In this article it is shown how optimized and dedicated microarray experiments can be used to study the thermodynamics of DNA hybridization for a large number of different conformations in a highly parallel fashion. In particular, free energy penalties for mismatches are obtained in two independent ways and are shown to be correlated with values from melting experiments in solution reported in the literature. The additivity principle, which is at the basis of the nearest-neighbor model, and according to which the penalty for two isolated mismatches is equal to the sum of the independent penalties, is thoroughly tested. Additivity is shown to break down for a mismatch distance below 5 nt. The behavior of mismatches in the vicinity of the helix edges, and the behavior of tandem mismatches are also investigated. Finally, some thermodynamic outlying sequences are observed and highlighted. These sequences contain combinations of GA mismatches. The analysis of the microarray data reported in this article provides new insights on the DNA hybridization parameters and can help to increase the accuracy of hybridization-based technologies.
were designed and used for hybridization to either statistical model (see Supplementary Data). The theory of Optimal Design provides some criteria of selecting an optimal set of measurements, which minimize the uncertainties in the parameters of a regression method [2]. The theory of Optimal Design provides selected from different sets of experiments were performed using the target sequences shown in Table I. These sequences were replicated separately on specific microarrays containing mismatched probes with up to two mismatches with respect to the target. Note that $t_1$ and $t_2$ share a common triplet of nucleotides AGC at the same sequence position (in bold characters). The mismatches centered around this triplet will be discussed in some details in the ‘Results’ section.

**TABLE I.** Target sequences used in the experiments. At the 3’ side of each sequence a 20-mer poly(A) is attached, terminating with a Cy3 fluorophore. The targets were selected from Optimal Design criteria [2] (Supplementary Data). Each target is hybridized separately on specific microarrays containing mismatched probes with up to two mismatches with respect to the target sequence and a probe sequence in $i$, $A$ is a parameter, which sets the intensity scale, $c$ the target concentration, $R$ the gas constant and $T$ the temperature (experiments are performed at $T = 65^\circ C = 338 K$, which is the value of the temperature used in the rest of the analysis). Although the data analyzed are background-subtracted from the Agilent scanner, there remains always some small aspecific signals, which we denote by $I_0$ in Equation (1). In the experiments $I_i$ is obtained from the average over typically approximately 15 replicated spots. One should note that Equation (1) is valid at sufficiently low target concentrations, i.e. when only a limited fraction of probes is hybridized in a spot, hence far from chemical saturation. On the other hand, at very low concentrations, the specific signal, i.e. the second term in Equation (1), can become comparable to $I_0$. Therefore, for the analysis of the data we restricted ourselves to intermediate concentrations and intensities for which we explicitly verified that the intensities scale linearly with concentrations, as predicted by Equation (1) (more details can be found in the Supplementary Data). In the intensity scale of the experiments $I_0 \approx 1$, whereas the values used in the analysis are $I_i \gtrsim 10$. In practice, the large majority of the intensities in experiments with target concentration $c = 100 \text{ pM}$ or higher are above this threshold value.

In the following, we will consider the logarithm of the intensities measured with respect to the perfect match (PM) intensity. Using Equation (1), for $I_i \gg I_0$ we get:

$$y_i \equiv \ln I_i - \ln I_{PM} = -\frac{\Delta G_i - \Delta G_{PM}}{RT}$$

which defines the free energy penalty of probe $i$ with respect to the perfectly matching probe. This penalty can be expressed as a sum of NN dinucleotide parameters. Consider, for instance, the example of a probe $i$ with a single mismatch of type A with respect to the target nucleotide G and with neighboring nucleotides G and T. We have:

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$$\Delta G \begin{pmatrix} GA \\ CG \end{pmatrix} + \Delta G \begin{pmatrix} AT \\ GA \end{pmatrix} - \Delta G \begin{pmatrix} GC \\ CG \end{pmatrix} - \Delta G \begin{pmatrix} CT \\ GA \end{pmatrix} \equiv \Delta \Delta G \begin{pmatrix} GAT \\ CGA \end{pmatrix}$$

**III. RESULTS**

**A. Nearest-neighbor parameters from linear regression**

Equilibrium thermodynamics predicts that the measured fluorescence intensity from a spot $i$ equals to:

$$I_i = I_0 + A e^{-\Delta G_i / RT}$$

where $\Delta G_i$ is the hybridization free energy between the target sequence and a probe sequence in $i$, $A$ is a parameter, which sets the intensity scale, $c$ the target concentration, $R$ the gas constant and $T$ the temperature (experiments are performed at $T = 65^\circ C = 338 K$, which is the value of the temperature used in the rest of the analysis). Although the data analyzed are background-subtracted from the Agilent scanner, there remains always some small aspecific signals, which we denote by $I_0$ in Equation (1). In the experiments $I_i$ is obtained from the average over typically approximately 15 replicated spots. One should note that Equation (1) is valid at sufficiently low target concentrations, i.e. when only a limited fraction of probes is hybridized in a spot, hence far from chemical saturation. On the other hand, at very low concentrations, the specific signal, i.e. the second term in Equation (1), can become comparable to $I_0$. Therefore, for the analysis of the data we restricted ourselves to intermediate concentrations and intensities for which we explicitly verified that the intensities scale linearly with concentrations, as predicted by Equation (1) (more details can be found in the Supplementary Data). In the intensity scale of the experiments $I_0 \approx 1$, whereas the values used in the analysis are $I_i \gtrsim 10$. In practice, the large majority of the intensities in experiments with target concentration $c = 100 \text{ pM}$ or higher are above this threshold value.

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| Target Sequences | Description |
|------------------|-------------|
| $t_1$: 5’-CTGGTCTTAGAGGACCGGACTGTGTTT-pol y(A)-3’-Cy3 | Primer with mismatches centered around AGC triplet |
| $t_2$: 5’-CTGCACAGTTCCCGACACGTTAGAATT-pol y(A)-3’-Cy3 | Primer with mismatches centered around AGC triplet |
| $t_3$: 5’-ATAATCGTCATTGGACACCGGGAA-pol y(A)-3’-Cy3 | Primer with mismatches centered around AGC triplet |
We use the following notation: the target sequence is the bottom strand and the probe sequence, which is oriented from 5'–3', is the top strand. This example corresponds to target $t_1$ or $t_2$ at position 10, counting from 3' end (the triplet of nucleotides are indicated in bold in Table 1). In Equation (3) $\Delta \Delta G$ is defined as the free energy penalty of an isolated mismatch in a DNA duplex. This penalty is expected to be a local effect. In the NN model this locality is inherent: the dots in Equation (3) indicate identical nucleotides in the two sequences, their contribution cancels out and leaves per isolated mismatch only four dinucleotide parameters around the mismatch position.

There are in total only 58 such dinucleotide parameters: 10 perfect match parameters and 48 single mismatch parameters (taking into account symmetries). The dinucleotide parameters are not directly experimentally accessible and are not unique [12], e.g. they can be shifted by some constant value such that the physically accessible $\Delta \Delta G$ remains unchanged (see Supplementary Data).

Equations (2) and (3) define a linear problem: each measured $y_i$ can be expressed by a linear combination of dinucleotide parameters. In order to extract the parameters from the data we combined the results of the three experiments and performed a least square minimization of Equation (2). Mismatches closer than five sites from the helix edges were excluded from the analysis, as well as pairs of mismatches with a distance smaller than 5 nt.

The 58 adjustable parameters were fitted on a set of about a thousand of experimental data points above the intensity threshold. The fitted parameters then applied to produce the plot as shown in Figure 1 for all available intensities of the experiments in which either sequence $t_1$, $t_2$ or $t_3$ was hybridized on its corresponding microarray at a concentration of $c = 100$ pM. The data are plotted as a function of the unique $\Delta \Delta G$ for triplets defined as in Equation (3). We note that there is very good agreement between the data and the thermodynamic model of Equation (1). The experiments follow the equilibrium isotherm (a straight line with a slope equal to $1/RT$) for a range of intensities of more than four orders of magnitude. A previous study [9] in which hybridizing strands were 30-mers did not provide a single straight line in a $\ln I$ versus $\Delta \Delta G$ plot. Deviations due to lack of thermodynamic equilibrium were observed in the high-intensity ranges, as discussed in [11] [13].

Further it is important to note that we do not only find internally consistent results, but that our microarray-derived free energy parameters also correlate to a fair degree with those reported in literature for hybridization in solution [8]. Figure 2 shows a correlation plot of the free energy penalties (i.e. the $\Delta \Delta G$ defined as in the example of Equation (3)) obtained from the microarray data analysis and those from SantaLucia et al. from [8]. The Spearman correlation coefficient is equal to 0.855. This clearly shows that free energy parameters for DNA features measured by the presented microarray approach also apply for thermodynamic properties in solution. This opens the highly paralleled microarray toolbox for the study of thermodynamics of DNA structures. An example is discussed in the next section.
FIG. 3. Schematic representation of hybridizing strands in the microarray experiment. From the appropriate ratios of intensities measured from these spots, the free energy parameters can be determined and the additivity principle can be tested. As in the rest of the article the lower strand is the fixed target sequence. The upper strand is the probe sequence. The filled triangles denote mismatching nucleotides. In the four examples from the top we show: (a) hybridization with a PM probe, (b,c) hybridization with a single mismatch probe where the mismatching nucleotides are m and n at positions x and x + Δx respectively, (d) hybridization with a probe carrying two mismatches. We use the notations I_x^m, I_x+Δx^m and I_x+Δx^m,n to denote the corresponding intensities measured in the experiment.

B. Nearest-neighbor parameters from ratios of intensities: probing additivity

The crucial assumption of the NN model is additivity of local free energy contributions. We probe here the limits of additivity of free energy penalties as a function of the distance between two mismatches. We will access the free energy parameters by comparing ratios of intensities measured from different spots in the microarray.

Here, we combine microarray spots that contain probes with zero, one or two mismatches with respect to the target and we denote the location of the mismatch by x or x + Δx as illustrated in Figure 3. The associated free energy penalties can then be derived from the intensity measurements as follows

\[ \Delta \Delta G_x^m = -RT \ln \left( \frac{I_x^m}{I_{PM}} \right) \]  
\[ \Delta \Delta G_{x+\Delta x}^m = -RT \ln \left( \frac{I_{x+\Delta x}^m}{I_{PM}} \right) \]  
\[ \Delta \Delta G_{x,x+\Delta x}^{m,n} = -RT \ln \left( \frac{I_{x,x+\Delta x}^{m,n}}{I_{PM}} \right) \]

in which the superscript m and n represent the three possible mismatching nucleotides at location x and x + Δx respectively. If the additivity of the NN model holds, the free energy penalty of Equation 4 should equal the sum of the individual penalties of Equations 4 and 5. To test this, we introduce

\[ \alpha = \frac{\Delta \Delta G_x^m + \Delta \Delta G_{x,x+\Delta x}^{m,n} - \Delta \Delta G_{x,x+\Delta x}^m}{\Delta \Delta G_x^m + \Delta \Delta G_{x,x+\Delta x}^m} \]  

which measures the relative deviation from additivity.

FIG. 4. Parameter α, the relative deviation from additivity, from the experiment of target t1, averaged over x, m and n as a function of the distance |Δx| between two mismatches. The inset shows the plot with α in log scale.

These results are setting some limitations on the additivity of the NN model. However, outside this interaction region of 4 nt we expect the NN model to hold i.e. α should be zero and mismatches can be considered as isolated. This can be explicitly checked in a very direct
way. When $\alpha = 0$ we get from Equation (7)
\[
\Delta \Delta G_{x}^m = \Delta \Delta G_{x,x+\Delta x}^m - \Delta \Delta G_{x+\Delta x}^m.
\]
(8)
The free energy penalty $\Delta \Delta G_{x}^m$ of a mismatch $m$ at location $x$, which we will call the focus mismatch $(m,x)$, can be estimated either directly using Equation (4) or via a second mismatch $(n,x + \Delta x)$ using Equations (5) and (6) for any choice of $n$ and $\Delta x > 4$. Hence, the free energy penalty of the focus mismatch can be estimated from measurements in many independent ways and they should provide the same answer if additivity holds. Note that, using Equations (5) and (6), $I_{PM}$ drops out in the right hand side of Equation (5).

Figure 5 illustrates how Equation (8) can be used to estimate the $\Delta \Delta G$ using different combinations of $n$ and $\Delta x$. In this specific example we consider $\Delta \Delta G_{10}^m$ which corresponds both for target $t_1$ and $t_2$ to $\Delta \Delta G \left( \begin{array}{c} GAT \\ CGA \end{array} \right)$ (in the Supplementary Data, we show other examples featuring additivity for different focus mismatches).

In the pane for target $t_2$, all the estimates of the free energy penalty are close the other, the $48 + 1$ estimates tightly lie around a median value, in this case $\sim 2.1$ kcal/mol, indicated by the dotted line. The picture in the right pane is a typical one which we observe for any focus mismatch $(m,x)$. This confirms that additivity holds in the regime $\Delta x > 4$, i.e. when mismatches are separated by $\geq 4$ nt. Moreover, it shows that the microarray measurement is internally consistent. Secondly, the left pane, i.e. experiment $t_1$, provides the same median value for the free energy penalty, showing also the robustness of the microarray approach to estimate free energies of DNA structures. However, this figure was chosen because it is atypical in the sense that one notices two pronounced outlying values. They correspond to a sequence where both the focus mismatch and the second mismatch are of type $AG$. Since they clearly deviate from an otherwise nicely consistent picture, we believe there must a physically underlying reason for it. We will come back to this point in the section where we discuss thermodynamic outliers.

Note that with this second method we accessed values for the free energy penalties of isolated mismatches without using any multiple regression or fitting procedure, but we simply compared the ratios of intensities, Equations (4)-(6), to get a consistent set of independent estimates. The free energy penalties are then obtained from the median over all data points. We compared the free energy penalties obtained from this method (median) with those obtained from linear regression as discussed in the previous section. The two sets of data are well-correlated with a Pearson correlation coefficient equal to 0.966 (see Supplementary Data). This correlation shows the equivalence of the two approaches. In this analysis, we restricted ourselves to mismatches in the bulk of the sequence, i.e. $x > 5$ nt from the border. Closer to the border we observe boundary effects, which are covered in the next section.

C. Boundary effects

The previous section ended by showing the equivalence of both approaches to access free energy penalties of an isolated mismatch, provided the data are restricted to bulk mismatches. The direct median method of the previous section can also assess penalties of mismatches close to the boundary, whereas on the contrary the fitting method cannot by construction. The latter, however, has the advantage of fitting a full parameter set of the NN model and as such can easily provide bulk values for the free energy penalty of any isolated mismatch. The combination of both methods now provides an elegant way to assess the effect of boundary proximity on an isolated mismatch. Hereto, we introduce the parameter $\beta$ as the relative reduction of free energy penalty of a mismatch when compared to its bulk value.

\[
\beta = \frac{\Delta \Delta G_{x}^m}{\Delta \Delta G_{x}^m_{\text{bulk}}}
\]
(9)

In Figure 6 the parameter $\beta$ is shown as a function of $x$ after averaging over $m$. It is clear that, as expected, $\beta$ is approximately equal to one in the bulk, whereas when approaching the boundary, a reduction of free energy penalty occurs which reaches up to 80%. Note that for mismatches at the boundary, $x = 1$ and $x = 25$, the NN model is not applicable and no data is presented. Figure 6 show that the range of the boundary effect is $\sim 4$ nt.

D. Thermodynamic outliers

As a final result of this article, we come back to the two outliers observed in Figure 5(a); the same deviations are found in replicated experiments at different concentrations: therefore, they are unlikely due to experimental errors. For these two cases we find $\Delta \Delta G_{10,15}^A - \Delta \Delta G_{15}^G \approx 1.2$ kcal/mol and $\Delta \Delta G_{10,17}^A - \Delta \Delta G_{17}^G \approx 3.1$ kcal/mol, strongly deviating from the median value ($\approx 2.1$ kcal/mol). The common feature of these two sequences is that they involve GA mismatches. The two set of mismatches are arranged in an antiparallel way i.e. one $G$ and one $A$ are on the same strand. Mismatches of GA type in DNA and RNA helices have been the subject of several studies in the past [14–21]. In the RNA folding, it is known that GA pairs contribute substantially to the RNA helix stability. Their contribution is comparable to that of a canonical AT pair. As AT pairs, GA form two hydrogen bonds, but can also assume four different conformations [14]. The microarray data suggest that the antiparallel combination of GA and AG pairs of mismatches have a long range interaction effect, which is probably a signature of some structural conformational change of a double helix containing these pairs. Next-nearest neighbor effects extending up to $4$ nt distance for antiparallel GA mismatches have been reported in the
FIG. 5. Free energy penalty $\Delta \Delta G_{10}^A$ for focus mismatch ($m = A, x = 10$) derived from experimental intensities according to Equation (8) as a function of the location $x + \Delta x$ of the second mismatch ($n, x + \Delta x$). For each $|\Delta x| > 4$ the three values, one per possible mismatch, are indicated by the letter representing the mismatching nucleotide $n$ of the probe. The target sequence is written in top of the x-axis in 3'-5' notation, $t_1$ in left pane, $t_2$ in right pane. The dotted line corresponds to the median value of the 48 estimates. The circled point is the estimate without second mismatch coming from Equation (4). For this particular mismatch, the free energy penalty for both $t_1$ and $t_2$ is identical and corresponds to $\Delta \Delta G\left(\frac{G_{AT}}{C_{GA}}\right)$.

FIG. 6. Boundary effect: $\beta$, the relative reduction of mismatch free energy penalty, as a function of location for experiment with target $t_1$. Each point is the average of three estimates, one per possible mismatch. Data are absent for the extremal locations $x = 1$ and $x = 25$, since no value can be calculated by the NN model.

FIG. 7. The free energy penalty of tandem mismatches, from experiment with target $t_1$: $\Delta \Delta G\left(\frac{x_{mn}y_{xy}}{x_{ab}y_{ab}}\right)$, where $x'$ and $y'$ are complementary to $x$ and $y$ respectively, as denoted above the x-axis are the fixed nucleotides in the target. $mn$ is a tandem mismatch in the probe and the vertical position of these letters in the plot gives the associated free energy penalty. Note the low free energy penalty for $3'_{a} - G_{AT} - 3'_{a}$ mismatches (encircled).

The case of RNA duplexes in [13] (longer distances were not considered that case). We investigated antiparallel GA and AG pairs of mismatches also in sequences $t_2$ and $t_3$, but found no anomalous behavior in those cases. This suggests that the nucleotide sequences between the two GA/AG pairs plays an important role in the overall stability of the duplex.

As a further proof of the outlying behavior of antiparallel GA/AG pairs we show in Figure 7 a plot of free energy penalties for tandem mismatches (neighboring double mismatches). These are again obtained from Equation (9) for different $m$ and $n$ mismatching nucleotides, where in the case of tandem mismatches, $\Delta x$ is equal to 1. On each location of the sequence our data set contains nine different types of tandem mismatch. A clear boundary effect is noticeable, but when looking at the full data points tandem mismatch of the type GA/AG are again outlying, they appear to be particularly stable with a free energy penalty $\sim 2$ kcal/mol below average.
IV. DISCUSSION AND CONCLUSION

In this article, we have analyzed DNA hybridization reactions in microarrays and quantified free energy penalties of single and double mismatches. We have shown that the experimental data are very precise and reproducible. The microarray data follow an equilibrium isotherm over a range of four orders of magnitude in the fluorescence intensities and allow the extraction of accurate thermodynamic parameters. First, the analysis provides a database with a large number of NN parameters for isolated mismatches. These parameters correlate well with those reported in the literature from hybridization experiments in solution. Second, the experiments contain systematic measurements of hybridization with two mismatches, which allowed us to probe the validity limit of the NN approximation. We showed that when two mismatches are separated by a distance of \( \geq 5 \) nt their effect is additive, allowing a standard approach with the NN model. However, for shorter distances, the additivity is no longer valid and we found that duplexes with neighboring mismatches are more stable than expected from additivity. This interaction was shown to decay exponentially as a function of the distance between mismatches. Further, we investigated the behavior of mismatches close to the helix edges, and showed that their free energy penalty is reduced up to \( \leq 80\% \) when compared to the bulk behavior. The boundary effect was observable up to 4 nt from the helix edge. Finally, we also found some thermodynamic outliers, sequences involving two antiparallel GA mismatches, in which the mismatch interaction appears to persist beyond 5 nt. These outliers were not related to experimental error indicating a signature of some structural conformational change of a double helix containing these mismatch pairs.

Overall, the analysis of the microarray data reported in this article provides new quantitative insights on the DNA hybridization parameters, on the NN model and its present limitations. Our study is in line with a number of recent articles, which have been dedicated to the investigations of fundamental physico-chemical properties of DNA arrays [22, 31]. Due to the relevance of hybridization in many technologies, going from PCR [1] to recent developments in biosensors, e.g. [22], a good thermodynamic model is also important from the application point of view. A precise quantification of interaction free energies involved in the hybridization will help to increase the accuracy of microarrays and other hybridization-based technologies, so that these devices could realize their full potential, for instance, for clinical applications [33]. For these applications, an increase in specificity and sensitivity is very important and can be achieved through better understanding of fundamental properties of hybridization in these devices.

There has been considerable attention in recent years [9, 22, 23, 34] in understanding the fundamentals of hybridization in DNA microarrays and its impact in data analysis. Here, we have shown that microarrays are a reliable and high-throughput tool to gain insight on DNA hybridization thermodynamics. The same method could be used to screen other types of defects, as bulges. Indeed, it was recently used for understanding loop conformations [22].

V. SUPPLEMENTARY DATA

Supplementary Data available in Appendix.

VI. ACKNOWLEDGEMENTS

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SUPPLEMENTARY DATA

Appendix A: Nearest neighbor model and linear regression

According to the nearest-neighbor model, the total hybridization free energy of a target to a probe can be expressed as a sum of the dinucleotide parameters $\Delta G_\alpha$ accounting for hydrogen bonding and stacking interactions. The index $\alpha$ covers all possible dinucleotide parameters. Some examples are:

\begin{align}
\Delta G\left(5'-AT-3'\right), \\
\Delta G\left(5'-AC-3'\right), \\
\Delta G\left(5'-A\bar{A}-3'\right), \\
\Delta G\left(3'-TA-5'\right), \\
\Delta G\left(3'-TG-5'\right)
\end{align}

where the underlined nucleotides indicate mismatches. In total there are 10 perfect match parameters (taking into account symmetries) and 48 parameters in the case of a single mismatch. These dinucleotide parameters are known not to be unique, see e.g. [1].

Thermodynamics predicts that the intensity measured from a spot $I_i$ is given by:

$$I_i = I_0 + A c e^{-\Delta G_i/RT}$$ (A2)

where $\Delta G_i$ is the total hybridization free energy between a target and a probe, $A$ is a parameter which sets the intensity scale, $c$ the target concentration, $R$ the gas constant and $T$ the temperature. $I_0$ is the aspecific signal that can be considered as background. In this paper the stability of duplexes was always compared to that of the perfect match, i.e.

$$y_i \equiv \ln I_i - \ln I_{PM} = -\frac{\Delta G_i - \Delta G_{PM}}{RT}$$ (A3)

which defines the free energy penalty of probe $i$ with respect to the perfectly matching probe. This penalty can be expressed as a sum of nearest-neighbor dinucleotide parameters:

$$y_i = \sum_{\alpha=1}^{58} X_{i\alpha} \frac{\Delta G_{\alpha}}{RT}$$ (A4)

where $X_{i\alpha}$ is the frequency matrix, which counts the number of times a given dinucleotide term contributes to $y_i$. As an example, for an isolated mismatch of type GA we have:

$$\Delta G_i \left(\ldots GAT \ldots \right) - \Delta G_{PM} \left(\ldots GCT \ldots \right) =$$

$$\Delta G\left(GA \atop CG\right) + \Delta G\left(A\bar{G} \atop T\bar{A}\right)$$

$$-\Delta G\left(GC \atop CG\right) - \Delta G\left(CT \atop GA\right)$$

$$\equiv \Delta \Delta G\left(GAT \atop CGA\right)$$ (A5)

For notational convenience we used, by symmetry, the equality of $\Delta G\left(\frac{A\bar{T}}{G\bar{A}}\right) = \Delta G\left(\frac{T\bar{A}}{A\bar{G}}\right)$ to have the mismatch on the right hand side of the dinucleotide. For any given $i$, the matrix elements $X_{i\alpha}$ are all zero except for the four dinucleotide terms of Equation (A4) which contribute by +1 for the two dinucleotides with mismatches and −1 for the two perfect matching dinucleotides. Equation (A4) defines a multiple linear regression, from which the 58 dinucleotide parameters can be fitted to match all the observed free energy penalties of mismatches. Note that it defines the dinucleotide parameters not in a unique way, e.g. the following transformation

$$\Delta G\left(\frac{x\bar{A}}{x'\bar{G}}\right) \to \Delta G\left(\frac{x\bar{A}}{x'\bar{G}}\right) + \varepsilon$$ (A6)

$$\Delta G\left(\frac{x\bar{G}}{x'\bar{A}}\right) \to \Delta G\left(\frac{x\bar{G}}{x'\bar{A}}\right) - \varepsilon$$ (A7)

in which the same constant $\varepsilon$ is added and subtracted to different dinucleotide parameters, leaves Equation (A5) invariant. The triplet parameters, such as defined in the last line of Equation (A5), are however unique as expected, since they are directly physically accessible.

Appendix B: Target sequence selection with Optimal design

As discussed above, the dinucleotide parameters can be obtained from a linear fit from $N$ independent experimental measurements. Such an approach always contains some uncertainties. These uncertainties can be lowered if one takes $N$ large. In our specific case $N$ equals the number of spots on the microarrays, and can be increased by combining data from more arrays (see main paper for experimental setup). Further, for a given fixed value of $N$ one can use some optimization criterion to select the best $N$ measurements which minimize the uncertainties on fitted parameters. In our case this comes down to the selection of a target sequence with good statistical properties. The theory of Optimal Design establishes some criteria for this purpose and we briefly discuss this theory here.

Before entering into the details of the optimization followed in the microarray experiment we discuss a one dimensional example, which illustrates the optimization...
method. Let us take the example of a simple linear regression with an intersect set to zero (corresponding to a one-dimensional system): \[ y_i = \beta x_i, \] (B1)

where \( \beta \) is the unknown of the problem, \( x_i \) and \( y_i \) are respectively the input and output of the experiment \( i \) and can take any real value. The parameter \( \beta \) can be obtained by the least square method:

\[
\beta = \frac{\sum(x_i - \bar{x})(y_i - \bar{y})}{\sum(x_i - \bar{x})^2},
\] (B2)

where the symbol \( \bar{\cdot} \) means the average over the \( N \) elements. The error on \( \beta \) is given by:

\[
\Delta \beta = \frac{S}{N} \sqrt{\frac{1}{\sum(x_i - \bar{x})^2}},
\] (B3)

where \( S \) is the cost function of the system. Equation (B3) implies that the error can be decreased by enlarging the sampled points (\( N \)) or, for \( N \) fixed, by increasing the variance of the variable \( x_i \). The latter criterion can be used in the design of the experiment by performing measurements \( y_i \) for a well spread set of points \( x_i \). Indeed, it is intuitively clear that when \( x_i \) are very close to each other (small variance) one has a large uncertainty on the estimate of the slope \( \beta \). In what follows we discuss about optional design criteria in higher dimensions, which roughly correspond to the idea of the maximization of the variance in the previous one-dimensional example.

We define first the so-called information matrix \( M = X^T X \), where \( X \) is the frequency matrix defined in Equation (A3) and where \( X^T \) denotes its transpose. In terms of matrix elements:

\[
M_{\alpha \beta} = \sum_{i=1}^{N} X_{i\alpha} X_{i\beta}
\] (B4)

which is thus in our case a square symmetric matrix of dimension \( 58 \times 58 \).

The information about the quality of the experimental design is encoded in \( M \) and in our case is defined by the sequence of the target oligo in the experiment (see main paper for experimental setup). The three most used criteria in optimal design are the A-, D- and E-optimality. A-optimality corresponds to minimizing the trace of \( M^{-1} \), D-optimality corresponds to minimizing the determinant of \( M^{-1} \) and E-optimality corresponds to maximizing the lowest eigenvalue of \( M \). Roughly speaking, these strategies amounts to maximize the information encoded in \( M \). We note that in the linear problem of Equation (A4) the information matrix has a minimum of 7 null eigenvalues (see the supplementary material of Ref. [3] for a detailed explanation). These come from unavoidable degeneracies of the problem, or equivalently from the fact that the dinucleotide parameters are not unique (see e.g.

**Appendix C: The linear regime**

As a measurement device the microarray technology is faced with a detection limit in the low measurement regime and a saturation in the high end: see sketch in
Equation (C1), or for high concentrations will consequently be higher than predicted by the theory of spot intensity. In both cases, $R$ can be close to saturation and consequently lower than expected. In the linear regime, however, for low concentrations, the intensity of a spot is identical (identical target sequence, identical probe sets, identical hybridisation conditions) except for the concentration $c_n$ of the target. If the data is in the linear regime, we expect the intensity of a spot to be

$$I_i \propto c_n \exp(-\Delta G_i/RT).$$

(C1)

If we now combine two experiments, one with target concentration $c_n$ and one with $c_{n+1} > c_n$, and define for each spot $i$ the quantity $R$ as

$$R_i(c_n, c_{n+1}) = \frac{I_i(c_n)}{I_i(c_{n+1})} \frac{c_{n+1}}{c_n}$$

(C2)

than we expect $R$ to be equal to 1 when both intensities are in the linear regime. However, for low $c_n$ the spot intensity $I_i(c_n)$ can be close to detection limit and consequently be higher than predicted by the theory of Equation (C1), or for high $c_{n+1}$ the intensity $I_i(c_{n+1})$ can be close to saturation and consequently lower than theoretically expected. In both cases $R$ will be above one. The result of this analysis is shown in Figure 9.

Figure 8. In our research, we want to limit ourselves to the intensity of a spot $i$, as a function of the quantity $c_n$, and define for each target the quantity $R$ as

$$R(c_n, c_{n+1}) = \frac{I_i(c_n)}{I_i(c_{n+1})} \frac{c_{n+1}}{c_n}$$

for the combinations $(c_1 = 20pM, c_2 = 100pM)$ and $(c_2 = 100pM, c_3 = 500pM)$ of target $t_2$. From this picture, it is clear that for a large part of the intensity range $R$ equals one and supports the linear regime. For the green dots, there is a deviation in the high intensity range due to the proximity of saturation of these spots in the $500pM$ experiment. For the red dots, a deviation is present due to proximity of the detection limit for these spots in the $20pM$ data. This approach gives a criterion to assess the validity of the linear regime per spot and the possibility to make a correction for the non-linear behaviour close to saturation or detection limit.

Appendix D: Free energy additivity of mismatches

In the main article, the additivity of free energy penalties of mismatches was shown when mismatches were separated by more than four nucleotides. For two examples, this was explicitly shown in Figure 5 of the main article. In this section, we add some further examples of the additivity with similar plots. These are shown in Figure 10.

Appendix E: Self-consistency in free energy penalties estimation of triplet nucleotides

In the main article, we present two different approaches that can be used to estimate free energy penalties of single mismatches in a triplet of nucleotides such as in Equation (3) of the main article. The first method, i.e. by linear fitting, produces a robust estimation provided that each of the 58 NN dinucleotide parameters are equally well-represented. This was achieved by the use of Optimal Design principle in the experiments. Another method is by taking the median of data points from ratios of intensities following Equations (4)-(6) of the main article. Figure 10 of this document shows six of these unique triplets in which the free energy penalties are indicated by the horizontal line from taking the median of each independent estimate. It is then imperative to see if these methods are equivalent in providing the estimates. Figure 11 shows that the free energy penalties calculated from the two methods are well-correlated with Pearson correlation 0.966 (such as mentioned in the main article). This is indicating the equivalence of the two methods. This is also a proof that our experiments are self-consistent from the different perspective of these two approaches.

[1] Gray, D.M. (1997) Derivation of nearest-neighbor properties from data on nucleic acid oligomers. I. Simple sets of independent sequences and the influence of absent nearest neighbors. Biopoly., 42, 783–793.

[2] Atkinson, A.C. and Donev, A.N. (1992) Optimum Experimental Designs. Clarendon Press, Oxford.

[3] Hooyberghs, J., Van Hummelen, P. and Carlon, E. (1992) The effects of mismatches on hybridization in DNA microarrays: determination of nearest neighbor parameters. Nucleic Acids Res., 37, e53.
FIG. 10. A few examples of different focus mismatches showing additivity as $|\Delta x > 4|$. Similar to Figure 5 in the main paper, the target shown in top of the x-axis is in 3’ to 5’ orientation, $t_1$ are on the left side, $t_2$ are on the right side.
FIG. 11. Comparison of estimates of free energy penalties for isolated mismatches, obtained in two different ways: from the linear model fit and from the median of independent estimates. The two sets of data are strongly correlated (Pearson’s correlation 0.966).