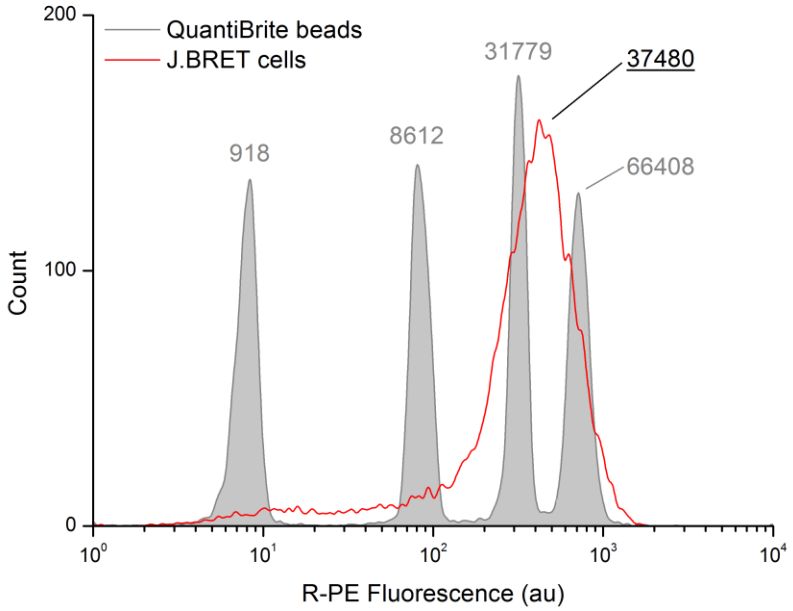
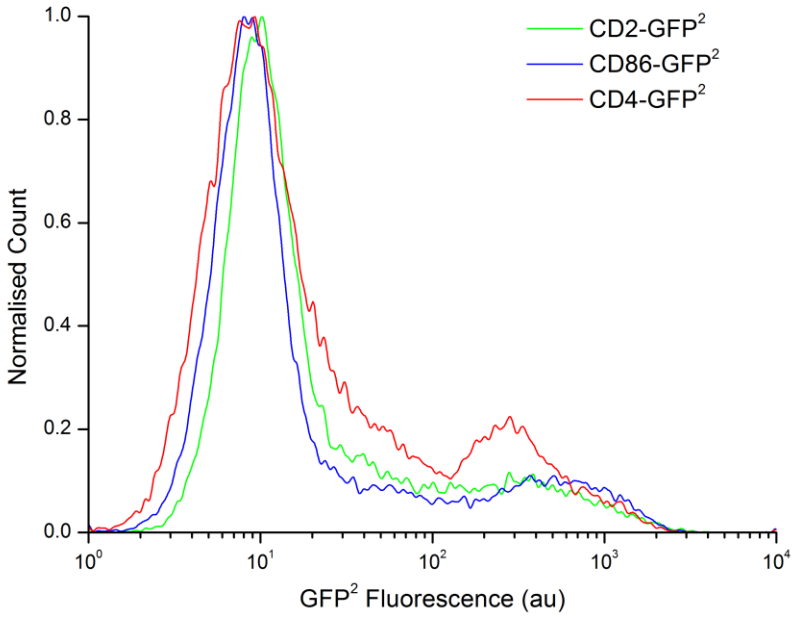
SUPPLEMENTARY FIGURE 1



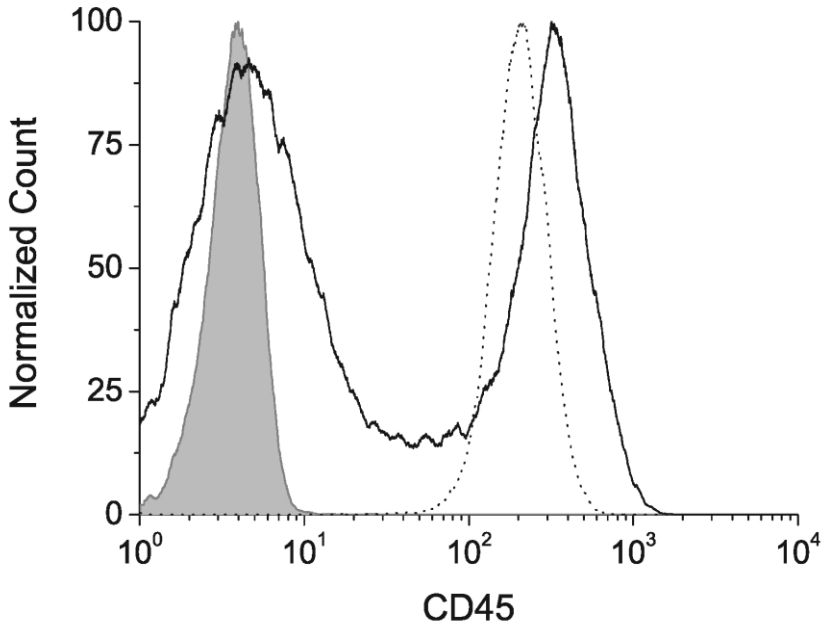
Quantitation of CD3ε expression on J.RT3 (TCRβ$^-$) Jurkat T cells after lentiviral transduction with the TCRβ cDNA sequence fused to Luc and GFP[2] (J.BRET). Quantibrite beads with known amounts of bound R-PE (gray values in figure) were used to calibrate the flow cytometer intensity values with number of fluorescent molecules, as described in ref. 17. The fluorescence intensity of Jurkat cells labeled with a saturating concentration of an α-CD3ε:RPE antibody can therefore be used to determine the geometric mean of the number of R-PE fluorophores per cell. On the assumption that the antibody can detect both CD3ε chains per TCR complex, there are ~19000 TCR complexes per cell.

SUPPLEMENTARY FIGURE 2



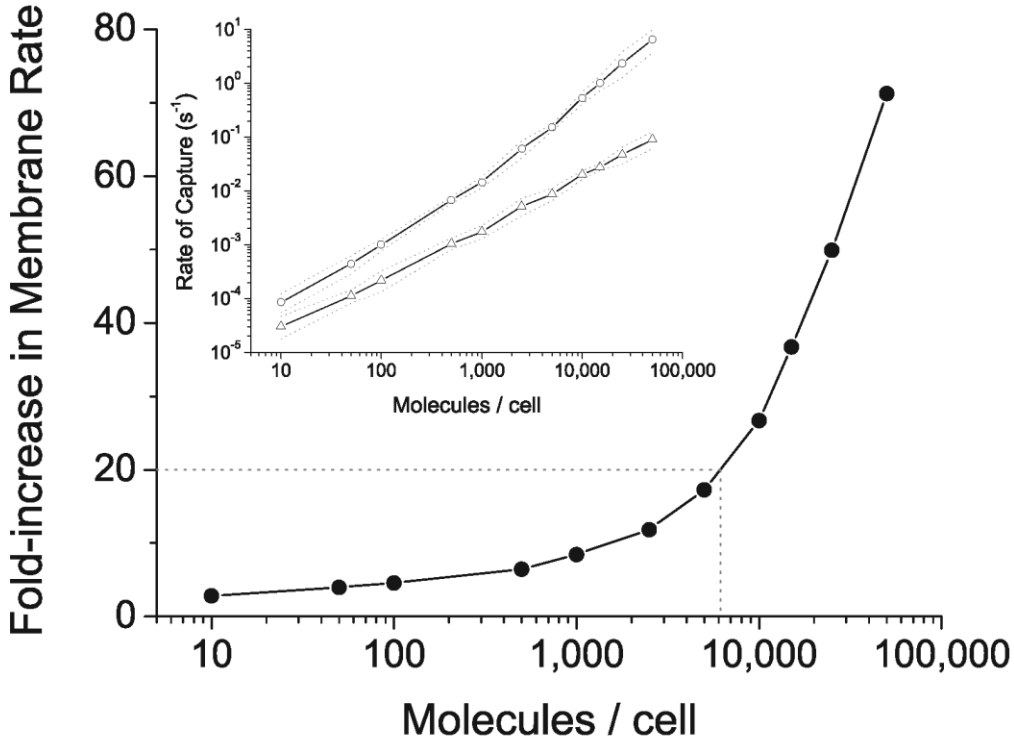
Transient expression of fluorophore-labeled CD4 is equivalent to previously quantitated membrane proteins. HEK-293T were transiently transfected with the plasmid encoding CD4-GFP^2 as per the BRET assay and then fixed and analyzed by flow cytometry for GFP^2 expression. By comparing this to two membrane proteins (CD2 and CD86) where the protein-independent correlation between fluorescence and surface expression has been established, we could show that CD4 expression is essentially equivalent and in the range of 10^3—10^5 molecules/cell. Based on the derived surface expression for CD2 and CD86, the geometric mean of the CD4-GFP^2 fluorescence indicates ~50,000 molecules per cell.

SUPPLEMENTARY FIGURE 3



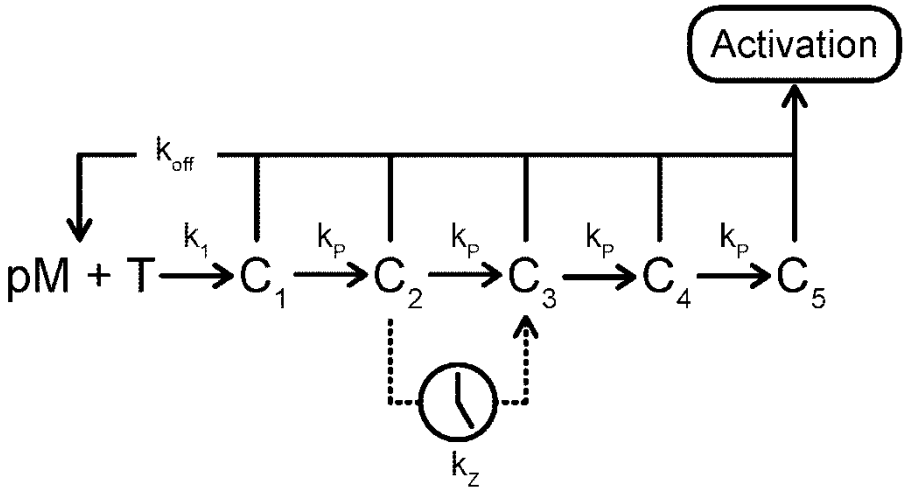
Transient expression of $CD45^{Ex}$ is comparable to that on T cells. 293T were transfected with the vector encoding $CD45^{Ex}{}_{GFP}$ for 48 hours, before harvesting cells with PBS. The transfected cells (solid line) were stained alongside Jurkat T cells (dotted line) with an Alexa647-labeled anti-CD45 antibody for 60 minutes on ice, washed and then fixed with 2% paraformaldehyde. An isotype control antibody-conjugate was also used to stain the Jurkat T cells to identify the background signal (filled line). The cells were than analyzed on a CyanADP flow cytometer and the detected Alexa647 fluorescence was plotted as a histogram. The transfected cells were approximately 60% positive and those that expressed CD45 did so at levels higher than the Jurkats. The larger size of 293T compared to Jurkats means the surface density of the phosphatase is likely to be essentially equivalent.

SUPPLEMENTARY FIGURE 4



Simulation of the effect of membrane confinement on reaction rates. Data from the molecular simulation. The inset shows the reaction rates for membrane-bound (circles) and cytosolic (triangles) proteins bounded by 99% confidence intervals, and the main graph shows the ratio between these values at different molecule concentrations.

SUPPLEMENTARY FIGURE 5



A rate-limiting step in Kinetic Proofreading leads to an intermediate state of activation. Schematic of the original KP model where pMHC (pM) binding to TCR (T) leads to complex (C) formation with a pMHC-specific koff value. Serial accumulation of intermediates (C1 – C4) with equal kinetics (k_P) leads to the fully-active complex (C5). In the revised model (dotted arrows), the C2-C3 step is significantly slower than the other steps (k_Z), which at certain k_{off} values leads to the frustration of the pathway and accumulation of C2.

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Vector Cloning

Construct		Oligonucleotide (5'->3')	Used in
TCRβ	1	TAGTAGCCGCGGCTCTGCCATGGACTCCTCGACC	Figure 2
	2	CTACTAGGATCCAAATCCTTTCTCTTGACCATGGC	
CD3ε	3	TAGTAGACGCGTGCCACCATGCAGTCGGGCACTCACTGG	Figure 2
	4	CTACTAGGATCCATGCGTCTCTGATTCAGGCC	
CD4wt	5	TAGTAGCTGCAGGGCAAGGCCACAATGAACC	Figure 4
	6	CTACTAGGATCCATGGGGCTACATGTCTTCTG	
$CD4^{K318E}$	7	CTAAGCTGATGCTGAGCTTGGAACTGGAGAACAAGGAGGC	Figure 4
	8	GCCTCCTTGTTCTCCAGTTCCAAGCTCAGCATCAGCTTAG	
$CD4^{Q344E}$	9	GGCGGGGATGTGGGAGTGTCTGCTGAGTGACTCGG	Figure 4
	10	CCGAGTCACTCAGCAGACACTCCCACATCCCCGCC	
$CD4^{\Delta Cys}$	11	GAGACTCCTCAGTGAGAAGAAGACCTCCCAGTCCCCTCACCGGTTTCAGAAGAC	Figure 4
	12	GTCTTCTGAAACCGGTGAGGGGACTGGGAGGTCTTCTTCTCACTGAGGAGTCTC	
$CD4^{Ex}$	13	TAGTAGACGCGTCTGCAGGGCAAGGCC	Figures 4,6
	14	CTACTAGGATCCCTTCGGTGCCGGCACCTGACAC	
$CD4^{Int}$	15	TAGTAGGGATCCAATGGCCCTGATTGTGCTG	Figure 4
$CD4^{TM}$	16	CTACTAGGATCCCTTCGGTGCCGGCACCTGACAC	Figure 4
Lck	17	TAGTAGACGCGTGCCACCATGGGCTGTGGCTGCAGCTCACAC	Figure 4
	18	CTACTAGGTACCCAAGGCTGAGGCTGGTAC	
Nck	19	TAGTAGACGCGTGCCACCATGGGCTGTGGCTGCAGCTCACACATGGCAGAAGAAGTGGTGG	Figure 4
	20	CTACTAGGATCCGATAAATGCTTGACAAGATATAA	
CD4Lck	21	TAGTAGAAGCTTGGCAAGGCCACAATGAACC	Figure 4
	22	CTACTAGGTACCGACACAGAAGAAGATGCCTAGCCC	
	23	TAGTAGGGTACCATGGGCTGTGGATGCAGC	
	24	CTACTAGGTACCCAAGGCTGAGGCTGGTAC	
CD2Lck	25	TAGTAGCTGCAGCCCCTAAGATGAGCTTTCC	Figure 4
	26	CTACTAGGTACCCCTTTTGGTGATATAGAAAACGAGCAG	
$CD45^{Ex}$	27	TAGTAGACCGGTCACCACCATCACCATCACCACCATCAAAGCCCAACACCTTCCCCC	Figures 5,6
	28	CATCTCCGTTTATCTCAGAGAG	
CD43	29	TAGTAGCTGCAGGCCTGGAAATGGCCACGCTTC	Figure 5
	30	CTACTAAAGCTTAGGGGCAGCCCCGTCTCCC	
ZAP70	31	TAGTAGACGCGTGCCACCATGCCAGACCCCGCGGCGCAC	Figure 6
	32	CTACTAGGATCCGCACAGGCAGCCTCAGCCTTC	

TCRβ The TCRβ chain was amplified from Jurkat cDNA using primers 1 and 2, and inserted into $pGFP^2$-N3 and prLuc-N3 (the BRET vectors) as a SacII/BamHI fragment.

CD3ε The CD3ε gene was amplified from a full length vector construct using primers 3 and 4 and inserted into the BRET vectors as a MluI/BamHI fragment.

CD4 The CD4 gene was amplified using primers 5 and 6 from a full length construct and inserted into the BRET vectors as a PstI/BamHI fragment.

$CD4^{K318E}$ The K318E mutation of CD4 was introduced by 2-stage chimeric PCR. Primers 5 and 8 were used to amplify the 5' fragment of CD4K318E and primers 6 and 7 for the 3' fragment. These PCR products were purified and then used as a template along with primers 3 and 4 to amplify the complete mutated gene. This PCR product was gel purified and cloned into the BRET vectors as a PstI/BamHI fragment.

$CD4^{Q344E}$ This CD4 mutant was cloned essentially as for CD4K318E, except the first stage PCRs were directed by primers 3 and 10, and 4 and 9.

$CD4^{\Delta Cys}$ This CD4 mutant was cloned essentially as for CD4K318E, except the first stage PCRs were directed by primers 3 and 12, and 4 and 11.

$CD4^{Ex}$ To remove the intracellular region of CD4, primers 13 and 14 were used to amplify CD4Ex, which was inserted as a MluI/BamHI fragment into the BRET vectors.

$CD4^{Int}$ To remove the extracellular region of CD4 and create $CD4^{Int}$, a convenient KpnI/BamHI fragment of CD3ζ was excised from a construct expressing the wild type gene and was cloned into the BRET vectors. This fragment encoded the signal peptide and small extracellular domain of CD3ζ and maintains the same reading frame as the N3 BRET vectors. The TM and intracellular regions of CD4 were then amplified using primers 15 and 6 and inserted into the CD3ζ -containing vectors as a BamHI fragment.

$CD4^{TM}$ To only express the TM region of CD4 at the cell surface, a similar strategy was used as for $CD4^{Int}$ but the insert was amplified using primers 15 and 16 and cloned as a BamHI fragment.

Lck Cloned from a full length construct using primers 17 and 18. The GFP in pGFP2-N3 was first replaced by mCherry and then the Lck PCR product was inserted into this vector as a MluI/KpnI fragment. Lck was fused to mCherry to facilitate quantitating its co-expression with the CD4 constructs.

Nck As a control for Lck activity, Nck1 was cloned from a full length construct using primers 19 and 20. Primer 19 incorporated the first 8 amino acids of Lck at the N-terminus of Nck. The PCR product was then inserted as a MluI/BamHI fragment to replace Lck in the mCherry-fused vector.

$CD4^{Ex}$:Lck CD4 was first amplified using primers 21 and 22 from the CD4WT GFP^2 vector and cloned into the BRET vectors as a HindIII/KpnI fragment. This removed the intracellular region of CD4. Lck was then amplified as a KpnI fragment using primers 23 and 24 and was inserted 3' of CD4Ex.

$CD2^{Ex}$:Lck CD2 was amplified from a full length construct using primers 25 and 26, which removed the intracellular region of the protein. The PstI/KpnI fragment of CD2Ex was then used to replace CD4 in the CD4Ex:Lck vector.

$CD45^{Ex}$ The extracellular and TM regions of CD45RO were first amplified using primers 27 and 28. Primer 27 fuses an eight amino acid stretch of histidine residues to the N-terminus of the mature protein sequence, and the signal peptide cleavage site was predicted using SignalP. The PCR product was cloned as an AgeI/SacII fragment into pLEX using a convenient SacII in the CD45 sequence present just after the end of the TM region of the gene. The pLEX vector incorporated a strong signal peptide sequence from gallus gallus that was 5' to the start of the His8 sequence. The HindIII/SacII fragment of this vector includes this signal sequence along with His_8-$CD45^{Ex}$ and was subcloned into the BRET vectors.

CD43 A PCR product was amplified from a full length construct using primers 29 and 30 and was cloned into the BRET vectors as a PstI/HindIII fragment.

ZAP70 A PCR product was amplified from a full length construct using primers 31 and 32 and was cloned into the BRET vectors as a MluI/BamHI fragment.

SmolDyn Simulation

SmolDyn is a computer program that models the reaction dynamics of a cell, where each reactive molecule is explicitly defined and simulated so that stochastic and spatial biochemical features can be recapitulated (Andrews and Bray, 2004). For the simulation used in this paper, a spherical cell of 5 μm radius was defined and the desired quantity of molecules randomly distributed either in the cytoplasm or cell surface, depending on the simulation scheme, along with a single "receptor" randomly inserted into the surface.

The diffusion coefficients (D) for the simulated molecules were taken from measured values of ZAP70 and the TCR (Sloan-Lancaster et al., 1998). Although estimates of D vary considerably, this paper measured both with the same assay, minimizing any associated experimental error. The value of D is probably an underestimate for the membrane diffusion as myristoylated proteins are likely to diffuse at a higher rate than the TCR, which would only increase the differences described in the main text. The reactions between molecules in the cytoplasm or membrane and the receptor had essentially equivalent reactive binding radii (defined by SmolDyn), ensuring any observed effect is due to localization alone. The reaction of the cytoplasmic molecule with the receptor was calculated to be "somewhat activation-limited", which is to be expected for ZAP70 activation, since it requires conformational changes both in its SH2 domains to bind to a doubly-phosphorylated ITAM and in the kinase domain to form the active conformer. Even if both reactions were completely diffusion-limited, there would still be a 5-fold improvement in the within-membrane reaction rate (Kholodenko et al., 2000).

The simulation was run until the reaction of receptor and molecule occurred and the simulated time taken for this was written to a file. The concentration of molecules was then adjusted to original conditions and the simulation continued. The time step defined in SmolDyn was selected for each concentration value so that essentially no reaction occurred within one time-slice, ensuring accuracy of measurement. A histogram of these "reaction times" gave an exponential distribution, and the process was repeated at various molecule concentrations. An exponential distribution is expected for a process in which events occur continuously and independently at a constant average rate. The inverse of the mean reaction time is the maximum-likelihood estimate for the true rate and this was plotted against the molecular concentration. Exact confidence intervals can be computed for the reaction rate, and are plotted (at an α-value of 0.01) with the data in Supplementary Figure 2 inset. The reaction ratio is simply the membrane-bound capture rate over the cytoplasmic rate, at a given molecule concentration.

Revision of the Kinetic Proofreading Model

The original Kinetic Proofreading model devised for antigen receptor signaling by McKeithan used serial (or distributive) steps of equal rates (k_P) to amplify weak changes in TCR/pMHC interaction. The more steps in the process of converting the initial binding event to a productive output, the greater the sensitivity in detecting subtle differences in ligand affinity. Following the original method, the equilibrium relationship between pMHC (M_{free}), TCR (T) and the complex formed between the two (C_{total}) is assumed to be in a steady state.

$$M_{free} + T \underset{k_{off}}{\overset{k_1}{\rightleftharpoons}} C_{total}$$

Since the total amount of pMHC (M_{total}) is conserved, the unligated fraction can be expressed as

$$M_{free} = M_{total} - C_{total}$$

At equilibrium, the forward and reverse rates are balanced, so

$$k_1 \cdot M_{free} \cdot T = k_{off} \cdot C_{total}$$

Assuming that the concentration of T is in large excess, remaining essentially constant and that $T = k_P/k_1$ allows calculation of M_{free} in terms of rate constants and M_{total}.

$$M_{free} = \left(\frac{k_{off}}{k_P + k_{off}}\right) \cdot M_{total}$$

With comparison to Supplementary Figure 3, we assume that C_{total} is comprised of 5 subcomplexes, where $\sum_{i=1}^{5} C_i = C_{total}$ and there are four modifying events of the nascent complex [C_1], all with a rate of k_P except the transition from [C_2] to [C_3], which is defined as k_Z or the "slow" step. By defining $\alpha = k_P/(k_P + k_{off})$, the fraction of M_{total} found as each subcomplex at equilibrium can be written as

$$M_{free} = (1 - \alpha)$$

$$C_1 = \alpha \cdot (1 - \alpha)$$
$$C_2 = \left(\frac{k_P}{k_Z + k_{off}}\right) \cdot \alpha \cdot (1 - \alpha)$$
$$C_3 = \left(\frac{k_Z}{k_Z + k_{off}}\right) \cdot \alpha^2 \cdot (1 - \alpha)$$
$$C_4 = \left(\frac{k_Z}{k_Z + k_{off}}\right) \cdot \alpha^3 \cdot (1 - \alpha)$$
$$C_5 = \left(\frac{k_P}{k_{off}}\right)\left(\frac{k_Z}{k_Z + k_{off}}\right) \cdot \alpha^3 \cdot (1 - \alpha)$$

These are the equations plotted in the main text with k_{off} varied over six orders of magnitude. For the original model, $k_P = k_Z = 1$ (Figure 6Bi), whilst for the revised model, k_Z was set to be $0.05\ x\ k_P$ based on the SmolDyn simulation (Supplementary Figure 2) and was plotted in a similar manner (Figure 6Bii).