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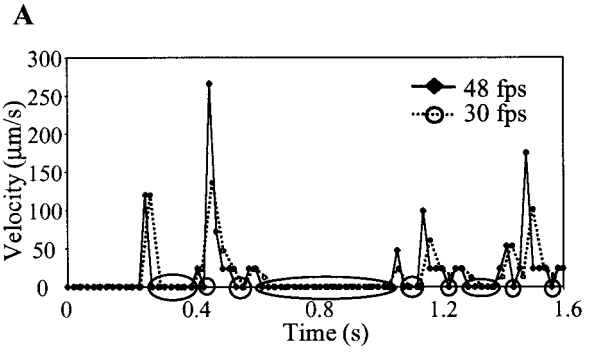
Response to: Measurement of Selectin Tether Bond Lifetimes

The concerns raised in the above letter can be grouped into two issues. First, there seems to be a failure to appreciate the measurement errors that might accrue when analyzing a high frequency signal with low frequency detector, a situation that potentially creates an aliased signal. Second, an issue is raised regarding the proper application of the Bell model to our data on the changes in selectin tether bond dissociation constant (k_{off}) with force. The approach we used to fit out measurements to the Bell model has been clearly described (Smith et al., 1999), although Dr. Springer and colleagues disagree with our analysis. To address these concerns, and also to allow us to correct the use of a formula that was erroneously applied to data in one of our figures, we have generated a table and several figures that we hope will clarify the main issues under discussion.

Measurement of the distribution of a leukocyte's adhesive event lifetimes is dependent to varying degrees on several parameters under experimental control. Critical parameters are the optical magnification necessary to measure a leukocyte's motion, the speed of the camera used to record that motion, the definition of the beginning and end of a transient adhesive event, and the number of events measured. All of the mentioned variables may influence the calculation of a selectin k_{off}. The values for several of these parameters are listed in Table 1, which essentially recapitulates the Table of data in Smith et al. (1999), with the exception of the added the reference to Ramachandran et al. (1999) that was in review at the same time as our manuscript. We hope this addition is satisfactory.

One of the key observations reported in our study (Smith et al., 1999) was that many L-selectin adhesive interactions may take place on a time scale well under the 1/30 s detection limit of video cameras normally used to record cell tethering events. Our use of a high-speed digital camera (as fast as 1/500 s) to analyze L-selectin mediated adhesive interactions during leukocyte rolling revealed a highly significant number of interactions that had previously been undetected. The additional interactions detected changed the distribution of adhesive event lifetimes considerably as forces increased, leading to a conclusion that L-selectin was a highly compliant bond rather than a highly non-compliant bond.



B

pause #	1	2	3	4	5	6	7	8	9
30 fps	0.13	none	0.03	0.25	0.07	none	0.07	none	none
48 fps	0.13	0.02	0.04	0.44	0.06	0.02	0.10	0.02	0.02

FIGURE 1 Velocity per frame of a leukocyte tracked while tethering and rolling on P-selectin. (*A*) The velocity tracked at 48 frames/s (*diamond*) revealed more leukocyte pauses than the 30 frames/s (*open circle*) sampling rate. The faster sampling rate also revealed very short pauses that were unobservable by the slower sampling rate. Nine pauses, in which the leukocyte came to a complete cessation of motion were circled. Both the accurate measure of longer pauses and the observance of very short pauses are important for the robust and reliable determination of the tether bond dissociation rate constant. (*B*) The length of time for each pause was compared for both sampling rates. The different sampling rates resulted in a different length of time measured for most pauses.

A difference between two experiments due to a technique with a more sensitive detection limit is not difficult to understand, especially in light of sampling theory. The Nyquist sampling theorem states that the sampling rate must be over twice the true signal rate to accurately reconstruct it from the measurement. Otherwise, as in the case under discussion, aliasing becomes possible. For analysis of leukocyte transient adhesive interactions (pauses) meditated by selectins, there must be sufficient sampling of the signal (the leukocyte velocity) to detect the peaks and valleys of velocity as bonds form and break, alternatively tethering and releasing the leukocyte to allow rolling. Fig. 1 *A* shows the actual measurement of leukocyte velocity through time while rolling and tethering on a P-selectin substrate at both 30 and 48 frames/s. By comparing the pauses (Fig. 1 *B*), in which the leukocyte comes to a brief but complete stop, we can see that even the slightly faster sampling rate of 48 frames/s more accurately measures longer pauses. More

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TABLE 1 Leukocyte pause times

Reference	Magnification	Image sampling rate (frames/s)	Pause time definition	No. of events analyzed
Kaplanski et al., 1993	40×	30	Motion<2μm/s	~70
Alon et al., 1995	10×	30	Motion<1μm/s	30–35
Alon et al., 1997	20×, 40×	30	Motion<2μm/s	30–40
Alon et al., 1998	20×	30	Motion<1μm/s	30–200
Puri et al., 1998	20×	30	Not stated	100–1600
Ramachandran et al., 1999	20×	30	Motion<6μm/s	~200
Smith et al., 1999	20×	48, 240	Motion = 0μm/s	30–545

Magnification, sampling rate, pause time definition, and the number of events measured for each distribution. The 20× magnification we used was an increase in spatial resolution for P-selectin data. The sampling rates we used, 48 frames/s and 240 frames/s, were increases in temporal resolution for all substrates. Our pause time definition of 0 μm/s leukocyte velocity was the most stringent definition used.

importantly, very brief pauses of one or two frames detected at 48 frames/s would not be observed at 30 frames/s (pauses 2, 6, 8, and 9). In this specific case of P-selectin mediated rolling, the mean pause times are such that the difference in detection ability at the two different capture rates may not be significant in estimating the k_{off}.

However, when we compare the motion of a leukocyte rolling via L-selectin captured at video capture rates (sampling rates) 240 frames/s versus 30 frames/s the difference in measured duration and lifetime distribution of pauses may be considerably greater. The velocity of a leukocyte rolling on L-selectin is shown in Fig. 2. The plot shows the instantaneous velocity of a leukocyte tracked using images captured at 240 or 30 frames/s during transient interactions (pauses) with L-selectin. A very liberal pause-time definition would set the beginning of the adhesive interaction with a drop in leukocyte velocity below 50 μm/s, resulting in the detection of one pause of 0.03 s when using a 30 frames/s camera. If the pause-time definition were made more conservative, i.e., beginning at slower velocities, there would appear to have been no adhesive interaction. The sampling rate of 240 frames/s shows that the single frame pause observed at 30 frames/s is accurate. But in this case, additional pauses are detected. Indeed, three more single frame pauses are observed at 240 frames/s that would have not been detected at the lower frame rate.

To further illustrate how more accurate detection of pauses could affect estimates of tether bond k_{off}, we reanalyzed some data on L-selectin mediated neutrophil pauses (Smith et al., 1999). Fig. 3 shows the analysis of data for L-selectin mediated tethering at 2.0 dyn/cm^2, (250 pN) when images are acquired from video at only 30 frames/s. The same image sequence originally analyzed in Smith et al. (1999) was modified to a lower temporal resolution by taking every eighth frame. At a 30 frames/s sampling rate, the same standard video rate used in all other references in Table 1, we calculated a k_{off} of 21 s^{-1} for L-selectin, which splits the difference between the values reported for similar

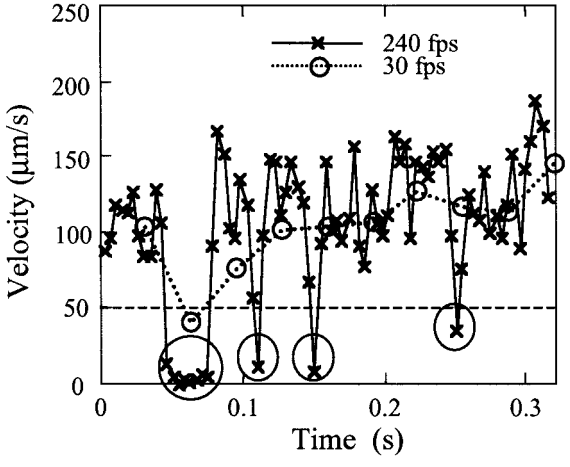

FIGURE 2 Velocity per frame for a leukocyte tracked while tethering on L-selectin. The faster sampling rate of 240 frames/s (*cross*) captured very short leukocyte pauses that were unobserved with a sampling rate of 30 frames/s (*open circle*). Definite pauses observed at the faster sampling rate were circled.

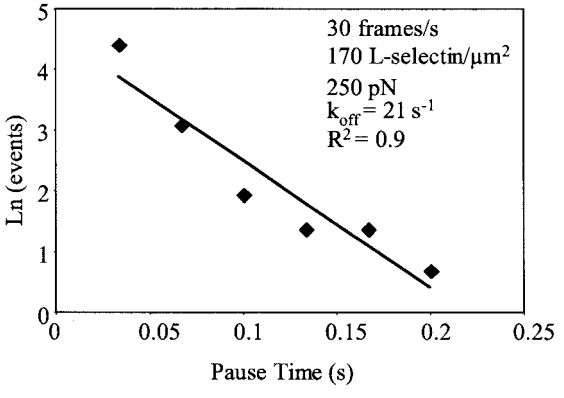

FIGURE 3 Reanalyzed data for L-selectin mediated pauses at 30 frames/s. The images for L-selectin mediated pauses at 250 pN and 170 sites/μm^2 were processed again at 30 frames/s. The dissociation rate constant, 21 s^{-1}, calculated at this lower sampling rate closely matched values from other reports with a similar low sampling rate. Therefore, the differences in dissociation rate constants appear to be due to shorter pauses unobservable at the slower sampling rate.

forces in Alon et al. (1998) and Ramachandran et al. (1999). Therefore, we believe our original conclusion, that the differences in estimated dissociation rate constants and bond reactive compliance between other reports and ours were due largely to improved temporal resolution, to be correct.

The question of whether it was appropriate to fit the Bell equation to tether bond k_{off} at forces above 125 pN was tested statistically as described in Smith et al. (1999), but apparently bears repeating. One of the most powerful uses of mathematical modeling is the identification of regimes where the fit to data breaks down, thereby indicating the existence of additional factors or processes not a part of the model's fundamental assumptions. This is exactly the approach we took when it became evident that the k_{off} of all three selectins deviated from an exponential fit at forces above 125 pN/bond. At the time it seemed illogical to try to fit the Bell model at forces where the detected k_{off} was clearly not increasing in the same exponential fashion as it was at lower forces.

At the time, there were several plausible reasons for not forcing the fit of the Bell model to k_{off} values derived at wall shear stresses much above 1.0 dyn/cm^2 (estimated $>$125 pN/bond). For example, the deformation of the leukocyte increases as flow rates are raised (Dong et al., 1999; Firrell and Lipowsky, 1989; Lei et al., 1999), changing both the contact area with the endothelium and possibly the number of bonds that might be formed and that might share the increased shear forces. Both of these effects could render dubious the fit of the Bell model to leukocyte pause time at higher forces. Additionally, recent insights into the ability of leukocyte microvilli, where selectins are presented during rolling, to stretch in response to forces as small as 60 pN could significantly change the resultant force on the selectin bond as flow increases (Park et al., 2002; Schmidtke and Diamond, 2000; Shao et al., 1998). Finally, reports of biotin-streptavidin (Merkel et al., 1999) bonds displaying an increased rupture strength at force-loading rates that might be expected on a selectin tether bond during rolling also cautioned against applying the Bell equation indiscriminately.

In regards to the formula we used to calculate k_{off} in Fig. 4 (see Fig. 4 legend, Smith et al., 1999), we fully acknowledge the mistake Dr. Springer and colleagues discuss. While at a conceptual level it was a significant error, in practice it made only a very small difference in the results. As described fully in Fig. 4 of Smith et al. (1999), we calculated the estimated k_{off} from the distribution of pauses lasting a specific bin length of time rather than from the distribution based on the number of remaining pauses lasting longer than each bin length of time. The mistake was based on a misreading of the analysis protocol (Alon et al., 1995) by one of the authors (M. Lawrence) and brought to Dr. Springer's attention when we shared raw data with him several years ago. Replotting the data using the corrected formula leads to an increase the k_{off} for each selectin at each of the forces measured, shifting our curves upward but not changing their shapes or relative position with each other. Because we made extensive measurements, the convergence of the two values that takes place at large sample number kept the effect on k_{off} to within experimental error ($<-10\%$, 101 s^{-1} versus 113 s^{-1}, for example). Despite our calculation error, the trend we observed of increasing tether bond k_{off} with increasing force remains the same. The bond reactive compliance values remain unchanged and so does our conclusion that tethers formed with L-selectin are more compliant than those formed with E-selectin or P-selectin, a finding in contrast with previous results (Alon et al., 1997, 1998). Subsequent studies from out laboratory have corrected our error (Park et al., 2002).

The L-selectin data at 0.5 dyn/cm^2 that particularly concerns Dr. Springer and colleagues was our "weakest" data point in that there were only 30 pauses measured, though that is approximately the number of events routinely used in earlier reports (Alon et al., 1995, 1998). This was also the lowest force measured and could easily include pauses composed of multiple bonds, thereby leading to slower k_{off} estimates. Our recalculation of the k_{off} for L-selectin at 0.5 dyn/cm^2 resulted in 12 s^{-1} (rather than our original erroneous calculation of 10.7 s^{-1}) with an R^2 value of 0.97, and which is still much less than the 113 s^{-1} L-selectin bond lifetime we measured at 2.0 dyn/cm^2 wall shear stress. Of course, even at a k_{off} of 12 s^{-1} (our calculation) or 16 s^{-1} (Dr. Springer's calculation from his laboratory's data) for L-selectin at the level of force at 0.5 dyn/cm^2, it is still possible that the true single bond dissociation kinetics could be much higher than any reference in Table 1 (Evans et al., 2001).

In conclusion, we stand by our original interpretation of the higher compliance of L-selectin mediated bonds relative to P- or E-selectin bonds, and the appropriateness of the Bell equation fit to the dissociation rate constants below 125 pN. It is interesting to note in support of these conclusions (Smith et al., 1999) that one of the authors of the above letter, Dr. Alon, has recently published two articles in which he used a high speed digital camera identical to ours for the analysis of L-selectin tether bonds (Dwir et al., 2002a,b).

We thank Dr. Springer and colleagues for stimulating the clarification of these issues and supporting a forum to further discuss sampling theory in biological applications.

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