odorant may react with many receptors. Thus, an odorant is not characterized by a single interaction but rather through a pattern of massively parallel responses yielding fingerprints characteristic for that specific odorant. Attempts to mimic the mammalian olfactory system have led to the development of "electronic noses" or arrays of cross-reactive sensors.[2] In cross-reactive arrays, instead of standard dose−response curves, analytical samples are matched through their characteristic fingerprints to available standards. However, the molecular frameworks suitable for introducing the incremental variations of structure needed to achieve differential cross-reactivity are currently limited. In this report, we demonstrate that biomolecular receptors based on nucleic acid three-way junctions can be adapted to yield cross-reacting arrays for fingerprinting of solutions containing hydrophobic molecules.

Nucleic acid junctions are formed at the intersection of three or more double helixes. We previously isolated the first cocaine-binding aptamers and characterized these structures through mutagenesis to be three-way junctions with mismatched stems.[3] We found that the fully matched analogue of our highest affinity aptamer bound cocaine less efficiently but was able to bind other hydrophobic molecules well. The capacity of various nucleic acid junctions to incorporate hydrophobic molecules (Figure 1) was reported during early footprinting studies[4] and confirmed by the isolation of antisteroid aptamers comprised of fully matched three-way junctions.[5] The three exposed aromatic surfaces of unstacked base pairs in three-way junctions form a lipophilic cavity approximately 11 Å in diameter, which is capable of binding a wide range of hydrophobic guest molecules.[4] We realized that the framework provided by the stems would ensure proper folding regardless of the modifications at the junctions. The ability to vary easily and systematically the structure of these receptors through the introduction of mutations, mismatches, and chemical modifications would represent an important advantage over other hydrophobic hosts,[6] such as cyclodextrins and calixarenes. According to our preliminary screening, each junction could interact with multiple guest molecules, and each guest could interact with multiple junctions. Thus, this system seemed suitable to test the usefulness of three-way junctions as the basis of arrays capable of generating fingerprints. In this case, the fingerprints would be characteristic for hydrophobic surfaces, and the resulting array would be a primitive solution-phase mimic of the olfactory system.

(1) Axel, R. Molecular Logic of Smell. *Sci. Am.* **1995**, *273*, 154.
(2) (a) Alberth, K. J.; Lewis, N. S.; Schauer, C. L.; Sotzing, G. A.; Stitzel, S. E. I.; Vaid, T. P.; Walt, D. R *Chem. Rev.* **2000**, *100*, 2595 and references therein. (b) Schauer, C. L.; Steemers, F. J.; Walt, D. R. *J. Am. Chem. Soc.* **2001**, *123*, 9443. (c) Lavigne, J. J.; Anslyn, E. V. *Angew. Chem., Int. Ed.* **2001**, *40* (17), 3118. (d) Rakow, N. A.; Suslick, K. S. *Nature (London)* **2000**, *406* (6797), 710. (e) McCleskey, S. C.; Griffin, M. J.; Schneider, S. E.; McDevitt, J. T.; Anslyn, E. V. *J. Am. Chem. Soc.* **2003**, *125*, 1114. (f) Goodey, A. P.; McDevitt, J. T. *J. Am. Chem. Soc.* **2003**, 125, 2870.
(3) (a) Stojanovic, M. N.; Landry, D. W. *J. Am. Chem. Soc.* **2002**, *124*, 9678. (b) Stojanovich, M. N.; de Prada, P.; Landry, D. W. *J. Am. Chem. Soc.* **2001**, *123*, 4928.
(4) Lu, M.; Guo, Q.; Mueller, J. E.; Kemper, B.; Studier, F. W.; Seeman, N. C.; Kallenbach, N. R. *J. Biol. Chem.* **1990**, *265*, 16778 and references therein.
(5) Kato, T.; Yano, K.; Ikebukuro, K.; Karube, I. *Nucleic Acids Res.* **2000**, *28*, 1963 and references therein.
(6) (a) Gellman, S., Guest Ed.) Introduction: Molecular Recognition. *Chem. Rev.* **1997**, *97*, special thematic issue. (b) Ariga, K.; Terasaka, Y.; Sakai, D.; Tsuji, H.; Kikuchi, J. *J. Am. Chem. Soc.* **2000**, *122*, 7835−7836. (c) Castellano, R. K.; Craig, S. L.; Nuckolls, C.; Rebek, J., Jr. *J. Am. Chem. Soc.* **2000**, *122*, 7876−7882. (d) ref 3a. (e) Breslow, R.; Dong, D. S. *Chem. Rev.* **1998**, *98*, 1997−2011.
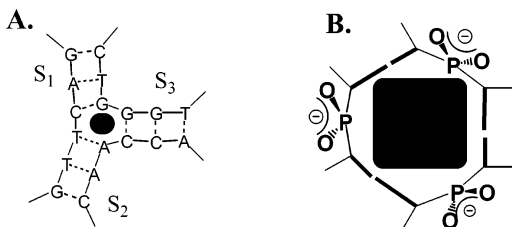
Figure 1. (A) Generic structure of the three-way junction region of a nucleic-acids-based receptor with a ligand guest (black circle). Further variations in junction structure could be introduced by mismatches and bulges (unpaired bases). (B) A schematic representation of junctions, with guest molecules (black square) shows the three aromatic unstacked surfaces separated by phosphodiester groups forming a hydrophobic binding pocket.
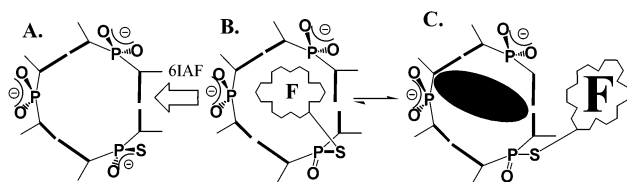

Figure 2. Schematic representation of the core structures of a three-way junction with (A) one out of three junctIonal phosphodiester groups substituted with a phosphorothioate group; (B) fluorescein (**F**) attached to the reactive sulfur through a reaction with 6-IAF (one diastereomer shown); and (C) fluorophore internally displaced from the cavity of the three-way junction by a hydrophobic molecule (black ellipse).

Our final consideration was the nature of the reporting event.[7] Inspired by the seminal work of Ueno and colleagues on cyclodextrins,[8] we decided to test the possibility that introduction of a fluorophore into the hydrophobic cavity of the junction would yield a molecular sensor based on the internal displacement of the fluorophore by a guest molecule. Fluorophores have been introduced stochastically *outside of* the binding pocket of an anti-ATP aptamer, by the individual substitution of standard bases with fluorescent dU analogues to yield ATP sensors.[9] While useful, this method provides us with only limited capabilities for the rapid testing of numerous sensors with fluorophores introduced *directly into* the hydrophobic pocket. We now report the synthesis of fluorescent-signaling sensors based on phosphorothioate-substituted three-way junctions and demonstrate that an array of such sensors is capable of fingerprinting hydrophobic molecules in solution.

Results and Discussion

Construction of Three-Way Junction-Based Sensor For Cocaine and Its Cross-Reactivity. We initially devised a sensor based on our cocaine-binding junction **MNS4.1**. For this purpose, we adapted a two-step method for the construction of the sensors. We first introduced a single phosphorothioate group into an aptamer and then selectively functionalized this group with a thiol-reactive fluorophore[10] (Figure 2). This method is especially convenient for rapid screening of various fluorophores as signaling components at various positions of oligonucleotide-
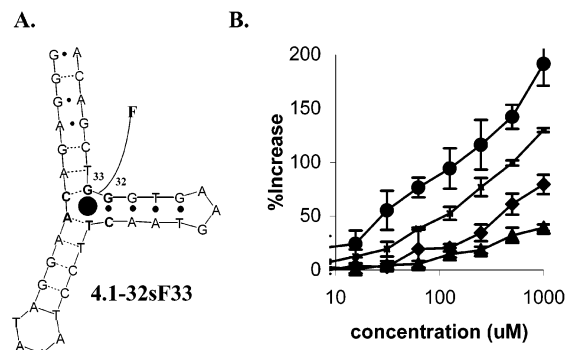
(7) De Silva, A. P.; Gunaratne, H. Q. N.; Gunnlaugsson, T.; Huxley, A. J. M.; McCoy, C. P.; Rademacher, J. T.; Rice, T. E. *Chem. Rev.* **1997**, *97* (15), 1515−1566 and references therein.
(8) Ikeda, H.; Nakamura, M.; Nobuyuki, I.; Oguma, N.; Nakamura, A.; Ikeda, T.; Toda, F.; Ueno, A. *J. Am. Chem. Soc.* **1996**, *118*, 10980−10988 and references therein.
(9) The aptamer-based molecular sensors for ATP with fluorophore in the proximity, but outside of the binding site, were reported by: Jhaveri, S. D. et al. *J. Am. Chem. Soc.* **2000**, *122*, 2469.
(10) Fidanza, J. A.; Ozaki, H.; McLaughlin, L. W. *J. Am. Chem. Soc.* **1992**, *114*, 5509.

Figure 3. (A) Structure of sensor **4.1-32sF33**. (B) Increase in fluorescence intensity (%) vs ligand concentration (μM) for **4.1-32sF33**. Ligands: cocaine hydrochloride, **1** (◆); deoxycorticosterone 21-glucoside, **2** (●); dehydro-isoandrosterone 3-sulfate sodium, **3** (■); and sodium deoxycholate, **4** (▲). All measurements were taken in triplicates, and the standard deviations are shown.

based sensors. The drawback of this method is that the sensors are obtained as mixtures of diastereomers at phosphorus that interact differently with ligands. Although diastereomers are separable by ligand-affinity chromatography (Supporting Information), for array work, we opted to use the mixtures directly. Hereafter, we will refer to each pair of diastereomers as a single sensor.

We constructed an analogue of **MSN4.1** in which a single phosphodiester bond between **G32** and **G33** at the rim of the putative three-way junction was substituted with a phospho-rothioate group. This derivative was coupled with a series of thiol-reactive fluorophores.[11] While many fluorophores yielded moderately successful cocaine sensors, comparable to the previously reported anti-ATP sensors (Supporting Information), we decided to focus on a fluorescein-modified derivative **4.1-G32FG33** (Figure 3A) which displayed an unusually strong 3-fold increase in fluorescence upon binding of cocaine (**1**) (Figure 4), with a dynamic range of 50 μM to 5000 μM. The magnitude of the increase in fluorescence compares favorably to all previously reported monofluorophoric aptameric systems, including those that were isolated through in vitro selection.[12] The excellent signaling of this aptamer could be rationalized by the possibility that several proximal guanosines in the noncanonical stem provide a potent quenching of fluorescein.[13] Although the affinity of the aptamer for cocaine diminished with fluorescent labeling, the sensor preserved selectivity for cocaine over its less hydrophobic metabolites benzoyl ecgonine and ecgonine methyl ester (cf. Supporting Information). This led us to consider the possibility that hydrophobic molecules could be sensed generally.

To characterize the affinity of **4.1-G32FG33** for hydrophobic ligands, we screened this junction for binding to three steroids, deoxycorticosterone 21-glucoside (**2**), dehydroisoandrosterone 3-sulfate (**3**), and deoxycholic acid (**4**) (Figure 4). These steroids are potential targets for "mix and measure" assays of urine samples. The first two steroids are conjugated members of the

(11) The following derivatives available from Molecular Probes were tested: 6-IAF, IAEDANS, BADAN, 5-TMRIA, mBBR, qBBR, Lucifer Yellow IA, Pyrene IA, PyMPO-maleimid.
(12) Jhaveri, S.; Rajendran, M.; Ellington, A. D. *Nat. Biotechol.* **2000**, *18* (12), 1293−1297.
(13) The distance-dependent quenching influence of guanosine residues has been used as a tool to probe conformation in DNA molecules: Knemeyer, J.-P.; Marne, N.; Sauer, M. *Anal. Chem.* **2000**, *72*, 3717−3724 and references therein.
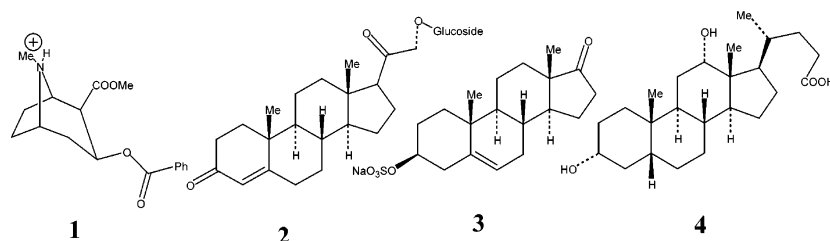
Figure 4. Structures of four ligands: cocaine (**1**), deoxycorticosterone 21-glucoside (**2**), dehydroisoandrosterone 3-sulfate (**3**), and deoxycholic acid (**4**).

17-ketosteroid (17-KS) and corticosteroid (including 17-hydroxycorticosteroid or 17-OHCS) groups. They have very similar hydrophobic shapes in solution and differ mostly in the position of the solubilizing groups. These steroids are of interest clinically because a change in their ratio indicates a gross abnormality in steroidogenesis and differentiates various forms of Cushing's disease. Current assays are cumbersome, multistep procedures. The third steroid is a representative bile acid, which is determined in clinical samples to diagnose abnormalities in liver function. Figure 3B shows the sensor response to the cocaine and the three steroids. The reference values for 17-KS, 17-OHCS, and bile acids in urine and bile are well within the sensitivity ranges of our sensor,[14] and **4.1-32GsFG33** clearly demonstrates the ability to react differentially with various hydrophobic molecules. This cross-reactivity is to be expected from a receptor with a primary recognition mechanism based on hydrophobic interactions. Whereas a low specificity of responses would typically invalidate a sensor, a panel of such sensors with different levels of cross-reactivity could be useful as an array.

Controling Shape of Hydrophobic Pockets Through Mismatches. We first varied the shape of the junction by introducing mismatches in the stems (Figure 5a). We began with a fully matched fluorescent analogue of our junction **fmtc-32sF33** and then methodically introduced mismatches at stem 3 to obtained three sensors: **A23-32sF33**, **G24-32sF33**, and **T25-32sF33**. All sensors were cross-reactive with various steroids and cocaine and displayed micromolar dissociation constants. We show, as an example, the dose−response curve for the **T25-32sF33** (Figure 5B), with a cross-reactivity pattern for hydrophobic molecules different from that of **4.1-32sF33** (cf. Figure 3B). The most striking difference between these two sensors is the weak interaction with cocaine and the strong interaction with deoxycholic acid, arguing for a larger and more symmetrical hydrophobic pocket being able to accommodate the bent molecules of the 5β-series.

Controling the Shape and Charge of the Hydrophobic Pockets through Fluorophore Positioning. In addition to junctional mismatches, we screened fluorophore positional isomers of **MNS4.1**: **4.1-7sF8**, **4.1-21sF22** (not shown), **4.1-22sF23**, **4.1-31sF32**, and **4.1-34FU** (Figure 6A), together with our initial **4.1-32sF33**. One of the sensors, **4.1-21sF22**, was poorly responsive to all ligands and was eliminated from further testing. Sensor **4.1-34FU**, shown in Figure 6B, incorporates a fluorescent dU analogue in place of base T34 by a published procedure[9,12] and is the only sensor in our array not synthesized through the phosphorothioate procedure.[15]

Changing the position of the flourophore has several effects on the sensors. First, the shape of the junction is influenced, as
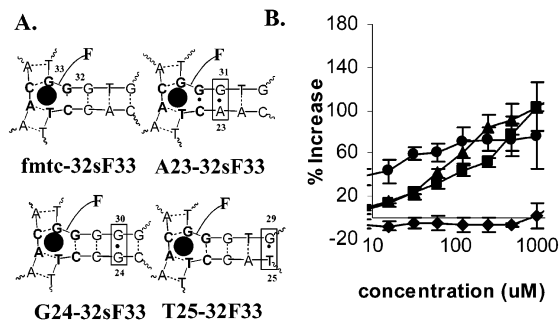

Figure 5. (A) Junctional structures of each sensor, with the position of fluoreophore attachment indicated (**F**, fluorescein). These four junctions differ in the position of mismatches (boxed) in the S_3 stem in regard to **4.1-32sF33**. (B) Dose response curves for **T25-32sF33**. Ligands: cocaine hydrochloride, **1** (◆); deoxycorticosterone 21-glucoside, **2** (●); dehydroisoandrosterone 3-sulfate sodium, **3** (■); and sodium deoxycholate, **4** (▲). All measurements were taken in triplicates, and the standard deviations are shown.
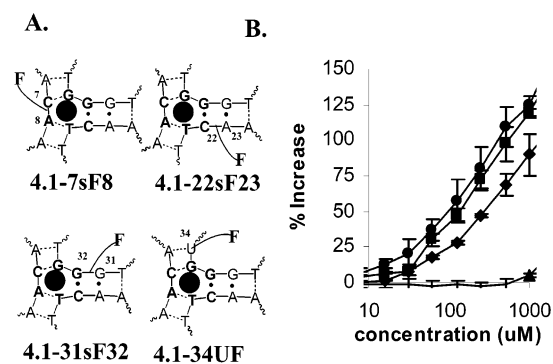

Figure 6. (A) Five isomeric sensors, based on the junction **MNS4.1**, with varying positions of fluorophore, as shown. The $G_{26}AA$ loop in the S_3 stem is not shown. (B) The dose−response curves for **4.1-33FU**. Ligands: cocaine hydrochloride, **1** (◆); deoxycorticosterone 21-glucoside, **2** (●); dehydroisoandrosterone 3-sulfate sodium, **3** (■); and sodium deoxycholate, **4** (▲). All measurements were taken in triplicates, and the standard deviations are shown.

could be clearly demonstrated by the stronger response of **4.1-34FU** than **4.1-32sF33** to the 3-sulfate **3**. Second, the introduction of a fluorophore within the hydrophobic pocket in the **32sF33** family of sensors reduces the cumulative negative charge in the junction; analogously, the removal of a fluorophore from the immediate junction region, as in the **4.1-22sF23** and **4.1-31sF32** sensors, increases the negative charge within the junction.

Other types of structural variations near the junction are also available to us, including the structure of fluorophore, the use of modified and unnatural nucleotides, the substitution of phosphodiester bonds with analogues, and the expansion of the

(14) Elin, R. J. Reference Intervals and Laboratory Values. In *Cecil Textbook of Medicine,* 20th ed.; Bennett, J. C., Plum, F., Eds.; W.B. Saunders Co.: Philadelphia, PA, 1996.

(15) We also tested this design by substituting our T21 with a fluoresceinated analogue and obtained changes in fluorescence of only up to 50% with cocaine.
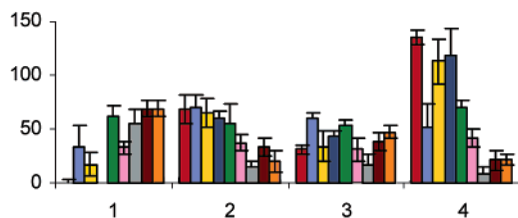
Figure 7. Fingerprints based on an array of eight sensors: cocaine, **1** (500 μM); deoxycorticosterone 21-glucoside, **2** (32 μM); dehydroisoandrosterone 3-sulfate, **3** (125 μM); and deoxycholic acid, **4** (2 mM). Red, **fmtch-32sF33**; blue, **A23-32sF33**; gold, **G24-32sF33**; deep blue, **T25-32sF33**; green, **4.1-32sF33**; pink, **4.1-7sF8**; gray, **4.1-22sF23**; brown, **4.1-31sF32**; orange, **4.1-FU**. The response from **4.1-32sF33** (green) was nearly identical to four ligands and was used as a reference point to choose concentrations. Triplicate measurements of fluorescence intensity were taken, with the standard deviations shown.

Figure 8. Fingerprints (fluorescence intensity, relative units) of urine (U), urine spiked with deoxycorticosterone 21-glucoside (U+2), and urine spiked with dehydroisoandrosterone 3-sulfate (U+3). Yellow, **4.1-7sF8**; light blue, **A23-32sF33**; red, **T25-32sF33**; brown, **4.1-32sF33**. Triplicate measurements of fluorescence intensity were taken, with the standard deviations shown.

framework to a four-way junction. We are preparing hundreds of variations and will shortly report some of these with a full characterization of the hydrophobic space of steroids.

Fingerprints of Ligands. Whereas a given sensor may weakly discriminate various hydrophobic ligands, an array of related sensors can actually achieve considerable specificity. This mode of identification is based on obtaining a series of fluorescence readouts characteristic for each concentration of a given ligand. The response of the panel provides a "fingerprint" for that concentration of ligand. Preferably, each concentration of each ligand of interest would have a unique fingerprint (shape, defined as a ratio of intensities) and/or intensity. We note that the shapes of fingerprints are not expected to be conserved over wide concentration ranges because individual sensors have dose−response curves that differ in slope and point of inflection (cf. Figures 3B, 5B, and 6B). Importantly, the conservation of fingerprint shape is not a requirement for array-based approaches, where individual arrays are usually incorporated with neural networks and trained to recognize exemplary solutions of interest.

The power and advantage of this approach in comparison to the classic sensor approach is clearly demonstrated by the following example: We took concentrations of the four ligands that provided a response of similar intensity (50−70%) to the sensor **4.1-32sF33** (green bar in Figure 7): **1**, 500 μM; **2**, 32 μM; **3**, 125 μM; and **4**, 2 mM. When a single sensor is presented with these four samples, it would not be able to distinguish them. On the other hand, the array clearly and reproducibly distinguished the solutions of the three steroids from each other and from cocaine (Figure 7). A physicochemical explanation for variations in the binding of a ligand to each sensor is not easily formulated, but fortunately, it is also not necessary. Thus, we were able to attribute characteristic fingerprints to all tested concentrations of all four ligands (Suplementary information), indicating that arrays are capable of both qualitative and quantitative analysis of samples with single interacting species. In these experiments, molecules widely different in hydrophobic properties are easily recognized with small subsets of sensors in arrays. For example, the fingerprint of any solution of cocaine can be easily visually distinguished from the fingerprint of any solution of deoxycholic acid or any solution containing two urinary metabolites based on the characteristic ratio of responses by, for example, **4.1-32sF33** and **T25-32sF33** (cf. Figures 3B and 5B). However, corticosterone and androsterone derivatives **2** and **3** with very similar hydrophobic shapes are more
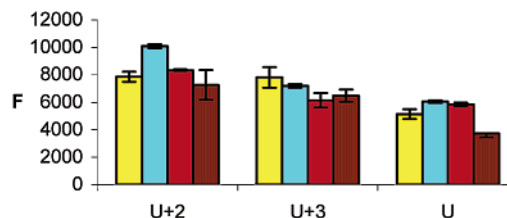
challenging to distinguish; while at some concentrations two or three sensors were sufficient, a subset of up to four sensors was needed to remove ambiguity at all concentrations tested (Supporting Information).

Fingerprints of Complex Mixtures. The fingerprints obtained for pure compounds are not necessarily an additive characteristic; that is, we do not expect to be able to recognize fingerprints of individual compounds upon adding them to complex mixtures. However, we would like to be able to fingerprint standard complex mixtures and then identify unknown mixtures through the comparison of their fingerprints with those of the standards.

Accordingly, as a final proof-of-concept experiment, we tested the ability of our sensors to obtain useful fingerprints in complex mixtures. A key test for this practical application would be the ability of a small array to obtain unique fingerprints from urine and from the same urine spiked with metabolites characteristic of particular diseases. For example, clinical urine samples contain large quantities of various steroidal metabolites. The ability to identify a urine sample with spiked steroids by means of its hydrophobic fingerprint would provide a proof-of-concept for fingerprinting gross deviations from clinical norms. Accordingly, we compared the fingerprints of a standard sample of urine (Sigma, lyophilized human male urine metabolites) to those of the same urine spiked with **2** or **3** (200 μM). We were able to differentiate unambiguously the three solutions based on fingerprints obtained through a subset of four sensors (Figure 8). Most of the other sensors were unresponsive under these conditions, perhaps as a result of saturation by steroids naturally present in urine. Importantly, this also demonstrated that a sensor which might have been initially considered redundant (i.e., one of the two sensors with an identical response to one ligand) can play a key role in the analysis of complex mixtures (cf. **A23-32F33** and **T25-32F33**).

Conclusions

The present approach to sensors differs fundamentally from the aptamer-based approaches previously reported.[16] In the traditional lock-and-key approach to sensors, the cross-reactivity of aptamers would be considered deleterious. However, as shown herein, we can transcend the relative lack of specificity of three-way junctions for hydrophobic molecules by organizing these receptors into cross-reacting arrays. We have also demonstrated that three-way junctions represent a construct suitable for the reliable, systematic variation of structure. Antibodies also utilize molecular scaffolds to facilitate the

(16) For example, see: Yamamoto, R.; Kumar, P. K. R. *Gen. Cell.* **2000**, *5*, 389. References 3, 9, 12 and references therein.

display of diverse binding domains, but the three-way junctions avoid the cost of large framework regions. Finally, we have shown that introduction of a fluorophore into the junction can reliably yield sensors. Thus, instead of isolating individual aptamers through in vitro selection and amplification of oligonucleotides from libraries, we can now systematically construct a series of incrementally varied fluorescent oligonucleotide receptors. The characteristic responses (i.e., the fingerprints) of a panel of samples to an array of these junctions permit the unambiguous identification of unknown samples through fingerprint matching. It is an intriguing possibility that these hydrophobic fingerprints are intrinsic characteristics of the hydrophobic regions of a molecule, similar to IR patterns or NMR spectra. In an effort to standardize the sensing of this characteristic, we are pursuing the preparative scale synthesis of sensors with large-scale affinity separation of diastereomers.

We are now constructing arrays of hundreds of nucleic-acid-based receptors based on modified and natural nucleotides capable of characterizing bodily fluids for metabolites and drugs. For this solution-based equivalent to olfaction, the detection of a novel compound through its distinctive fingerprint would be analogous to detecting a new odorant molecule. Finally, we have shown that even an array of relatively small size is able to fingerprint instantaneously the hydrophobic surfaces of urinary metabolites characteristic for certain endocrinopathies. Clinical trials that would characterize fingerprints of normal urine samples and correlate abnormal fingerprints to disease states are in progress.

Materials and Methods

Materials. All oligonucleotides were custom-made and HPLC purified by Integrated DNA Technologies Inc. (Coralville, IA) or TriLink Biotechnologies (San Diego, CA) and used as received. Liophilized human male urine metabolites and steroids were purchased from Sigma. Cocaine was obtained through the National Instituted of Drug Abuse.

Instrumental. Initial characterization of fluorescent spectra for **MNS4.1-32F33** and **fmtch-32F33** were performed on a Hitachi Instruments Inc. (San Jose, CA) F-2000 fluorescence spectrophotometer with a Hamamatsu xenon lamp. Experiments were performed at the excitation wavelength of 480 nm and emission scan at 500−600 nm. All assays were performed using a Wallac Victor2 1420 multilabel counter (PerkinElmer Instruments, Shelton, CT) in 96-well plates (F96 Maxisorb, Nunc-immunoplates), using appropriate filters ($\lambda_{em} = 530 \pm 10$ nm, $\lambda_{exc} = 480 \pm 10$ nm).

Synthesis of Sensors. Procedures: Aptamer (5 nmol) in 20 μL of binding buffer (TRIS•HCl 20 mM, pH = 7.4, NaCl 140 mM, 6mM KCl), 40 μL of deionized water, and 5 μL of 6-iodoacetamido fluorescein (Molecular Probes, Eugene, OR) in DMSO (1 mg/10 μL) were incubated at room temperature (for mismatched junctions) or at 50 °C (fully matched junctions). After 90 min for heated and 180 min for room temperature mixtures, the reactions were applied to Sephadex G-25 columns (1.8 mL) and fluorescent macromolecular fractions (total of 400 μL) were isolated. The solutions (mixtures of diastereomers and starting materials) were used directly in assays. In a control reaction without a phosphorothioate group on a three-way junction, only negligible fluorescence was observed in these fractions.

Characterization of Sensors with Ligands. Solutions of sensors were diluted in binding buffer with 2 mM $MgCl_2$ to achieve responses between 300 and 2000 fluorescence units on the plate reader. Then, standard dilutions of ligand concentrations were made in the solution of sensors on 96-well plates. All measurements were performed in triplicates.

Characterization of Urine. Urine metabolites were dissolved in 35 mL of water, and the pH was adjusted to 7.4 by the addition of 300 μL of 10 N NaOH and 1 mL of 1 M TRIS buffer (pH 7.4). Urine was spiked with deoxycorticosterone 21-glucoside **2** and dehydroisoandresterone 3-sulfate **3** to a 200 μM concentration. Samples of urine or spiked urines (25 μL) were diluted with buffer containing sensors (5 μL of sensor solution in 75 μL of binding buffer) followed by reading on the plate reader.

Supporting Information Available: (1) Fluorescence spectra changes of **4.1-32sF33** in the presence of corticosterone and cocaine. (2) Selectivity of **4.1-32sF33** for cocaine over cocaine metabolites. (3) Response of sensor **4.1-32sBIMAN33** to cocaine. (4) Response of sensors **4.1-7sEDANS8**, **4.1-21sE-DANS22**, and **4.1-32sEDANS33** to cocaine. (5) Separation of diastereomers of **4.1-32sF33** on a cocaine affinity column. (6) The fingerprints of four ligands at all tested concentrations. This material is available free of charge via the Internet at http://pubs.acs.org.

JA0289550