# Reply to "Comment on 'Solving the riddle of the bright mismatches: Labeling and effective binding in oligonucleotide arrays'"

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In their Comment, Carlon *et al.* correctly observe that hybridization experiments in solution have shown an asymmetry which we failed to notice. However, contrary to their statement, the stacking free energies of *unlabeled* nucleic acids in solution are *not* compatible with our (and other authors') measurements, even qualitatively. Furthermore, to bolster our paper's assertion that labeling of the nucleic acids materially modifies the observed affinities, we present experimental data in which a change of labeling protocol results in a clear change in the affinities in question. We must therefore agree with extant studies suggesting that the energies of hybridization in solution are a superficial approximation to the much more complex physicochemical process of hybridization of labeled nucleic acids in high-density oligonucleotide arrays.

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We appreciate Carlon *et al.*'s observation that the Sugimoto stacking energies show an asymmetry which we failed to note, and we stand corrected in this matter: we should not have been surprised to find this asymmetry. Carlon *et al.* thus correctly assert the asymmetry is not evidence implicating the labels. However, the Comment appears to misinterpret other findings in our paper. Our claim that the labeled nucleotides are involved in the "bright mismatch" phenomenon was not based exclusively on this one observation: the hybridization energies in solution are a poor approximation to the data, as we shall now demonstrate.

The quantitative argument of the Comment is summarized in Table II, which shows in Column 4 the affinities from the model Eq. 1, using Sugimoto *et al.*'s hybridization energies, and in Column 5 our measured affinities from our Fig. 3, at the *center nucleotide* of the strands. While this table shows that there is indeed an asymmetry, it can scarcely be said to match the data, and if anything it is strong evidence against the model Eq. (1). First, a matter of scale: the energies are too large by factors ranging from 3.5 (C) to 35 (T). Second, a matter of pattern: the model predicts G and T to differ from each other in affinity by roughly the same amount as the A or C energies, while our data show these differences to be 10 times smaller. Third, a matter of geometry: our Fig. 3 shows unambiguously a spatially dependent curve, having extrema at the center of the strands for C and A [12]; the Comment's Table II uses this maximum value (rather than the mean) and fails to discuss that neither the model nor Sugimoto's energies are position dependent. Finally, a matter of sign: our Fig. 4 plots the affinities, including fits to the sequence *outside of the hybridization region, where there is no duplex*, and shows a large change of sign near the duplex boundary and statistically significant nonzero residuals outside the duplex area. Carlon *et al.*'s Comment does not address three out of these four important differences between their model and our measurement; the only disagreement noted in the Comment, the factor of 4 between the model's predictions and our measurements for the A and C letters, is not explained on any physical grounds, just described as "to be expected" given the difference between hybridization in solution and microarray hybridizations.

Our results are in agreement with published results of other groups, who have found effects similar, and in close quantitative agreement, to our own: Binder *et al.* reported reduced affinity due to labeled nucleotides and also similar affinities for the G and T letters [1], and several groups have reported position-dependent affinities [2–7].

The Comment closes by stating that we have not *shown* that fluorescent labels interfere with binding, or are the cause of bright mismatches. Therefore, we shall now provide direct experimental proof that the labeling process *does affect* the binding and the bright mismatches. If the labels did not affect the binding affinities, changing the letters being labeled may result only in an overall change of scale, but relative energies (e.g., the difference in affinity between PM and MM) should be unchanged. We modified the labeling protocols such that we could compare the standard labeling, in which the RNA strands to be hybridized incorporate both biotinilated U and C's during transcription, to labeling using only biotinylated C's; we labeled with the two different protocols the *same* RNA sample and carried out otherwise identical hybridizations onto separate chips. Strikingly, the sample where only the C nucleotides were labeled (Fig. 1, right column) showed substantially different PM-MM distributions from the sample where both pyrimidines were labeled (left column), in particular showing a more pronounced "bright mismatch" effect for probes with a middle letter (PM) of G (whose complement is the labeled C), completely consistent with our argument. This experiment provides strong direct evidence that labels influence the relative binding affinities of the PMs versus MMs.

Our paper was published briefly after Affymetrix was first made available to the public the probe sequences. Since that

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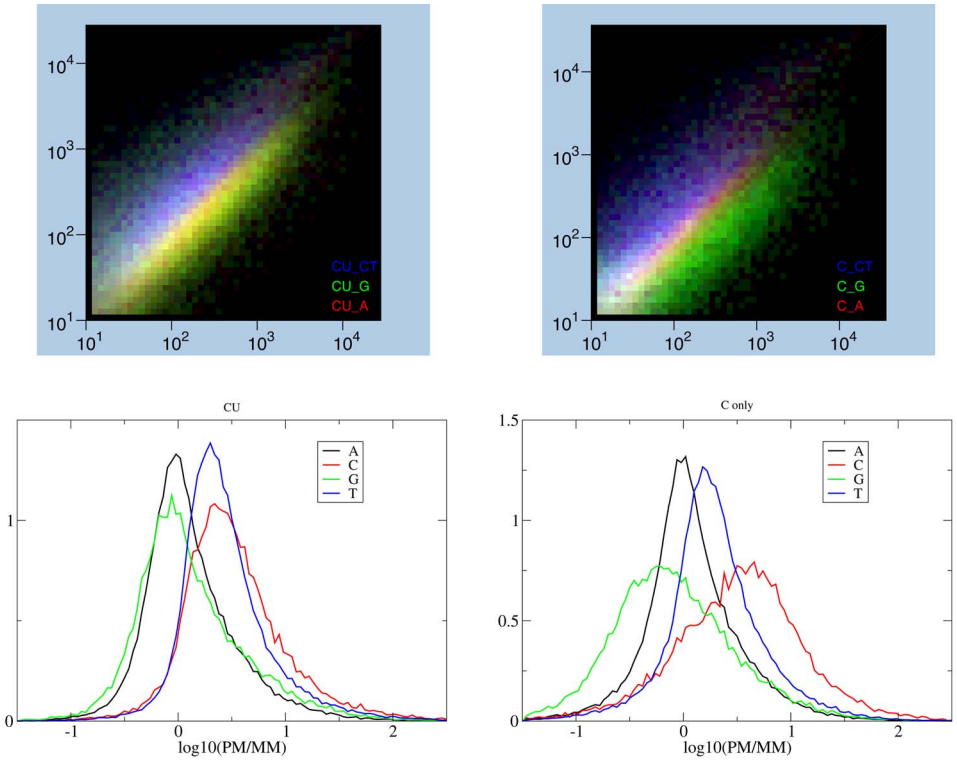


FIG. 1. (Color online) Our experimental results. In the left column, the RNA sample was labeled using both Cs and Us as per the standard protocol ("CU mix"); in the right column, the sample was labeled using only Cs ("C-only mix"). Top row, two-dimensional histograms as in our Fig. 2; bottom row, histograms of log(PM/MM) according to the middle letter of the PM. Anything below the diagonal (top row) or to the left of 0 (bottom row) is a "bright mismatch," which can be seen to exist in great abundance. Labeling mixtures: (i) Our CU mix was constructed to mimic the ENZO standard kit and consists of 35 mM rATP, 35 mM rGTP, 26.25 mM UTP, 26.25 mM rCTP, 8.75 mM bio-11-rCTP, and 8.75 mM bio-16-UTP. This constitutes a 10× mix for the *in vitro* transcription reaction. We modified this for the (ii) "C-only mix" (bio-11-rCTP only) by using 35 mM UTP and 23.3 mM rCTP plus 11.7 mM bio-11-rCTP [15]. Drosophila chips hybridized with *Drosophila* head RNA preparation as described [16].

release, a number of papers have shown the involvement of various nontrivial effects in the brightness of the probes and the bright mismatch effect, such as complexity of the background [4,14], chemical saturation of the probes at much lower levels than expected from *in vitro* studies [8,9], competition effects [1], sequence-specificity of "nonspecific" binding [10], nonequilibrium effects [11,13], etc. Although the energies encapsulated in Sugimoto's parameters contribute to our reported asymmetry, it is naïve and misleading to convey the message that they are sufficient for understanding GeneChip hybridizations, even at a qualitative level. In this Reply we showed that a strict analysis of these parameters leads to erroneous predictions regarding scales and relative letter affinities, and we reported experimental results that clearly demonstrate the influence of labels on hybridization affinities. These results, taken together with the abundant literature, support the widely shared view that no good model for GeneChip hybridization is currently available, and that the equilibrium free energies of hybridization in solution, as in the Comment's Eq. (1), are a superficial approximation to a much more complex physicochemical process.

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[12] We pose a caveat: Sugimoto's free-energy parameters for RNA-DNA hybrids are derived from a single experiment com-

prising only 68 different sequences of small varying length. Furthermore the fits are position independent, so it is plausible that some of the parameters would be modified substantially by including more sequences.

[13] The Sugimoto energies predict melting temperatures in the 90 °C range for the probes. The Affymetrix hybridization protocol prescribes a hybridization temperature of 50 °C. It is unlikely that at this temperature most RNA strands would have the chance to bind and unbind enough times to establish equilibrium, and hence we should expect that the free-energy contributions to brightness differences should be matched by equally important kinetic terms.

[14] We want to correct a misstatement in the Comment: our paper was *not* based on the analysis of the Affymetrix Latin Squares calibration datasets. It was based on using several research-grade datasets using human, mouse, drosophila, and yeast chips. We did work based on the calibration datasets in [8]. Analysis of the single spiked-in genes from the calibration datasets shows a surprisingly low proportion of bright mismatches, in agreement with the contention in [4,10] that the structure of the background is deeply influential for this effect.

[15] The fraction of C or U nucleotides which are biotinylated in the solution in the standard protocol is 25%, and in our modified protocol 33%. It should not be assumed that biotinylated nucleotides are incorporated into the strands at this rate by the polymerase during the *in vitro* transcription, or that the efficiency of incorporation is the same for bio-11-rCTP or bio-16-UTP.

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