Modeling of microarray data with zippering

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The ability of oligonucleotide microarrays to measure gene expression has been hindered by an imperfect understanding of the relationship between input RNA concentrations and output signals. We argue that this relationship can be understood based on the underlying statistical mechanics of these devices. We present a model that includes the relevant interactions between the molecules. Our model for the first time accounts for partially zippered probe-target hybrids in a physically realistic manner, and also includes target-target binding in solution. Large segments of the target molecules are not bound to the probes, often in an asymmetric pattern, emphasizing the importance of modeling zippering properly. The resultant fit between the model and training data using optimized parameters is excellent, and it also does well at predicting test data.

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Oligonucleotide microarrays have had a profound impact on medical diagnosis and molecular biology. These devices have thousands of cells, each containing numerous copies of a specific DNA probe attached to the substrate. After amplification, eRNA (or DNA) derived from a biological sample is fluorescently labeled and then fragmented into targets that are allowed to bind to the probes. The targets hybridize with their complementary DNA probes with high specificity. The fluorescence allows an optical readout of the concentration of thousands of RNA transcripts simultaneously.

There are often orders of magnitude difference in the concentrations of the various kinds of targets and many important genes are expressed only in very small concentrations. These can be hard to measure by this method for reasons we now discuss.

In Affymetrix GeneChips, 16 kinds of probe, all with the same concentration and probing different segments of a single mRNA, are employed for every transcript. There can be order of magnitude variations in the signal intensities between these 16 probes. The specific signal intensities are reproducible and so cannot be explained as statistical error. Instead, they occur because the interactions between the probes and the target molecules are complex. In addition to the binding between a probe and its complementary target (specific binding), there are binding of targets to noncomplementary probes (non-specific binding) and target-target binding. In addition, even when a target binds to its complementary probe, it is possible that it is only partially hybridized, with the ends unzipped. This is likely to be an important effect in microarrays, since their operating temperature is close to the melting temperature of the hybrids, so that substantial fluctuations in binding can be expected.

Various statistical techniques have been used to reduce the errors caused by these effects. For example Affymetrix, in addition to using 16 probes for every transcript, has added another set of “mismatch” probes that differ from the original “perfect match” probes by the alteration of the middle nucleotide. These are compared to reduce the error from nonspecific binding. Although these techniques reduce the uncertainties in predicted input concentrations, by analyzing 32 numbers, they still have difficulty in detecting small concentrations of RNA at biologically significant levels. Alternative statistical approaches have been proposed.

As recognized by several previous authors, the statistical techniques used by Affymetrix and others do not utilize the probe sequence information. The hope is that including these should greatly increase the reliability of predictions.

Held et al modeled the binding of each target molecule to its complementary probe using a Langmuir adsorption model. If \( c \) is the concentration in solution of the target, the fraction of probe molecules that are hybridized is

\[
J_h = \frac{1}{1 + \exp[\beta(\Delta G - \mu)]}
\]

where \( \exp(\beta\mu) \propto c \). The binding energy \( \Delta G \) was obtained from previously measured stacking free energies. All target-probe pairs were treated independently, with nonspecific binding included phenomenologically by adding a \( \Delta G \) dependent constant to the measured signal. To be of the form \( a + b\exp(c\Delta G) \). Partially hybridized probes were not considered, although the authors comment that they might be of importance. Perhaps because of this, in their Boltzmann factors \( \exp(\beta\Delta G) \), the best fit effective temperature was approximately seven times the actual temperature.

Hekstra et al also used a Langmuir adsorption model with an additive background from nonspecific binding. However, the resultant three parameters for each probe-target pair were not evaluated using previously determined stacking energies, but were fitted to linear combinations of the number of each nucleotide.

Zhang et al attempted to include the effects of partial binding of target molecules to probes. The model...
they came up with was the “positional dependent nearest neighbor model” (PDNN) where the binding energy for a probe-target pair was taken to be of the form

\[ \Delta G = \sum_k \omega_k \epsilon(b_k, b_{k+1}), \]

where \( \epsilon \) is the stacking energy for the adjacent bases \( b_k \) and \( b_{k+1} \), and \( \{ \omega_k \} \) was a set of weights depending only on the position on the probe \( k \), and not on the specific probe molecule. The probe intensities were assumed to be linear in the target concentrations, i.e., saturation effects were not included.

The above methods incorporating sequence information have different approaches to data fitting, with various physical effects included. In this paper we present a comprehensive approach that includes what we believe to be all the most important effects, to construct a physical model for microarrays. In particular we have included “zippering effects”, i.e. target molecules partially hybridized to probes, by using a full statistical mechanical approach rather than ad-hoc position-dependent weights. This reduces the number of fitting parameters enormously, because partial binding can be understood completely as a consequence of the stacking energies. We also include the effect of target-target binding in solution. As pointed out by Held et al [2], the saturation intensities of different probes that correspond to target fragments from the same mRNA molecule can be different by an order of magnitude. It is likely that this is because of these target target interactions, reducing the effective concentration in solution of different targets by different amounts. Since RNA-RNA interactions are stronger than RNA-DNA [1], this effect can be substantial. We also include non-specific binding for each probe similarly.

We present our model in three parts: specific binding, including zippering, non-specific binding, and finally target-target interactions in solution.

Consider a target molecule consisting of a sequence of bases \( \{b_1 \ldots b_N\} \) (where \( N = 25 \) for the Affymetrix GeneChip). According to the stacking energy description of hybridization, if this target molecule is fully bound to its complementary probe, the resultant change in free energy is of the form

\[ \Delta G(1, N) = \sum_{k=1}^{N-1} \epsilon(b_k, b_{k+1}) + \epsilon_i, \]  

(2)

where \( \epsilon_i \) is the initiation energy of attachment. However, it is also possible for the target to be partially bound to the probe, with only the bases \( n \) to \( m \) being bound. This configuration would have a \( \Delta G(n, m) \) given by Eq. (2) but with the sum from \( k = n \) to \( k = m-1 \). Because the local stiffness is large, and the target-probe hybrid is in a helical structure, we only need to consider configurations for which the unbound parts start at the ends, rather than forming isolated islands in the middle. Thus a target-probe pair can be viewed as a double-ended zipper.

The resultant partition function for the bound state that includes all partially bound configurations is

\[ Z = \sum_{n < m} \exp(-\beta \Delta G(n, m)) = \exp(-\beta \Delta G) \]  

(3)

where \( \Delta G \) is the total binding free energy. Naively, this takes \( O(N^3) \) operations to compute, which is prohibitively expensive, because \( \Delta G \) is computed repeatedly when optimizing the model. However, \( Z \) can be computed in \( O(N) \) operations using recursion relations. Define \( Z(i) \) as the analog of \( Z \) in Eq. (3), but with only the bases from \( i \) to \( i \) included. \( Z(i) \) is the sum of two terms: \( Z_u(i) \), which considers configurations that are unbound at the site \( i \) (but have a bound segment somewhere before \( i \)), and \( Z_b(i) \), which considers configurations that are bound at the site \( i \). The recursion relations for these are

\[ Z_u(i + 1) = Z_u(i) + Z_b(i) \]  

\[ Z_b(i + 1) = Z_b(i) \exp(-\beta \epsilon(b_i, b_{i+1})) + \exp(-\beta \epsilon_i). \]  

(4)

In the first of these equations, configurations that are attached at \( i \) can detach at \( i + 1 \). However, in the second equation, because we allow only one bound segment in our zipper model, and configurations in \( Z_u(i) \) have already had a bound segment before the base \( i \), there is no contribution from \( Z_u \). These recursion relations are a simplification of those in Ref. [10, 11]. The \( \Delta G \) from Eq. (5) can now be used in Eq. (3).

The next effect we consider is nonspecific binding. In principle, this could be accomplished with an approach very similar to the one we have constructed for specific binding. This would require a complete knowledge of all molecular fragments present in the solution; these include a background of human RNA, and the additional “spiked-in” target molecules that the experiment tries to measure. It would also be necessary to determine the stacking energies for all \( 4^4 \) possible mismatched (or matched) sequences of two base pairs. Since neither of these is fully known, we use a statistical approach to model non-specific binding. We assume that the RNA giving rise to non-specific binding is sufficiently diverse that it can be treated as a ‘bath’ of random sequences. We also make the approximation that stacking energies can be defined in terms of the nearest neighbors on the probe. If \( \{b_k\} \) is the probe sequence and \( \{c_k\} \) is a (non-complementary) target sequence, this approximation is

\[ \sum_{\{c_k\}} \prod_k e^{-\beta \epsilon(b_k, b_{k+1}; c_k, c_{k+1})} = \prod_k e^{-\beta \epsilon(b_k, b_{k+1})} \]  

(5)

where \( \epsilon' \) is an effective stacking energy. In the absence of experimental knowledge of the 256 stacking energies, not using this approximation would result in so many adjustable parameters in our model so as to make any results meaningless.

Finally, we include target-target binding in solution. As mentioned earlier, there are substantial variation in the saturated probe intensities for different target fragments from a single mRNA molecule. Further, the intensities saturate at a much higher concentration of target molecules than one would expect from the number
of probe molecules in a single cell [12]. This strongly suggests that the target concentration in solution is significantly depleted, by an amount that differs from one target species to another. We model the fraction of any target species that is lost to target-target binding by using the analogs of Eq. (1). For reasons similar to those for non-specific binding [12], we model this statistically.

We performed numerical simulations using the model described above using Affymetrix’s Series 1532 Latin Square [15] data. In these experiments, a cocktail of human cRNA was “spiked in” to a background of human RNA of unknown composition. In any experiment, the transcripts had concentrations 0, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, and 16.0 pM. The transcripts whose concentration was large varied from one experiment to another cyclically, in a pattern that formed a Latin Square matrix. Each cRNA transcript could hybridize with 16 different probe sequences at different positions along the original transcript.

In the simulations, we varied the parameters of the model in order to minimize the fitness, the log mean square difference between the measured signal intensities and the model predictions:

$$F = \sum \frac{(\ln I_{\text{meas}} - \ln I_{\text{pred}})^2}{M}$$

where the sum runs over probes and experiments, and M is the total number of data points used. We followed the common practice of using the logarithm in this definition, because the intensities vary over orders of magnitude, and doing otherwise would discount low concentration transcripts, which are important biologically. The data from probes 407 at and 36889 at was not used, following Affymetrix’s recommendation. The minimization of F as a function of model parameters is a computationally intensive optimization problem. In order to increase convergence to the solution, we used several techniques, of which parallel tempering [16] was found to work best.

The parameters in the model were 16 stacking energies and one initiation energy for specific, non-specific and target-target binding (51 parameters), the number of probe molecules for each species, a scale factor converting from hybridization to signal intensity, and a (small) uniform background to the signal. The total number of parameters was 54. Although DNA-RNA stacking energies for matched bases are known [17], we allowed these to vary as free parameters. This was because the addition of fluorescent tags to the RNA molecules has been shown [18] to change the stacking energies. The final optimized stacking energies were of the same order of magnitude as experimental results for untagged molecules [19].

Although 54 fitting parameters might seem to be a large number, the model was used to fit 2464 data points, and our number of parameters compares favorably with prior work [4]. Importantly, when we randomly shuffled the sequences associated with the different probes and redid the optimization, the function F in Eq. (1) increased by a factor of more than two, indicating that our results were not an artifact of having too many parameters.

Apart from being able to fit the given data accurately, the purpose of a model is to be able to analyze new experiments with unknown concentrations of transcripts, and accurately predict these concentrations. Accordingly, we left out some some (one or three) of the transcripts during parameter estimation (in addition to transcripts 407 at and 36889 at mentioned earlier). After this data had been used to train the model, we tried to predict the concentrations of the transcripts that had been left out.

Figure 1 shows the measured signal intensities for all the probes in a typical experiment, except for those corresponding to three transcripts: one to be used in the subsequent prediction stage, and the two that were known to be unreliable. The figure also shows the signal intensities from the model, with optimized parameters, and the input concentrations. The model reproduces the measured intensities quite well. The model parameters were optimized once for all the experiments simultaneously. The residual error F, defined in Eq. (1), was 0.19.

With the optimal parameters obtained above, the model was used to predict the signal intensities for the probes corresponding to the transcript that was left out in the training stage, as shown in Figure 2. The same figure shows results (with the same procedure) using the PDNN model of Ref. [4]. As seen in the figure, the predictions with our model are much better.

We also show the prediction capabilities of the model with a different procedure, which is similar to the one used by Ref. [4]. Three transcripts were left out (in addition to the two faulty ones) in the training stage.
In the prediction stage, for each experiment, the sixteen measured probe intensities were used collectively to predict the input concentration of each of the three excluded transcripts. The results are shown in Figure 3.

When target-target interactions were omitted from our model, the residual error increased to 0.27. However, the significance of this is hard to interpret, since the number of parameters is thereby reduced from 54 to 37. Therefore, we assessed this reduced model for prediction. We found a noticeable degradation in predictive power at low input concentrations compared to Figure 3.

Lastly, in Figure 4 we present some data on partial zippering. The figure displays the fraction of the time a base pair is unbound as a function of position on the probe. As is evident, these probes are only partially bound. The places they bind are not symmetric about the middle, and depend strongly on the probe sequence. This kind of behavior differs from that of previous models. Evidence for this kind of asymmetric partial binding can be seen from careful experiments on Agilent microarrays.

In this paper, we have constructed a physical model for hybridization of RNA transcript targets to probes in microarrays, in order to predict experimental signal intensities. Our model includes for the first time the effect of partial hybridization of probes (zippering) derived from fundamental statistical mechanics, and also the effect of target-target interactions. The prediction capabilities of our model appear to surpass those of other approaches.

**References**

[1] C. Li and W.H. Wong, Proc. Natl. Acad. Sci. 98, 31 (2001).
[2] G., Held, G. Grinstein and Y. Tu, Proc. Natl. Acad. Sci. 100, 7575 (2003).
[3] D. Hekstra, A.R. Taussig, M. Magnasco and F. Naef, Nucl. Acids Res. 31, 1962 (2003).
[4] L. Zhang, M.F. Miles and K.D. Aldape, Nature Biotech. 21, 818 (2003).
[5] P.W. Atkins, Physical Chemistry 5th ed., Oxford University Press (Oxford, UK, 1994).
[6] Although DNA-DNA stacking energies appear to have been used instead of the appropriate DNA-RNA.
[7] J. Santa Lucia, Proc. Natl. Acad. Sci. 95, 1460 (1998).
[8] S.M. Freier et al, Proc. Natl. Acad. Sci. 83, 9373 (1986); L. He et al, Biochem. 30, 11124 (1991).
[9] N. Sugimoto et al, Biochem. 34, 11211 (1995).
[10] T. Garel and H. Orland, e-print cond-mat/0402037.
[11] D. Poland, Biopolymers 13, 1859 (1974).
[12] The number of probe molecules can be estimated from their density, about 1 molecule every 40 square Å, and the solution volume (200 µL). This gives ≈ 0.2pM.
[13] E. Southern, K. Mir and M. Shchepinov Nature Genetics Suppl. 21, 5 (1999).
[14] We have verified that all the mismatched RNA-RNA stacking energies are not known, unlike often believed.
[15] R.A. Irizarry, B.M. Bolstad, F. Collin, L.M. Cope, B. Hobbs and T.P. Speed Nucl. Acids Res 31, e15 (2003).
[16] E. Marinari and G. Parisi, Europhys. Lett. 19, 451 (1992); K. Hukushima and K. Nemoto, J. Phys. Soc. Jpn. 65, 1604 (1996).
[17] F. Naef and M.O. Magnasco, Phys. Rev. E 68, 011906 (2003).
[18] After optimization, the nonlinear relation between input concentration and output signal was inverted. When the measured signal exceeded the saturation value, a large positive value was arbitrarily assigned. The training data was run through this inversion procedure to create a calibrated prediction of input concentration. This calibration function was used on the test data.
[19] T.R. Hughes et al, Nature Biotechnology 19, 342 (2001).