Programmable pH-triggered DNA nanoswitches

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Supplementary Information

MATERIALS AND METHODS

Oligonucleotide probes employed in this work were synthesized, labelled, and purified (HPLC) by IBA GmBH (Göttingen, Germany) and used without further purification.

The Triplex pH-triggered Nanoswitches probes are labeled with BHQ-2 (Black hole quencher 2) at position 13 and with Alexafluor 680 (AF680) at the 3'end. The sequences of the switches are as follows:

0% TAT:

5'-GGGGG-GGGGG-**TTT(BHQ-2)A**-CCCCC-CCCCC-CTTTG-CCCCC-CCCCC (AF680)-3'

20% TAT:

5'-GGAGG-GGAGG-**TTT(BHQ-2)A**-CCTCC-CCTCC-CTTTG-CCTCC-CCTCC (AF680)-3'

50% TAT:

5'-AAAAA-GGGGG-**TTT(BHQ-2)A**-CCCCC-TTTTT-CTTTG-TTTTT-CCCCC (AF680)-3'

60% TAT:

5'-AAGGA-AGAAG-**TTT(BHQ-2)A**-CTTCT-TCCTT-CTTTG-TTCCT-TCTTC (AF680)-3'

80% TAT:

5'-AAGAA-AAGAA-**TTT(BHQ-2)A**-TTCTT-TTCTT-CTTTG-TTCTT-TTCTT (AF680)-3'

100% TAT:

5'-AAAAA-AAAAA-**TTT(BHQ-2)A**-TTTTT-TTTTT-CTTTG-TTTTT-TTTTT (AF680)-3'

For all the sequences above the bases in bold represent the loop for the duplex portion and the underlined bases represent the loop for the parallel triplex region.

The duplex probes used as controls were labeled with BHQ-2 at the 5' end and at the 3'end with AF680. The sequences of the control duplex probes were as follows:

Control duplex 80% TA:

5'-(BHQ-2) AAAAG-AAGAA-**CTTG**-TTCTT-CTTTT (AF680)-3'

Control duplex 100% TA:

5'-(BHQ-2) AAAAA-AAAAA-**CTTG**-TTTTT-TTTTT (AF680)-3'

For all the sequences above the bases in bold represent the loop portion.

The reference probe that we employed to verify the pH dependence of the fluorophore was only labeled with AF680 at the 3'end of the 50%TAT triplex probe described above:

Reference probe 50% TAT:

5'-AAAAA-GGGGG-**TTTA**-CCCCC-TTTTT-<u>CTTTG</u>-TTTTT-CCCCC (AF680)-3'

For the sequence above the bases in bold represent the loop for the duplex portion and the underlined bases represent the loop for the parallel triplex region.

pH titration curves were obtained using a Varian Cary Eclipse Fluorometer with excitation at 679 (±5) nm and acquisition between 690 and 712 nm at a temperature of 25°C. Switches were first diluted in phosphate buffer 10 mM, 50 mM NaCl, 4 mM MgCl (pH 7.4) at the concentration of 1 μM. This stock solution was then diluted to 20 nM in a universal citrate/phosphate/borate buffer whose pH was adjusted to the desired value (Östling and Virtama, 1946). The solutions of universal buffer at the required pH were prepared following the method reported elsewhere (Östling and Virtama, 1946). The pH titration curves were fitted using the following equation:

$$F = F_{triplex} + \left(\frac{\left[H^+\right] \cdot \left(F_{duplex} - F_{triplex}\right)}{\left[H^+\right] \cdot K_a^{app}} \right)$$

Where $F_{triplex}$ and F_{duplex} represent the fluorescence intensities of the probe in the triplex state (closed) and duplex state (open), respectively and where $[H^+]$ represents the total concentration of hydrogen ions and K_a^{obs} is the observed acid constant for the switch.

Extended pH titration curves were obtained using a mixture of two or more nanoswitches in the same solution (total switch concentration =20 nM). The ratio of each individual nanoswitch presents in the solution was optimized by performing a simple simulation that takes in consideration the slightly different background and amplitude signal provided by each of the nanoswitches (Vallée-Bélisle et al., 2012). More specifically, to achieve a pH dynamic range from 5.5 to 9.5 we used the 50%TAT and the 80%TAT nanoswitches at a concentration of 9 and 11 nM respectively (see Fig. 3, center). Similarly, to achieve a pH dynamic range from 7.5 to 11.0 we used the 80%TAT and the 100%TAT nanoswitches at a concentration of 10.8 and 9.2 nM respectively (Fig. S5). Finally, to achieve a pH dynamic range from 5.5 to 11.0 we used the 50%TAT, the 80%TAT and the 100%TAT nanoswitches at a concentration of 7.2, 6.8 and 6 nM respectively (Fig. 3, right).

Folding and unfolding reactions of the switches were carried out in an Applied Photophysics SX-20 stopped flow instrument at 25°C. For unfolding and folding reactions of the switches, stopped flow mixing experiments were carried out by mixing 100 nM of the relative nanoswitches in a universal citrate/phosphate/borate buffer at different pH values (Östling and Virtama, 1946) to varying concentrations of NaOH (0.005, 0.0075, 0.01, 0.015, 0.02, 0.025 M) or HCl (0.005, 0.0075, 0.01, 0.015, 0.02, 0.025 M) in a 1:1 ratio. Folding (or unfolding) experiments were initiated at a pH where the nanoswitches are completely unfolded (or folded) and the folding (or unfolding) reaction of the triplex nanoswitch were monitored as a function of time by following the fluorescence of AF680 (excitation at 679 nm ±10 nm and the emission monitored above 692 nm using a high pass filter). The folding/unfolding rate constants were determined by using the best fit to a single exponential function.

References

Östling, S.; Virtama, P. Acta Phys. Scandinav. 1946, 11, 289-293.

Vallée-Bélisle, A.; Ricci, F.; Plaxco, K.W. J. Am. Chem. Soc. 2012, 134, 2876-9

SUPPORTING FIGURES

I. Study of pH sensitive range of the fluorophore (AlexaFluor 680)

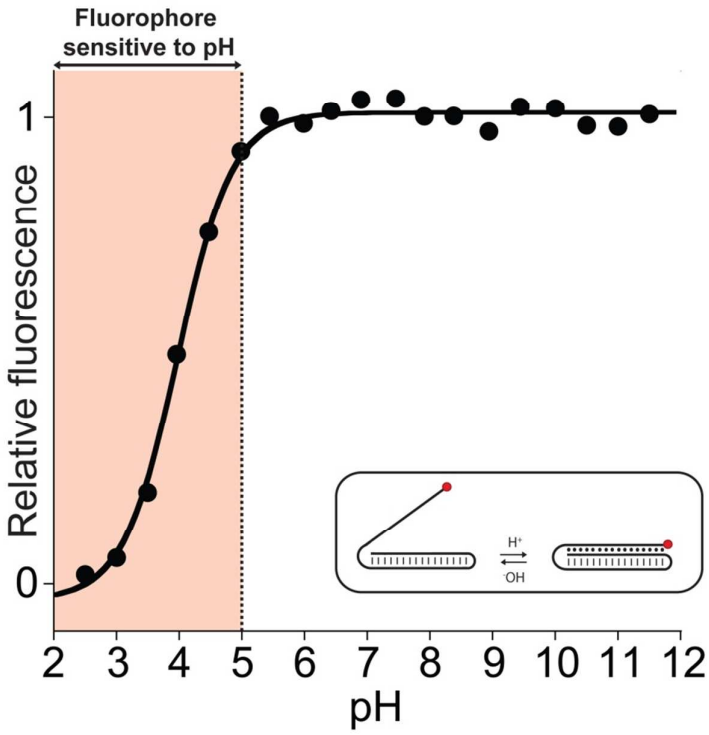

Figure S1. To confirm that the fluorophore used in this work (AlexaFluor 680, AF680) is insensitive to pH in the range covered by the triplex pH-triggered nanoswitches, we have designed a reference probe that contains only the fluorophore (at the 3' end) (see sequence in materials and methods section). This fluorophore is positioned at the same location in all the other switches. The fluorescence intensity of this AF680 remains constant between pH 5 to pH 12 , the pH range at which our nanoswitches perform best.

II. pH titration curves of triplex nanoswitches with high CGC content (<50% TAT)

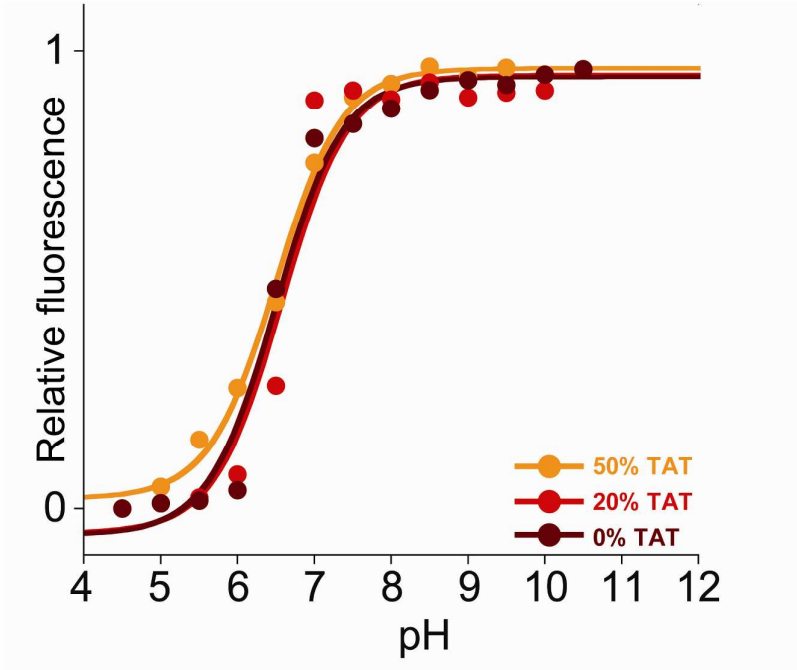

Figure S2. By changing the relative content of CGC/TAT triplets in the sequence of our switches it is possible to tune their pH dependency over a window of more than 5 units of pH. Switches with CGC triplets content higher than 50% (<50% TAT), however, display the same pH-dependence. For example, here we show the pH titration curves for the switches with 50%, 20% and 0% TAT triplets content. These pH-titration curves were performed using 20 nM of nanoswitch in a universal citrate/phosphate/borate buffer (Östling and Virtama, 1946) at 25°C.

III. Control to demonstrate pH range sensitivity of the "Watson-Crick" duplex portion of the nanoswitches

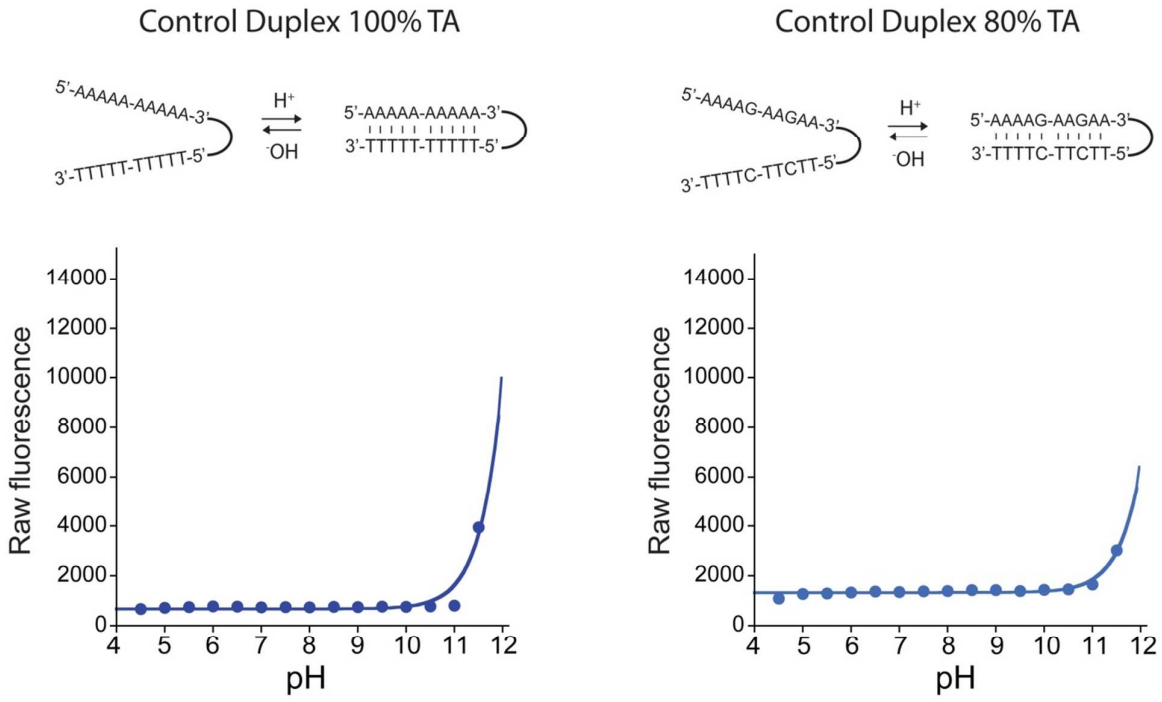

Figure S3. The Watson-Crick duplex portion of the switches remains close over all the pH window investigated in the present study. To demonstrate this we designed two control probes that contain only the duplex portion of the switches with a content of 100% and 80% TA base pairs. As expected (AT and GC Watson-Crick base pairing are known to be relatively insensitive to pH), the unfolding transition from the duplex state to random coil state only starts to occur at very high pH (> 11.5). These pH-titration curves were performed using 20 nM of nanoswitch in a universal citrate/phosphate/borate buffer (Östling and Virtama, 1946) at 25°C.

IV. Effect of the sequence on the observed pK_a for all the tested triplex pH-triggered nanoswitches

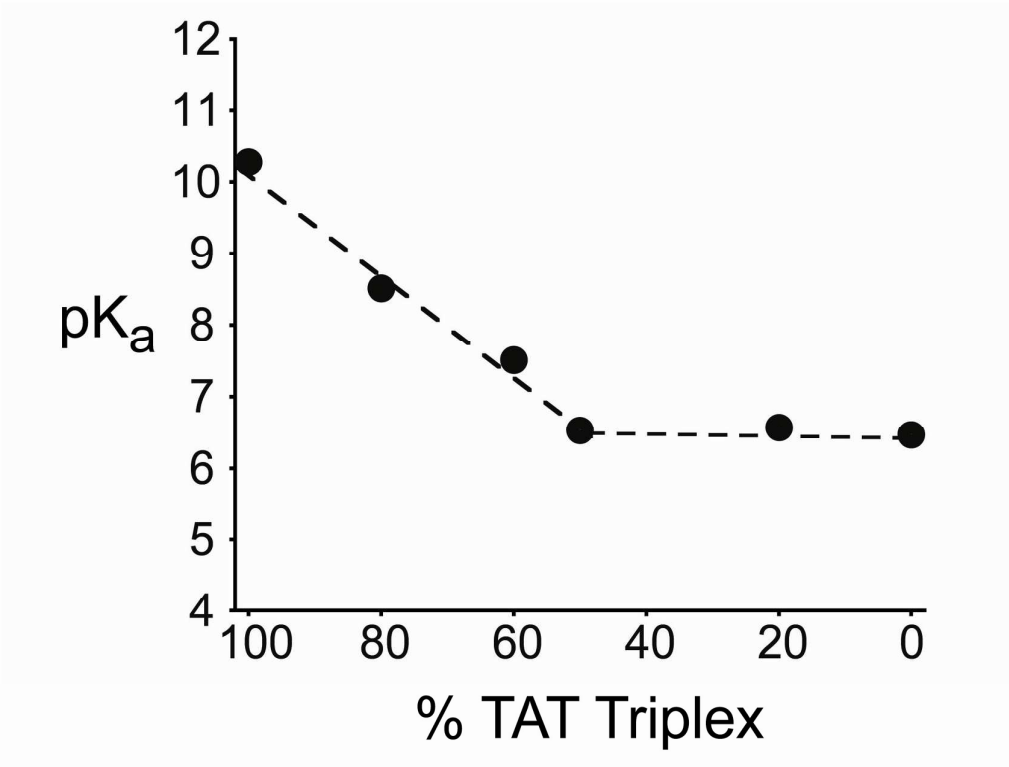

Figure S4: The pH of semiprotonation of our switches (here defined as pK_a^{obs}, the average pK_a due to several interacting ionizing groups), drops from 10.2 (100% TAT) when no cytosines are present in the structure, to 6.5 (50% TAT) when five cytosines are inserted in the sequence (see Figure 2, SI4). Of note, a CGC content higher than 50% (*e.g.* 20%TAT and 0%TAT) does not lead to a different pH sensitivity (see also Figure S2). Observed pKa values were obtained using pH-titration curves.

V. Extended dynamic range obtained with switches 80%TAT and 100%TAT

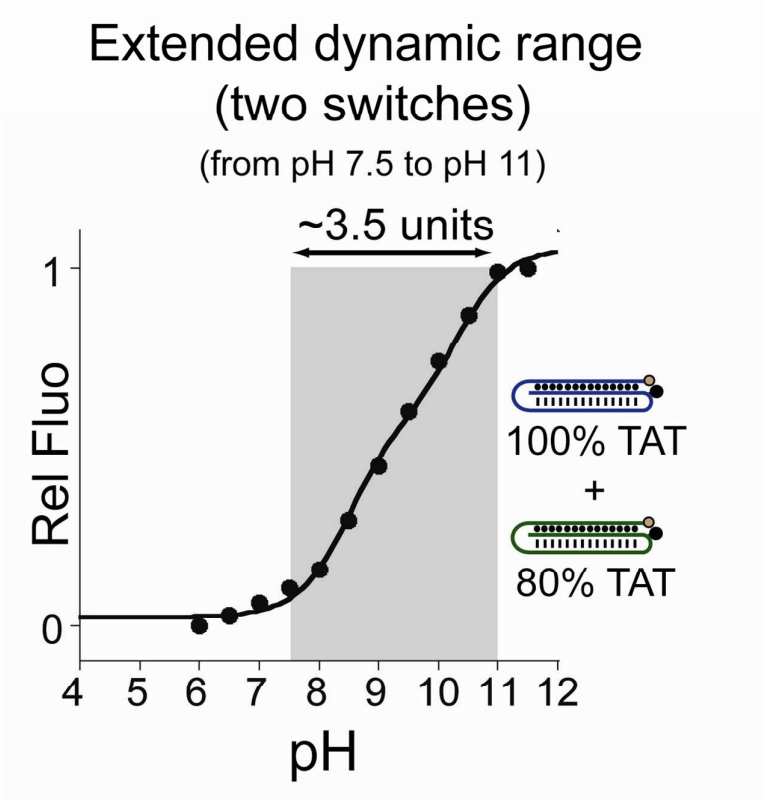

Figure S5. By combining in the same solution two triplex-nanoswitches, each triggered over a different pH window, it is possible to extend the useful dynamic range to ca. 3.5 units of pH (shown is the range of response observed using in the same solution the triplex-nanoswitches 80%TAT and 100%TAT). Shown are the pH-titration curves achieved using a universal citrate/phosphate/borate buffer at different pH values (Östling and Virtama, 1946) with a total concentration of probe of 20 nM.

VI. Kinetic data

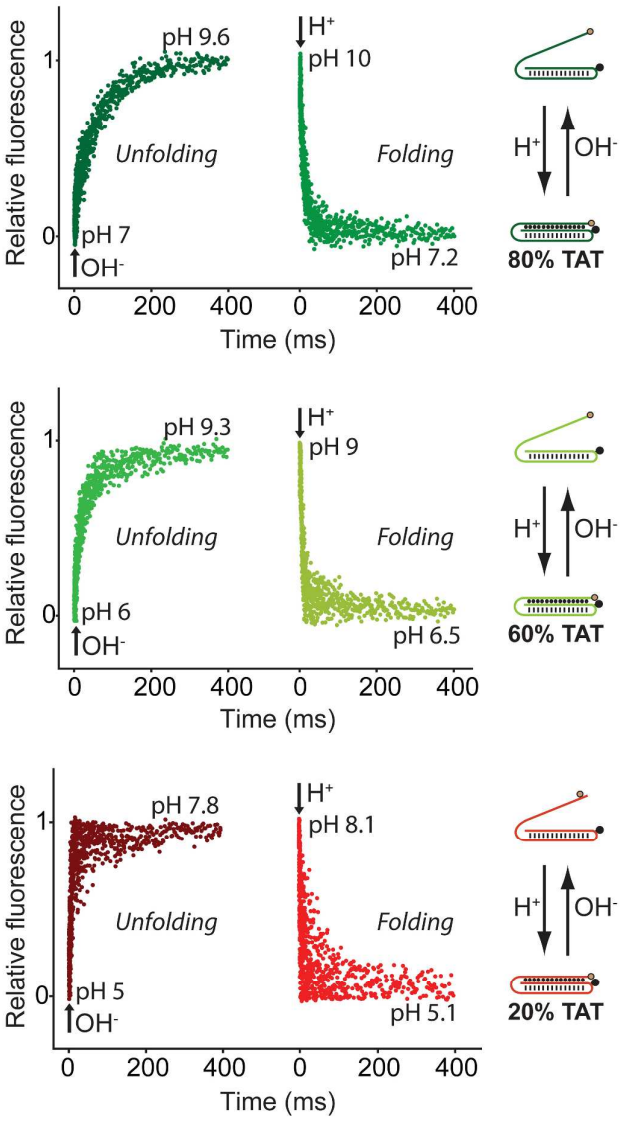

Figure S6. Our triplex pH nanoswitches respond to pH variation within milliseconds. For example, a switch containing 80% TAT triplets (top) displays an unfolding time constant (tunfolding) of 61 ms and a folding time constant (tfolding) of 13 ms. Switches containing 60% of TAT (middle) and 20% of TAT (bottom) show even faster folding/unfolding rate constants (see table S1 for detailed rates). The experiments shown in this figure were performed by rapidly mixing (1:1) a buffered switch solution (final switch concentration = 100 nM) with a NaOH (0.015 M) or HCl (0.015 M) solution, to obtain a nearly 3-unit pH change (see experimental details above). Each kinetic trace shown here is an average of 10 acquisitions.

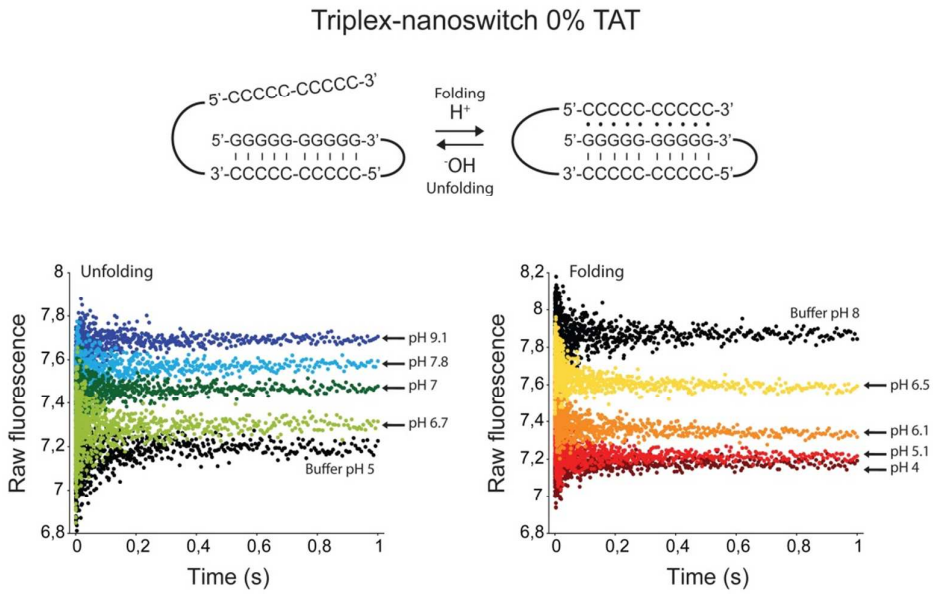

Figure S7. The 0%TAT switch responds to pH variation within milliseconds. For the unfolding experiments (left) the switch was first equilibrated at pH 5.0 in the universal buffer (condition at which the switch is completely folded). The pH was then rapidly changed to the values indicated in the figure using a 1:1 mixing experiment with NaOH (0.0075, 0.01, 0.015, 0.02 M). For the folding experiment (right) the switch was first equilibrated at pH 8.0 in the universal buffer (condition at which the switch is completely unfolded). The pH was then rapidly changed to the values indicated in the figure using a 1:1 mixing experiment with HCl (0.0075, 0.01, 0.015, 0.02 M). Each kinetic trace shown is an average of 10 measurements. The final nanoswitch concentration is 100 nM.

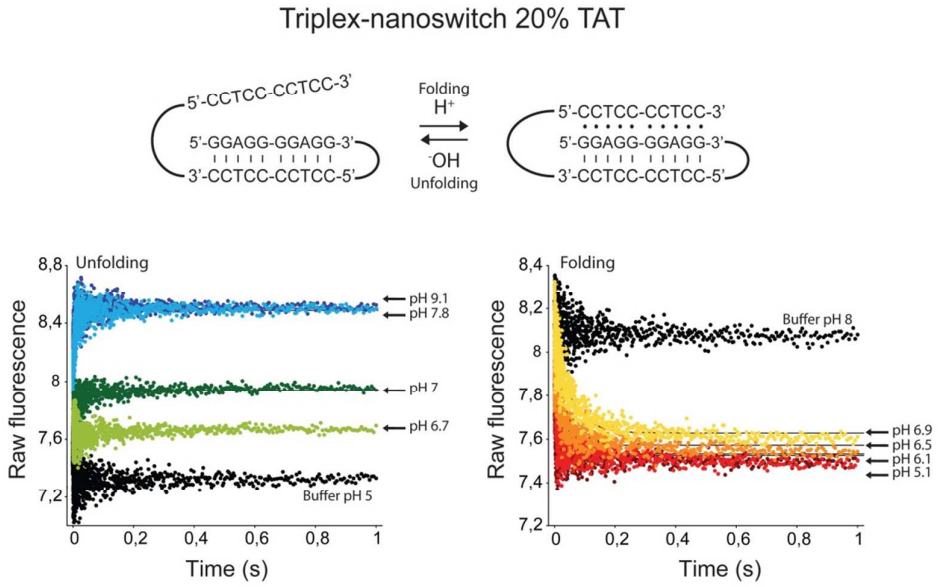

Figure S8. The 20% TAT switch responds to pH variation within milliseconds. For the unfolding experiments (left) the switch was first equilibrated at pH 5.0 in the universal buffer (condition at which the switch is completely folded). The pH was then rapidly changed to the values indicated in the figure using a 1:1 mixing experiment with NaOH (0.0075, 0.01, 0.015, 0.02 M). For the folding experiment (right) the switch was first equilibrated at pH 8.0 in the universal buffer (condition at which the switch is completely unfolded). The pH was then rapidly changed to the values indicated in the figure using a 1:1 mixing experiment with HCl (0.0075, 0.01, 0.015, 0.02 M). Each kinetic trace shown is an average of 10 measurements. The final nanoswitch concentration is 100 nM. Folding and unfolding rate constants are reported in Table S1.

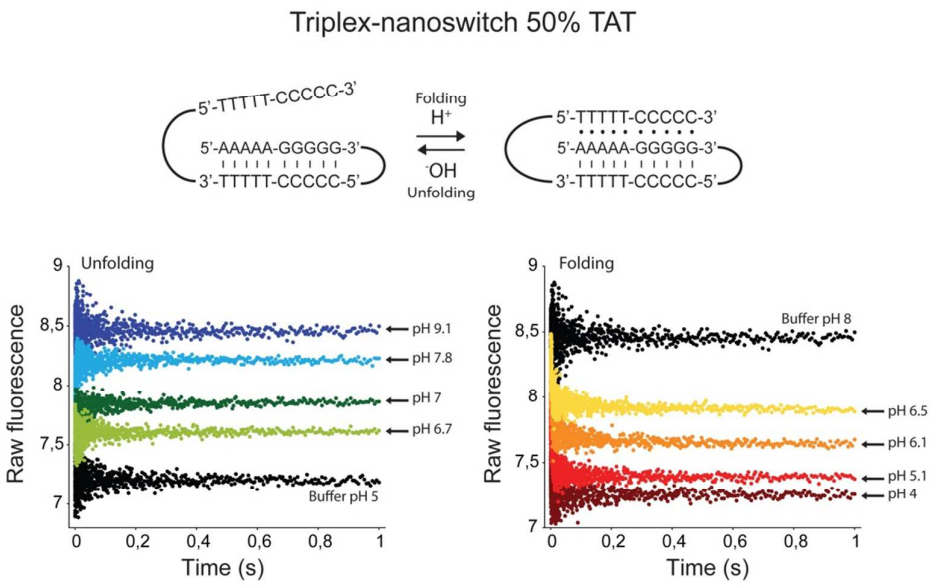

Figure S9. The 50% TAT switch responds to pH variation within milliseconds. For the unfolding experiments (left) the switch was first equilibrated at pH 5.0 in the universal buffer (condition at which the switch is completely folded). The pH was then rapidly changed to the values indicated in the figure using a 1:1 mixing experiment with NaOH (0.0075, 0.01, 0.015, 0.02 M). For the folding experiment (right) the switch was first equilibrated at pH 8.0 in the universal buffer (condition at which the switch is completely unfolded). The pH was then rapidly changed to the values indicated in the figure using a 1:1 mixing experiment with HCl (0.0075, 0.01, 0.015, 0.02 M). Each kinetic trace shown is an average of 10 measurements. The final nanoswitch concentration is 100 nM.

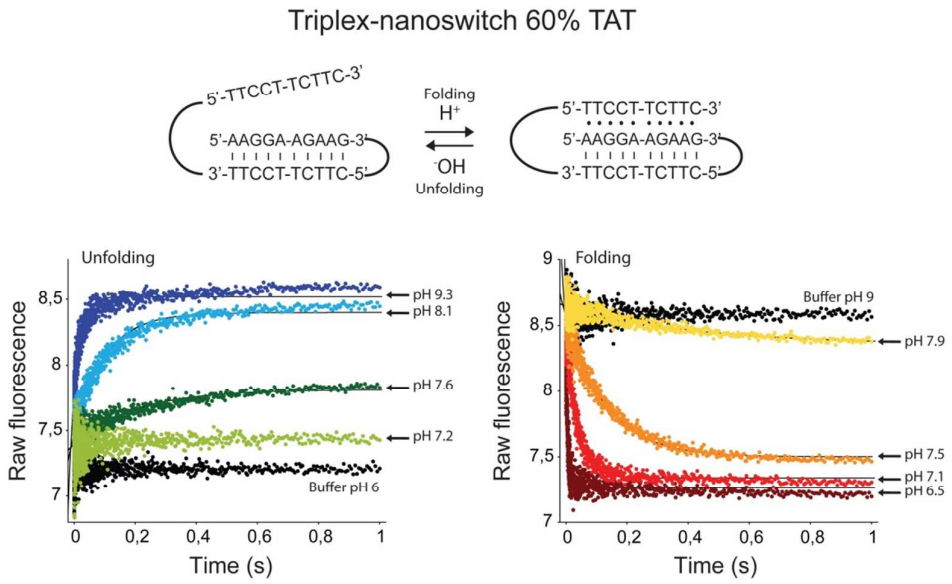

Figure S10. The 60% TAT switch responds to pH variation within milliseconds. For the unfolding experiments (left) the switch was first equilibrated at pH 6.0 in the universal buffer (condition at which the switch is completely folded). The pH was then rapidly changed to the values indicated in the figure using a 1:1 mixing experiment with NaOH (0.005, 0.0075, 0.01, 0.015 M). For the folding experiment (right) the switch was first equilibrated at pH 8.0 in the universal buffer (condition at which the switch is completely unfolded). The pH was then rapidly changed to the values indicated in the figure using a 1:1 mixing experiment with HCl (0.005, 0.0075, 0.01, 0.015 M). Each kinetic trace shown is an average of 10 measurements. The final nanoswitch concentration is 100 nM. Folding and unfolding rate constants are reported in Table S1.

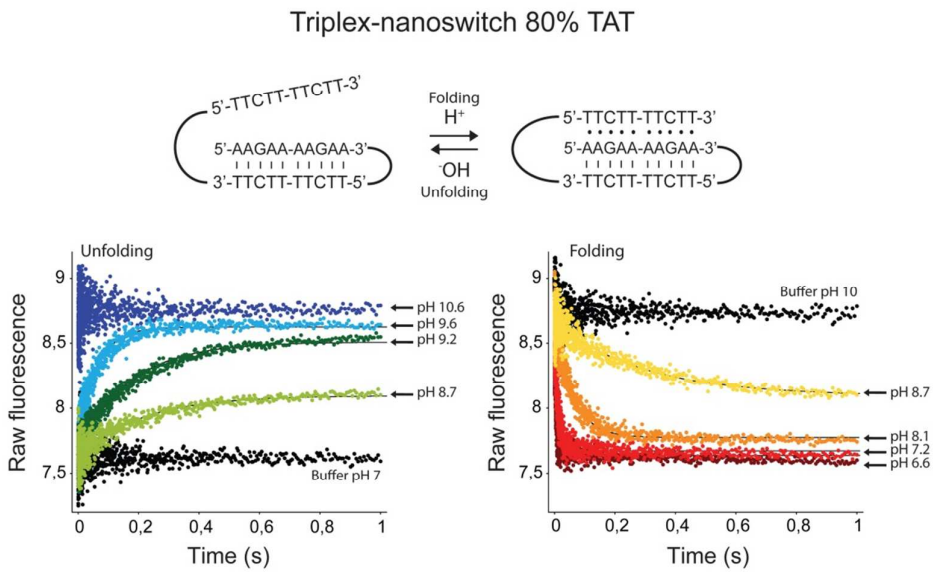

Figure S11. The 80% TAT switch responds to pH variation within milliseconds. For the unfolding experiments (left) the switch was first equilibrated at pH 7.0 in the universal buffer (condition at which the switch is completely folded). The pH was then rapidly changed to the values indicated in the figure using a 1:1 mixing experiment with NaOH (0.005, 0.0075, 0.01, 0.015 M). For the folding experiment (right) the switch was first equilibrated at pH 10.0 in the universal buffer (condition at which the switch is completely unfolded). The pH was then rapidly changed to the values indicated in the figure using a 1:1 mixing experiment with HCl (0.0075, 0.01, 0.015, 0.02 M). Each kinetic trace shown is an average of 10 measurements. The final nanoswitch concentration is 100 nM. Folding and unfolding rate constants are reported in Table S1.

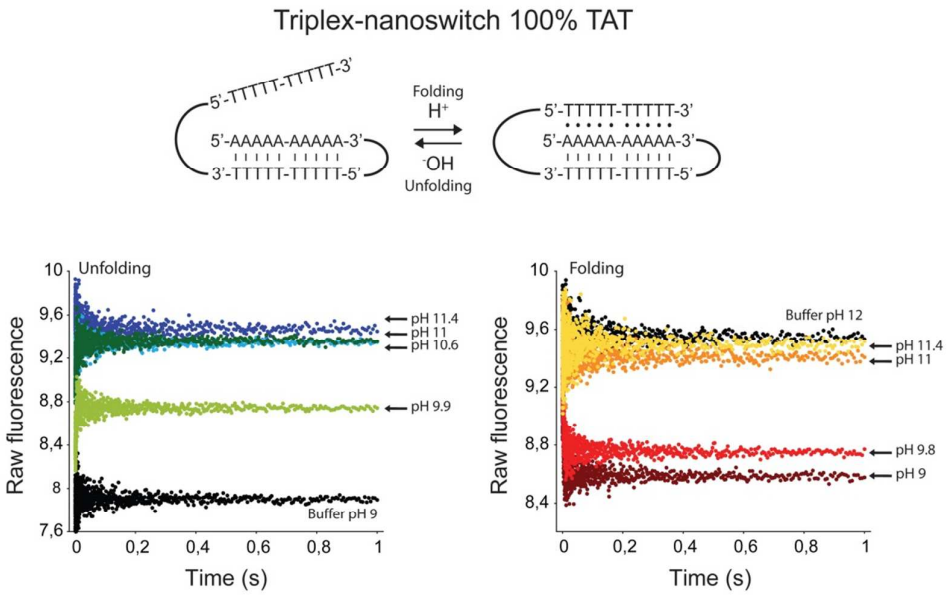

Figure S12. The 100% TAT switch responds to pH variation within milliseconds. For the unfolding experiments (left) the switch was first equilibrated at pH 9.0 in the universal buffer (condition at which the switch is completely folded). The pH was then rapidly changed to the values indicated in the figure using a 1:1 mixing experiment with NaOH (0.005, 0.0075, 0.01, 0.015 M). For the folding experiment (right) the switch was first equilibrated at pH 12.0 in the universal buffer (condition at which the switch is completely unfolded). The pH was then rapidly changed to the values indicated in the figure using a 1:1 mixing experiment with HCl (0.01, 0.015, 0.02, 0.025 M). Each kinetic trace shown is an average of 10 measurements. The final nanoswitch concentration is 100 nM.

VII. Kinetic values

Triplex-nanoswitch	Unfolding			Folding		
% TAT	pH	t (msec)	ΔpH jump	pH	t (msec)	ΔpH jump
20	7	(21.4 ± 1.4)	2	6.9	(53.8 ± 2.0)	-1.1
	7.8	(7.3 ± 0.3)	2.8	6.5	(24.9 ± 1.0)	-1.5
	9.1	(4.1 ± 0.3)	4.1	6.1	(7.6 ± 0.3)	-1.9
				5.1	(5.4 ± 0.3)	-2.9
60	7.6	(196.1 ± 11.5)	1.6	7.9	(416.7 ± 52.1)	-1.1
	8.1	(98.1 ± 1.9)	2.1	7,5	(129,9 ± 1.7)	-1.5
	9.3	(25.1 ± 0.6)	3.3	7.1	(37.6 ± 0.6)	-1.9
				6.5	(6.9 ± 0.2)	-2.5
80	8.7	(204.1 ± 12.5)	1.7	8.7	(294.1 ± 17.3)	-1.3
	9.2	(169.5 ± 5.7)	2.2	8.1	(58.1 ± 1.4)	-1.9
	9.6	(61.0 ± 1.1)	2.6	7.2	(12.6 ± 0.3)	-2.8
				6.6	(6.2 ± 0.2)	-3.4

Table S1. Unfolding (left) and (folding) rate constants measured following pH-jumps. Only with switches containing a TAT% content of 20%, 60% and 80% we observe unfolding/folding at a rate slow enough to be measured using our stopped-flow mixing instrument (dead time of our instrument is 3.2 msec). For the 20%TAT switch the unfolding rate constants have been experimentally measured using pH jumps from 5.0 to 7.0, 7.8 and 9.1. Folding experiments were done using pH-jumps from 8.0 to 5.1, 6.1, 6.5, and 6.9. For the 60%TAT switch the unfolding rate constants have been experimentally measured using pH jumps from 6.0 to 7.6, 8.1 and 9.3. Folding experiments were done using pH-jumps from 9.0 to a value of 6.5, 7.1, 7.5, and 7.9. For the 80%TAT switch the unfolding rate constants have been experimentally measured using pH jumps from 7.0 to 9.6, 9.2 and 8.7. For folding experiments were done using pH-jumps from 10.0 to 6.6, 7.2, 8.1, and 8.7.

VIII. Reversibility studies

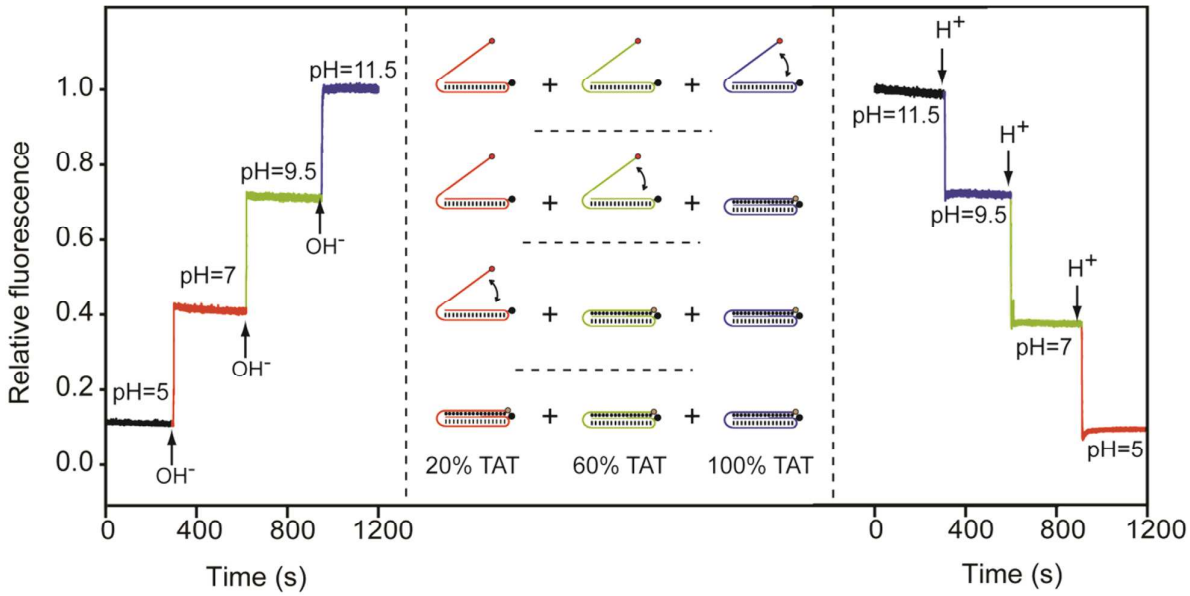

Figure S13. The response of our triplex pH-triggered nanoswitches is fast and reversible. We demonstrate this by sequentially and gradually changing the pH of a solution containing three switches that are triggered within three different pH windows. At pH 5.0 all three switches are closed in a triplex structure and a low background fluorescence signal is observed. At pH 7.0 we observe a signal that is comparable to that expected for the opening of only the 20%TAT switch. The other two switches remaining in the triplex/closed conformation. Increasing the pH of the solution sequentially to 9.5 and to 11.5 leads to the sequential opening of the 60%TAT and the 100%TAT switches, respectively (green and blue lines). Decreasing the solution pH using the same pH steps highlights the perfect reversibility of the nanoswitches.This experiment was performed in a universal citrate/phosphate/borate buffer (Östling and Virtama, 1946) using 20 nM of each switch (20%, 60% and 100% TAT). The initial pH of the buffer solution (pH = 5, left and pH= 11.5, right) was gradually changed by adding small aliquots of NaOH and HCl respectively.

IX. Drift of the fluorescence signal.

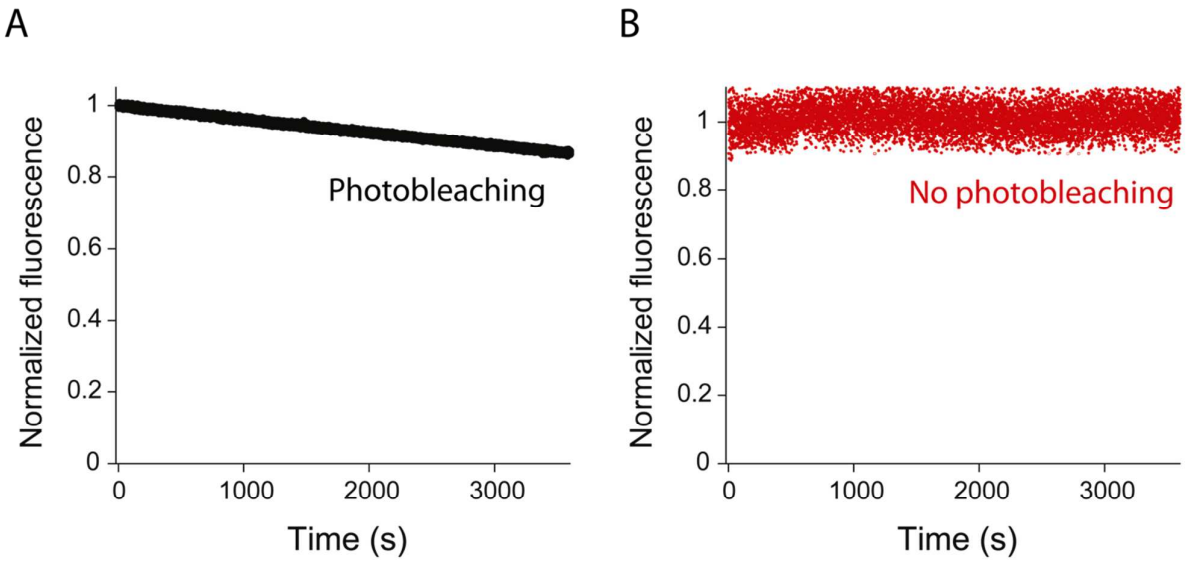

Figure S14. The fluorophore used in our triplex pH-triggered nanoswitches is subject to photobleaching under the experimental conditions we have used in this work. **Left**: by continuously measuring the pH nanoswitches and using a excitation bandwidth (5 nm) and an acquisition time of 0.2 sec we observe a drift of the signal of about 15% after about 30 minutes. Right: the same continuous measurement using a smalled excitation bandwidth (0.2 nm) and an acquisition time of 0.4 sec showed no drift of signal after 30 minutes. The experiments shown in this figure were performed using the reference switch used in figure S1 in a buffered solution at pH 7.0.