(Supporting Information)

# Configuring robust DNA strand displacement reactions for *in situ* molecular analyses

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## Table S1. List of oligonucleotide sequences used in design of DNA-circuits.

**3 Strand Probe System ($PC_{3s}$)**

| **Strand** | **Sequence (5'-3')** |
|---|---|
| TS | CAT TCA ATA CCC TAC GTC TCC ATT TTT TTT TT/AmMC6/ |
| Output A (OA) | CCA CAT ACA TCA TAT TCC CTC ATT CAA TAC CCT ACG/IAbRQSp/ |
| Output B (OB) | CTT TCC TAC A CC TAC GTC TCC AAC TAA CTT ACG G |
| Cy5LB-1 | TGG AGA/Cy5/ CGT AGG GTA TTG AAT GAG GGC CGT AAG TTA GTT GGA GAC GTA GG |
| Eraser (E) | CCT ACG TCT CCA ACT AAC TTA CGG CCC TCA TTC AAT ACC CTA CG/IAbRQSp/ |

**2 Strand Probe System ($PC_{2s}$ 3-way)**

| **Strand** | **Sequence** |
|---|---|
| TS | /AmMC6/TTT TTT TTT TCG AGA TGC CTT ACA GTA GGT TGG A |
| Output A (OA) | CGA GAT GCC TTA CAG TAG/IAbRQSp/ |
| Cy5_Dye | /Cy5/ACG ATG TCC AAC CTA CTG TAA GGC ATC TCG |
| Eraser (E) | CGA GAT GCC TTA CAG TAG GTT GGA CAT CGT/IAbRQSp/ |

**2 Strand Probe System (4-way)**

| **Strand** | **Sequence** |
|---|---|
| TS | /AmMC6/TTT TTT TTT TCG AGA TGC CTT ACA GTA GGT TGG A |
| Output A (OA) | /IAbRQ/AGA TGC CTT ACA GTA G |
| Cy5_Dye | ACG AAC TCC AAC CTA CTG TAA GGC ATC T/Cy5Sp/ |
| Eraser (E) | /IAbRQ/AGA TGC CTT ACA GTA GGT TGG AGT TCG T |
| EraserP (EP) | ACT CCA ACC TAC TGT AAG GCA TCT |

**2 Strand Probe System2 ($PC_{2s}$ 4-way)**

| **Strand** | **Sequence** |
|---|---|
| TS | /AmMC6/TTT TTT TTT TGT GTA CCG GAA ACA TCG GCG AAT TAG |
| Output A (OA) | /IAbFQ/GTG TAC CGG AAA CAT CGG |
| Cy3_Dye | CTT GTC AAT TCG CCG ATG TTT CCG GTA CAC/Cy3Sp/ |
| Eraser (E) | /IAbFQ/GTG TAC CGG AAA CAT CGG CGA ATT GAC AAG |
| EraserP (EP) | CTA ATT CGC CGA TGT TTC CGG TAC AC |

**2 Strand Probe System3 (4-way)**

| **Strand** | **Sequence** |
|---|---|
| TS | /AmMC6/TTT TTT TTT TGG CCA CCG AGA CAA TAC GCA GGA CCC |
| Output A (OA) | /IAbFQ/GGC CAC CGA GAC AAT ACG |
| Cy5_Dye | CCT TAA GTC CTG CGT ATT GTC TCG GTG GCC/Cy5Sp/ |
| Eraser (E) | /IAbRQ/GGC CAC CGA GAC AAT ACG CAG GAC TTA AGG |
| EraserP (EP) | GGG TCC TGC GTA TTG TCT CGG TGG CC |

**2 Strand Probe System4 (4-way)**

| **Strand** | **Sequence** |
|---|---|
| TS | /AmMC6/TTT TTT TTT TGA TAT CAA GCT GCT CTG GGT ATG C |
| Output A (OA) | /IAbFQ/GAT ATC AAG CTG CTC T |
| Cy5_Dye | AAT CCT ATA CCC AGA GCA GCT TGA TAT C/Cy3Sp/ |
| Eraser (E) | /IAbFQ/GAT ATC AAG CTG CTC TGG GTA TAG GAT T |
| EraserP (EP) | GCA TAC CCA GAG CAG CTT GAT ATC |

*/AmMC6/ represents an amino modifier; /Cy5/ or /Cy5Sp/ indicate a Cy5 fluorophore; /Cy3/ or /Cy3Sp/ indicate a Cy3 fluorophore; and /IAbFQ/ or /IAbRQ/ represent an Iowa Black Quencher for the green to pink or red spectral ranges, respectively.

**Table S2.** Calculated standard free energies of the probe complexes ($\Delta G^{o}_{comp}$) and their labeling and erasing reactions ($\Delta G^{o}_{net}$) that were determined using NUPAC.

| | | |
|---|---|---|
| **2-Strand Probes (3-way)** | | |
| **Complex** | **Strands** | **$\Delta G^{\circ}_{comp}$ (kcal/mol)** |
| Probe Complex ($PC_{2s}$) | Cy5_Dye, OA | -26.90 + 2.38 = -24.52 |
| Reporting Complex ($I_{R2s}$) | Cy5_Dye, TS | -37.04 + 2.38 = -34.66 |
| Waste (W) | Cy5_Dye, E | -45.99 + 2.38 = -43.61 |
| **Reaction** | **$\Delta G^{\circ}_{net}$ (kcal/mol)** | |
| Label ($PC_{2s}$ + TS -> $I_{R2s}$) | -34.66 – (-24.52) = -10.14 | |
| Erase ($I_{R2s}$ + E -> TS + W) | -43.61 – (-34.66) = -8.95 | |
| **3-Strand Probes (3-way)** | | |
| **Complex** | **Strands** | **$\Delta G^{\circ}_{comp}$ (kcal/mol)** |
| Probe Complex ($PC_{3s}$) | Cy5LB-1, OA, OB | -68.73 + 2(2.38) = -63.97 |
| Reporting Complex ($I_{R3s}$) | Cy5LB-1, TS, OB | -70.49 + 2(2.38) = -65.73 |
| Waste (W) | Cy5LB-1, E | -67.78 + 2.38 = -65.40 |
| **Reaction** | **$\Delta G^{\circ}_{net}$ (kcal/mol)** | |
| Label ($PC_{3s}$ + TS -> $I_{R3s}$) | -65.73 – (-63.97) = -1.76 | |
| Erase ($I_{R3s}$ + E -> TS + W) | -65.40 – (-65.73) = +0.33 | |
| **2-Strand Probes (4-way)** | | |
| **Complex** | **Strands** | **$\Delta G^{\circ}_{comp}$ (kcal/mol)** |
| Probe Complex ($PC_{4w}$) | Cy5_Dye, OA | -22.96 + 2.38 = -20.58 |
| Reporting Complex ($I_{R4w}$) | Cy5_Dye, TS | -32.94 + 2.38 = -30.56 |
| Eraser Complex (EC) | E, EP | -35.32 + 2.38 = -32.94 |
| Duplexed TS (DT) | TS, EP | -32.94 + 2.38 = -30.56 |
| Waste (W) | Cy5_Dye, E | -42.01 + 2.38 = -39.63 |
| **Reaction** | **$\Delta G^{\circ}_{net}$ (kcal/mol)** | |
| Label ($PC_{4w}$ + TS -> $I_{R4w}$) | -30.56 – (-20.58) = -9.98 | |
| Erase ($I_{R4w}$ + EC -> DT + W) | (-30.56 + (-39.63)) – (-30.56 + (-32.94)) = -6.69 | |

## Thermodynamic Analysis for Erasing Reactions

Standard free energies of all complexes ($\Delta G_{comp}$) were calculated using NUPACK (Table S2) (24,25). Standard free energies for each probes labeling and erasing reaction ($\Delta G_{net}$) were estimated using these calculations and an extension of Hess's Law. The distance probe reactions are away from equilibrium for a given ON/OFF ratio was examined using the following expressions:

$$IR_i + E_i \rightleftharpoons W_i + O_i + T_i$$

$$\Delta G_i = \Delta G^{\circ}_{T_i} + \Delta G^{\circ}_{O_i} + \Delta G^{\circ}_{W_i} - \Delta G^{\circ}_{E_i} - \Delta G^{\circ}_{IR_i} + RTlnQ_i$$

$$where\ i = P3s, P2s3w, P2s4w$$

$$\Delta G_i \stackrel{\text{def}}{=} \Delta G^{\circ}_{net_i} + RTlnQ_i$$

$$where\ Q_i = \frac{\prod_j a_{j_i}}{\prod_k a_{k_i}}, a = concentration\ of\ species, j = W, T\ or\ O, k = IR, E$$

Here, the reaction quotient, Q, and reflects how far the system is from its equilibrium distribution of reactant and product complexes (at equilibrium $Q = K_{eq}$). Standard conditions are 25 °C, [NaCl] =0.05 M, [MgCl]=0.0125 M and all DNA strands at 1 M. Complex free energies were calculated by setting the dangles parameter in NUPACK to ALL, and were taking as the Minimum Free Energy (MFE) secondary structure of each complex plus a configurational energy factor (n-1)x(2.38 kcal/mol), where n is the number of strands in a complex (8). Thus, the standard free energies of erasing reaction of the 3-strand probes ($\Delta G_i$) includes a -2.38 kcal/mol contribution that accounts for the fact that a single eraser strand displaces two strands in a $I_{R3s}$ complex, thus resulting in an increase in the entropy of the system.

**Estimates of erasing performances assuming homogenous reaction conditions.**

To determine whether the thermodynamic properties of the different probe complexes should, in principle, facilitate efficient erasing (≥ 20:1 ON / OFF), one can examine whether the quantity $RTlnQ_i$ for a reaction that has reached a 20:1 ON / OFF ratio is less than $\Delta G^{\circ}_{net}$. If so, that reaction should be able to reach an equilibrium distribution that would produce even higher ON/OFF ratios. For these analyses, one must first estimate the concentrations of all reactant and product species of the probe reaction within 100 μL reaction chamber / well volume. The concentrations of $I_R$ complexes within the cells can be estimated by first determining the analog-to-digital units per photon for each illumination setup (*excitation power, integration time, and filter set*) on our microscope (26). This relationship can then be used to convert measured fluorescence intensities into IR concentrations. Here, we assume each pixel corresponds to a cubic voxel with dimension of 200nm on each side. For cells that have been labeled fully (*i.e., their labeling reaction has saturated*) $I_R$ concentrations within a voxel were found to range between 10 - 250 μM. An ON/OFF ratio of 20:1 therefore corresponds to a voxel concentration of 0.5-12.5 μM.

If the conditions of the *in situ* erasing reactions mimic those of a homogeneously-mixed solution, Q can be calculated using the concentration of $I_R$ within the total 100 μL reaction volume of the well. The cells in our samples are typically at ~90% confluence (yielding ~20,000 cells/well), and roughly half the cells are transfected successfully with GFP targets. Cells dimensions are approximately 15 μm x 15 μm x 4 μm (length, width, height), which yields a volume/cell $9x10^{-13}$ L. The total volume of the cells containing labeled GFP is therefore taken to be ~$9x10^{-9}$ L. Thus, prior to the erasing reaction, a mean concentration of 25 μM corresponds to a the total $I_R$ concentration within the 100 μL reaction volume $[IR]_{well}$ = 2,500 nM x ($9x10^{-9}$ L / $1x10^{-4}$ L) = 2.25 nM. The total concentration of $I_R$ when 95% of the labeled targets have been erased is therefore 1.125 nM. Using this estimate of $I_R$, the concentrations of TS, O and W can be calculated using the appropriate mass balance for an erasing reaction. The E concentration was 1 μM in each reaction, and, given its large excess, its concentration is considered to remain constant during the reaction. With these considerations, the quantity *RTlnQ* for the 2-strand (3-way), 3-strand (3-way), and 2-strand (4-way) probes are as follows:

**2-strand (3-way):** RTlnQ = -1.81 kcal/mol

$\Delta G^{o}_{net}$ = -7.22 kcal/mol

$\Delta G$ = -9.03 kcal/mol

**3-strand (3-way)**: RTlnQ = -13.12 kcal/mol
$\Delta G^{o}_{net}$ = +0.33 kcal/mol
$\Delta G$ = -12.79 kcal/mol

**2-strand (4-way):** RTlnQ = -1.81 kcal/mol
$\Delta G^{o}_{net}$ = -6.69 kcal/mol
$\Delta G$ = -8.50 kcal/mol

The above calculations show that all of the probes should be able to reach ON / OFF ratios that are better than 20:1, and that the $PC_{3s}$ system is the furthest away from its equilibrium one this ratio is reached. Furthermore, despite better measured performance of the 2-strand (4-way) probes compared to the 2-strand (3-way) probes, the 4-way probe system is closer to its equilibrium, despite $\Delta G^{o}_{net}$ being smaller than that of the 2-strand (3-way) probes. Considering this behavior and our observation that fully-duplexed waste products can label TS strands on cells (Figure 5), we conclude that the differences in our erasing performances cannot be attributed to difference in the thermodynamic properties of the probe complexes alone. Instead, our results imply that the 2-strand (3-way) probes erase more slowly than our other probe constructions due to the crowded reaction environment of the cells, the occurrence of non-toehold mediated, and the resultant slow diffusion of its waste complexes out of the cells.

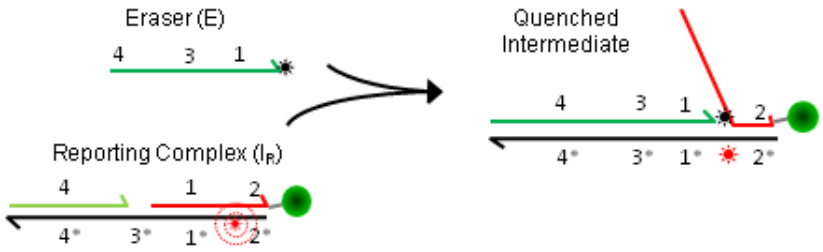


**Figure S1:** Scheme depicting an intermediate state in the $PC_{3s}$ erasing reaction where E has displaced output B and TS from $IR_{3s}$, but where TS and W have not yet dissociated. The similarity of the erasing responses of the PC3s probes with and without the quencher indicates this intermediate state complex is not present within the cells at an appreciable level after the erasing reactions.