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2010 Phys. Biol. 7 048002

(http://iopscience.iop.org/1478-3975/7/4/048002)

Phys. Biol. **7** (2010) 048002 (6pp) doi:10.1088/1478-3975/7/4/048002

REPLY

# Reply to 'Linking probe thermodynamics to microarray quantification'

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Received 12 July 2010
Accepted for publication 30 September 2010
Published 12 November 2010
Online at stacks.iop.org/PhysBio/7/048002

**Abstract**
We defend Langmuir-like models of microarrays from accusations by Li *et al* (2010 *Phys. Biol.* **7** 048001) that they fail to link sequence-specific properties to hybridization signals. We argue that existing Langmuir-like models based on accepted principles of physical chemistry, together with a model of post-hybridization washing, are entirely consistent with various controlled experiments. Li *et al*'s competitive hybridization model on the other hand is not verified experimentally using designs which allow for an unambiguous differentiation with respect to Langmuir-like models and exhibits no benefit in fitting microarray probe intensities.

## Alternative models of microarray hybridization

Plots of measured fluorescence intensities against spike-in concentration for a given probe can be fitted well by a monotonically increasing, convex-downwards response function which asymptotes to a probe-sequence-dependent constant at high spike-in concentration. The majority of physical models proffered for explaining this response curve have their origins in Langmuir adsorption theory, which leads to a hyperbolic response function [10].

The central point of contention raised by Li *et al* [14] concerns the physical origin of differing saturation asymptotes of hybridization response curves in oligonucleotide microarray spike-in experiments. The Langmuir-based model cannot explain the probe-sequence dependence of this difference, but instead predicts that all probes must saturate completely to the same asymptote. Burden *et al* [8] and Held *et al* [11] have proposed that the problem is resolved by considering the post-hybridization washing, which dissociates bound probes in a probe-sequence-specific manner. Subsequent experiments by Skvortsov *et al* [17] and Binder *et al* [3] are entirely consistent with this scenario.

An alternate explanation is proposed by Li *et al* [13, 14], who assume that the adsorption reaction rate is independent of target concentration in bulk solution, but instead depends on the relative fraction of specific targets.

To compare the consistency of both models with experiment, we set out the mathematics of each model using a notation closely aligned with that of Li *et al* [13]. In the conventional Langmuir-based model of competitive hybridization as proposed by various authors including Binder [1] and Burden [6] (hereafter referred to as the BB model), the coverage fraction of specific ($\alpha$) and non-specific ($\beta$) probe–target duplexes, *before* washing and ignoring secondary effects such as target–target hybridization in the supernatant solution, is

$$\alpha = \frac{K_T[T]}{1 + K_T[T] + K_N[N]},$$
$$\beta = \frac{K_N[N]}{1 + K_T[T] + K_N[N]} \quad \text{(BB model)}. \tag{1}$$

Here $[T]$ and $[N]$ are the specific and effective non-specific target concentrations in the supernatant solution, and $K_T$ and $K_N$ are the specific and effective non-specific equilibrium constants for hybridization reactions.

Li *et al*'s model (hereafter referred to as the LPB model) gives the analogous coverage fractions, after a little algebra

 

from equations (8) and (9) of [13], as

$$\alpha = \frac{K_T[T]}{(1+K_T)[T]+(1+K_N)[N]},$$
$$\beta = \frac{K_N[N]}{(1+K_T)[T]+(1+K_N)[N]} \quad \text{(LPB model)}, \qquad (2)$$

where for convenience we have absorbed Li *et al*'s rate constants into two equilibrium constants $K_T = k_b/k_d$ and $K_N = k_b/k_n$.

For both models, the total coverage fraction before washing has the functional form of a rectangular hyperbola:

$$\theta^{\text{before wash}} = \alpha + \beta = \theta_0 + (\theta_\infty - \theta_0)\frac{K[T]}{1+K[T]}. \qquad (3)$$

The models differ in how the parameters $\theta_0$, $\theta_\infty$ and $K$ are related to the underlying physical constants, namely

$$\theta_0 = \frac{K_N[N]}{1+K_N[N]}, \quad \theta_\infty = 1,$$
$$K = \frac{K_T}{1+K_N[N]} \quad \text{(BB model)}, \qquad (4)$$

or

$$\theta_0 = \frac{K_N}{1+K_N}, \quad \theta_\infty = \frac{K_T}{1+K_T},$$
$$K = \frac{1+K_T}{1+K_N}.\frac{1}{[N]} \quad \text{(LPB model)}. \qquad (5)$$

Note that the saturation asymptote $\theta_\infty$ is independent of probe properties for the BB model, but decreases with increasing specific target dissociation rate $k_d$ in the LPB model. This is essentially the source of Li *et al*'s resolution of the asymptote problem.

In the BB model we have made the assumption that the fractions $w_T$ and $w_N$ of specific and non-specific bound targets respectively are dissociated during the post-hybridization stringent washing, where $0 < w_N < w_T < 1$. If this assumption is applied, the total coverage fraction after washing is easily calculated to be

$$\theta^{\text{after wash}} = w_T\alpha + w_N\beta$$
$$= w_N\theta_0 + (w_T\theta_\infty - w_N\theta_0)\frac{K[T]}{1+K[T]}. \qquad (6)$$

This formula is equally valid for both models. In the LPB model as proposed in [13] the approximation $w_T = 1$, $w_N \approx 0$ is used.

Target depletion from the supernatant solution is accounted for in [13] and [16] by making the substitution $[T] = \hat{T} - \alpha p$, where $\hat{T}$ is the nominal spike-in concentration and $p$ is an effective probe concentration, or a slightly more elaborate substitution for Affymetrix Genechips with a perfect match(PM)/mismatch(MM) design [7]. An important point to note is that the same reparameterization $[T] \to \hat{T}$ of the horizontal axis of the isotherm $\theta^{\text{after wash}}$ as a function of $\hat{T}$ applies to both the BB and LPB models.

In summary, the basic hybridization isotherm predicted by both BB and LPB models can be transformed into a common mathematical form (equations (3) and (6)) despite the different underlying physical assumptions. It can be complemented by the same appropriate depletion mechanism in both cases [7, 13]. Consequently, simply fitting of data obtained from typical spike-in experimental designs, e.g. in the Affymetrix Latin square U133 dataset, will not allow one to decide unambiguously between one model or the other.

## Results of calibration experiments

To settle the issue requires additional experimental efforts which independently prove the relevance of the special assumptions made by the BB and LPB models, such as post-hybridization washing and/or target depletion. Also the results of alternative experiments designed according to special conditions using, for instance, spikes without background or a dilution series, can provide substantial information about the underlying hybridization mechanism.

Table 1 judges the essential results of a series of calibration experiments within the light of predictions made by both alternative models. This analysis provides the following results.

(a) The main purpose of [13] is to fit the LPB model with local specific target depletion to the U133 latin square dataset. The claim is made in Li *et al*'s comment [14] that the LPB model 'clearly agrees better with the real data exemplified in figure 2C'. Setting aside that the example is obviously cherry picked to prove a point, both the BB and LPB models happen to be trying to fit a curve of the *same* functional form describing the intensity response to changes in the specific target concentration in the presence of significant non-specific background. The fits in [7] consequently apply equally well to the BB or LPB models. Note also that the analysis of the U133 dataset in [7] is a test of whether target depletion is a significant effect and not a test of the washing hypothesis *per se*.

(b) Both of the U95a spike-in data sets (with and without complex background) were analysed in [6]. The saturation asymptote was observed to be unaffected by the presence of a complex non-specific background (see figure 3 of [6]). While it is consistent with the predictions of the LPB and BB models, different physical origins are proposed, and determining the correct model requires independent experiments (see g).

(c-f) The selected calibration experiments study the intensity response in the special cases that the non-specific background changes in the absence of specific binding (c), that the concentrations of both specific and non-specific targets are equally diluted (d and e) and that the specific target concentration is changed in the absence of complex background (f). In each case one observes significant changes in the probe intensities which are in agreement with the predictions of the BB model. In clear contrast to this the LPB model predicts an invariant probe intensity. Note that the predictions of both models are not related to the washing hypothesis but originate from different assumptions concerning the hybridization mechanism.

For example, one gets for the response curve in the absence of specific targets

$$\theta^{\text{after wash}}([T]=0) = \frac{w_N K_N[N]}{1+K_N[N]} \quad \text{(BB model)} \qquad (7)$$

$$\theta^{\text{after wash}}([T]=0) = \frac{w_N K_N}{1+K_N} \quad \text{(LPB model)}. \qquad (8)$$

**Table 1.** Comparison of the results of selected calibration experiments with the predictions/assumptions made by both alternative models.

| | Experiment | Result | Prediction/assumption | | Comment | Conclusion |
|---|---|---|---|---|---|---|
| | | | BB model | LPB model | | |
| a | Affy-LS U133 with complex background | The intensity increases nonlinearly with increasing sp-in concentration; the saturation asymptote depends on the probe sequence and differs typically for PM and MM probes (figure 3 of [6]). | Acceptable fit by equation (6) with $0 < w_N < w_T < 1$; the asymptote is governed by the washing yield and depends on the probe sequence: $\theta([T]\rightarrow\infty)\rightarrow w_T$ (see equation (6)) | Acceptable fit by equation (6) with $w_N = 0$, $w_T = 1$; the asymptote is governed by the specific binding constant and depends on the probe sequence: $\theta([T]\rightarrow\infty)\rightarrow K_T/(1+K_T)$ (see equation (5)) | Both, the BB and LPB models predict an identical dependence of the intensity as a function of the specific target concentration at constant non-specific background (equation (6)). Consideration of target depletion identically applies to both models. | This experimental design is not suited to differentiate between the alternative models by means of the quality of fit. |
| b | Affy-LS U95A with and without complex background | The saturation asymptote is not significantly affected by the presence/absence of a complex background (figure 3 of [6]) | The asymptote is independent of the presence/absence of background/non-specific binding (see a) | The asymptote is independent of the presence/absence of background/non-specific binding (see a) | Both models properly describe the experimental result. The assumed physical origin of the predicted probe-specific asymptotic intensity level is however different, namely washing (BB) versus concentration-independent binding (LPB). | Independent experiments such as 'washing' experiments are required to clarify the physical origin of the probe-specific asymptote (see g). |
| c | As above | The background intensity strongly depends on the presence of the complex background in the absence of spikes (figure 4 of [6]) | The background intensity depends on the amount of non-specific transcripts: $\theta([T]\rightarrow 0)\rightarrow w_N K_N[N]/(1+K_N[N])$ (see equations (4), (6) and (7)) | The background intensity is independent of the amount of non-specific transcripts: $\theta([T]\rightarrow 0)\rightarrow w_N K_N/(1+K_N)$ (see equations (5), (6) and (8)) | The BB model properly explains the observed change of the non-specific background intensity whereas the LPB model does not. | The predictions of the BB model are in agreement with the experimental results whereas the predictions of the LPB model fail. Therefore the LPB model must be rejected for these experimental situations which essentially agree with the typical conditions of microarray applications. |

**Table 1.** (Continued.)

| | Experiment | Result | Prediction/assumption: BB model | Prediction/assumption: LPB model | Comment | Conclusion |
|---|---|---|---|---|---|---|
| d | GeneLogic dilution experiment | Upon dilution the intensity of the non-specific background decreases (figure 3 of [2]) | The same prediction as in c | The same prediction as in c | See c | See c |
| e | As above | Upon dilution the intensity component due to specific target binding decreases (figure 3 of [2]) | For the conditions of the dilution experiment ($[T]/[N]\sim$const) the intensity due to specific binding decreases with decreasing specific target concentration: $\theta\sim w_T K_T[T]/(1+K_T[T])$ (see equations (4) and (6)) | For the conditions of the dilution experiment ($[T]/[N]\sim$const) the intensity due to specific binding is independent of the specific target concentration: $\theta\sim w_T K_T/(1+K_T)$ (see equations (5) and (6)) | The BB model properly explains the observed change of the specific signal whereas the LPB-model does not. | See c |
| f | Hoybergs equilibration experiment [12] | The intensity increases with increasing specific target concentration at virtually zero non-specific background concentration (figure 1 of [12]) | For $[N]=0$ the intensity increases with $[T]$: $\theta\sim w_T K_T[T]/(1+K_T[T])$ (see equations (4) and (6)) | For $[N]=0$ the intensity does not change with $[T]$: $\theta\sim w_T K_T/(1+K_T)$ (see equations (5) and (6)) | The BB model properly explains the observed change of the probe intensity whereas the LPB model does not. | See c |
| g | Washing experiments [3, 17] | Progressive washing decreases the intensity of the probes in a sequence dependent fashion. MM probes are more strongly washed off than the paired PM upon specific binding. The washing rate decreases with the number of washing cycles. | The model assumes washing survivals according to $0<w_N<w_T<1$. MM probes are more strongly affected by washing than the PM probes due to the weaker probe/target binding of the MM. | The model essentially assumes washing survivals according to $w_N=0$ and $w_T=1$. The model does not consider differences of the washing yield between PM and MM probes. | The BB model assumes washing survival factors in agreement with the experiment whereas the LPB model overestimates washing of non-specific targets and underestimates washing of specific targets. | The washing hypothesis inherent in the BB model is experimentally verified (see b). |

The response of the BB model is determined by non-specific target concentration, and can also be related to probe sequence via $K_N$ (see figure 8 of [6]). By contrast, the LPB response also depends on the equilibrium constant $K_N$ but not on the non-specific target concentration $[N]$, in contradiction to the experimental results.

To put it another way, the LPB model predicts that the response of a probe targetting a non-expressed gene will depend on the composition of the hybridized RNA or DNA solution, but not on its absolute concentration. Our recent analysis of the well-known GeneLogic dilution experiment (figure 3 of [2]), in which a fixed RNA target sample is progressively diluted in a series of hybridizations, shows behaviour consistent with equation (7) and not (8). The analogous result is obtained for specifically hybridized probes (replace $T$ by $N$ and *vice versa* in equations (7) and (8)).

The LPB model also breaks down in the absolute limit of zero non-specific target concentration because of the way it relies on the existence of a non-specific target to set the forward specific target reaction rate through the $[T]/[N]$ ratio (e, see equations (6) and (7) of [13]).

The Hooyberghs dataset [12] (f) is also relevant to the current discussion because it is designed to address the very phenomenon that the LPB model sets out to tackle, namely non-equilibrium hybridization effects due to congestion at the microarray surface. Hooyberghs *et al* present a two-step model in which hybridization initially proceeds to a quasi-equilibrium state in which targets are not fully zipped, and then relaxes at a much slower rate to a fully hybridized duplex. One finds that on timescales long compared with the relaxation step the model behaves as a Langmuir model with an equilibrium constant $K$ referring to the fully zipped duplex. However, on intermediate timescales which are long compared with the initial hybridization step, but short compared with the subsequent relaxation step, the model *also* behaves as a standard Langmuir model, but with a reduced effective equilibrium constant $K$ referring to partially zipped duplexes. This is consistent with an observation made for Langmuir models of microarrays, namely that the equilibrium constant generally corresponds to the Arrhenius equation acting at a higher effective temperature. Hooyberghs *et al* noted that in general standard protocols followed for microarray applications operate within the second of these two regimes. Thus the BB model, when interpreted appropriately, is fully consistent with this dataset, whereas the LPB model is not.

(g) Li *et al* cite as their primary evidence that 'the 'washing hypothesis' is dead' [14] the work of Skvortsov *et al* [17]. Taken out of context, this quote is a misrepresentation of Skvortsov *et al*'s position. The point being made by Skvortsov *et al* is simply about the form of the survival function and not about the relevance of washing. Both empirically and theoretically it is not a single exponential in time, as in the original Held model, but is a weighted sum of exponentials reflecting the complex nature of the ensemble of specific and non-specific targets binding to the probes of a given feature. This is made clear further down in the same respective paragraph of Skvortsov *et al* [17]. A more recent washing experiment performed by the current authors [3] confirms this result.

The ratio $I(t)/I(0) \equiv \theta^{\text{after wash}}/\theta^{\text{before wash}}$ of the observed fluorescence intensities (with optical background subtracted) after $t$ washing cycles is plotted in figure 4 of [3]. The consistent pattern that emerges, irrespective of whether probes are PM or MM, is that the survival fraction drops most rapidly for those probes for which the initial fluorescence intensity is the lowest. Given that most of the variation in intensity is due to probe–target binding energy rather than specific target concentration, this is simply the observation that washing most strongly affects the most weakly bound probes. We also observe that the slope of the plot of $\log[I(t)/I(0)]$ levels off with time, consistent with the BB models expectation that the survival function should be a multi-phase sum of exponentials.

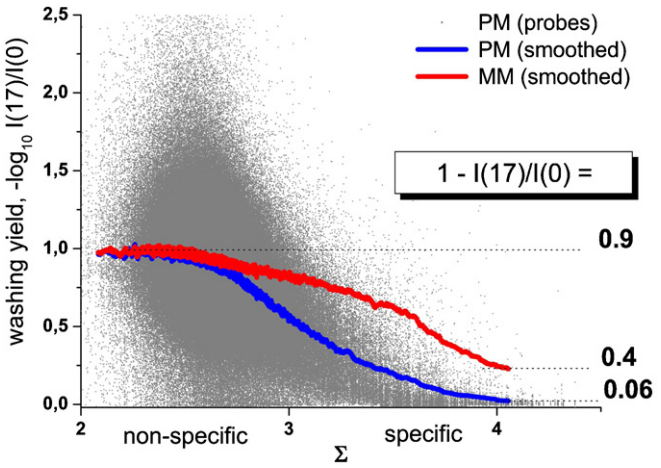


**Figure 1.** Washing yield as a function of the logged intensity $\Sigma = \frac{1}{2}\langle I^{\text{PM}} + I^{\text{MM}}\rangle$ mean averaged over the probe sets. $I(0)$ and $I(17)$ are the probe intensities before and after 17 cycles of stringent washing. The curves are calculated from smoothed logarithmic averages of the PM and MM intensities.

Finally, in figure 1 we plot the washing yield for all PM probes on a microarray after 17 wash cycles. This is a measure of the portion of fluorescent label carrying targets removed by washing. We observe that, on average, washing removes about 90% of the non-specific background and up to 40% and 6% respectively of specifically hybridized PM and MM probes. This is entirely consistent with the BB-model washing explanation of the differing PM/MM saturation asymptotes in the Latin square experiments [8].

## Application to expression analysis

The 4-parameter algorithm of Li *et al* [14] is claimed to be 'the first model of DNA microarray hybridization

that explains probe signal intensities through sequence-based thermodynamic properties without excessive parameter fitting'. We dispute this claim. Firstly, the inverse Langmuir method (ILM) of Mulders *et al* [15], which is based on an earlier model of Carlon and Heim [9], achieves these ends with precisely the same number of parameters, including probe-specific binding energies. Secondly, Li *et al* sidestep the difficult problem of non-specific background correction at low target concentration by subtracting zero-spike-in intensities (though these are unknowns in any practical application) and present their algorithm as being only applicable to high target concentrations, whereas the ILM contains a robust probe-sequence-dependent procedure for handling this subtraction.

An alternative method for analysing data from Affymetrix GeneChips with a PM/MM design, the hook curve method of Binder *et al* [4, 5], is based on the BB model. It uses the distributional properties of intensities over the whole chip to infer relative transcript abundances, including chip-to-chip normalizations [2]. Furthermore it uses essentially no externally specified parameters, robustly accounts for probe-specific affinities and is applicable over the whole range of specific target concentrations. The basic intention of the hook method is to bypass single-probe properties using a representative hybridization isotherm which reveals the basic aspects of the physico-chemical mechanism of hybridization. This approach has been verified, and it was successfully applied to hundreds of GeneChip hybridizations [4, 5]. Hence, the averaging *per se* is no argument against this approach. We make use of this method in [7] to differentiate between the limiting cases of local and global depletion because the hook curve is predicted to have characteristically different shapes in these two cases. The hook curve approach was applied to the U133 spike-in data and the Suzuki data set (figures 9 and 10 in [7]) to complement and support single-probe fitting results.

## Conclusions

Langmuir-like models have been proven in microarray analysis as a first-order correction to simple linear intensity-versus-target concentration models. More elaborate models which better account for the complex system of reactants and conditions at the chip surface together with practicable parameter adjustments are required to extract more precise and robust expression measures from microarray intensity data. However we maintain that the physical assumptions behind the LPB model are not confirmed experimentally, and thus it constitutes no progress in this sense. Furthermore we suspect that any claimed success in inferring target concentrations in a cross validation analysis of the Affymerix U133 spike-in dataset [13] was likely the result of a fortuitous agreement with the functional form of the Langmuir-based response function (6), and that the setting of fitting parameters was simply a heuristic exercise.

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