Förster Resonance Energy Transfer to impart Signal-on and -off capabilities in a single microRNA biosensor

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KEY WORDS:

Toe-hold, Strand displacement, Polyethylene glycol spacers, FRET pairs, Sensitivity, Limit of detection

Section S1 Sequence Information:

Name	Sequences 5' to 3'
IAbRQ|Cy5 Pair 0 Spacers	/5Cy5/CGATGCCTGTTCT+TGATTACT+TTCGGACATC+G/3IAbRQSp/
IAbRQ|Cy5 Pair 18 Spacers	/5Cy5//iSp18/CGATGCCTGTTCT+TGATTACT+TTCGGACATC+G/iSp18//3IAbRQSp/
Cy3|Cy5 Pair 0 Spacers	/5Cy5/CGATGCCTGTTCT+TGATTACT+TTCGGACATC+G/3Cy3Sp/
Cy3|Cy5 Pair 18 Spacers	/5Cy5//iSp18/CGATGCCTGTTCT+TGATTACT+TTCGGACATC+G/iSp18//3Cy3Sp/
6-FAM|ATTO 633 Pair 0 Spacers	/5ATTO633N/CGATGCCTGTTCT+TGATTACT+TTCGGACATC+G/3FAM/
6-FAM|ATTO 633 Pair 18 Spacers	/5ATTO633N//iSp18/CGATGCCTGTTCT+TGATTACT+TTCGGACATC+G/iSp18//3FAM/
Probe mmu-miR26a-2-3p	GAAACAAGTAATCAAGAACAGG
mmu-miR26a-2-3p (as DNA)	CCTGTTCTTGATTACTTGTTTC

Table S1. List of DNA sequences and modifications. The + symbols indicate locations of Locked Nucleic Acids (LNAs™). The location for a hexaethylene glycol spacer is indicated by iSp18. '5ATTO633N' is a ATTO 633 dye

on the 5-prime end, ‘5Cy5’ is a Cy5 dye on the 5-prime end, ’3FAM’ is a 6-FAM (6-carboxyfluorescein) dye on the 3-prime end, ‘3Cy3’ is a Cy3 dye on the 3-prime end, and ‘3IAbRQSp’ is an Iowa Black Red Quencher on the 3-prime end. Since DNA is more stable it was used instead of actual microRNA. ‘0’ or ‘18’ refers to the type of spacers on the 5-prime and 3-prime ends of the DNA sequences.

Section S2 Signal to Background Analysis:

	Excite 740		Excite 935/945 nm	
	520/565 nm Emission Reporter ± STDEV (RSD %) Reporter+Probe ± STDEV (RSD %)	660/670 nm Emission Reporter ± STDEV (RSD %) Reporter+Probe ± STDEV (RSD %)	520/565 nm Emission Reporter ± STDEV (RSD %) Reporter+Probe ± STDEV (RSD %)	660/670 nm Emission Reporter ± STDEV (RSD %) Reporter+Probe ± STDEV (RSD %)
miR-26a-R IAbRQ|Cy5 Pair 0 Spacers	N/A	597988.79 ± 8699.04 (1.45 %) 35277788.44 ± 289225.84 (0.82 %)	N/A	338.85 ± 255.45 (75.39) 10830.22 ± 522.32 (4.82 %)
miR-26a-R IAbRQ|Cy5 Pair 18 Spacers	N/A	2223216.33 ± 24899.85 (1.12 %) 35400552.56 ± 341280.62 (0.96 %)	N/A	794.07 ± 359.09 (45.22 %) 11207.44 ± 366.93 (3.27 %)
miR-26a-R Cy3|Cy5 Pair 0 Spacers	16372.74 ± 1792.66 (10.95 %) 116421.63 ± 4764.45 (4.09 %)	13469963.18 ± 111434.72 (0.83 %) 36917823.41 ± 294813.20 (0.80 %)	5816.67 ± 381.15 (6.55 %) 69513.33 ± 1256.26 (1.81 %)	35480.48 ± 773.32 (2.18 %) 30513.00 ± 470.84 (1.54 %)
miR-26a-R Cy3|Cy5 Pair 18 Spacers	39933.65 ± 2110.714 (5.29 %) 102277.44 ± 4476.76 (4.38 %)	21408227.80 ± 1230160.78 (5.75 %) 37568744.16 ± 1048953.21 (2.79 %)	14635.63 ± 623.94 (4.26 %) 59778.93 ± 1099.95 (1.84 %)	46985.67 ± 695.71 (1.48 %) 31926.04 ± 850.49 (2.66 %)
miR-26a-R 6-FAM|ATTO 633 Pair 0 Spacers	1398.93 ± 391.42 (27.98 %) 14554.89 ± 907.26 (6.23 %)	2793295.33 ± 120614.49 (4.32 %) 8311403.17 ± 281857.10 (3.39 %)	5313.30 ± 468.76 (8.82 %) 83554.57 ± 2948.90 (3.53 %)	19262.07 ± 988.59 (5.13 %) 15439.30 ± 613.60 (3.97 %)
miR-26a-R 6-FAM|ATTO 633 Pair 18 Spacers	3029.96 ± 483.43 (15.96 %) 24072.41 ± 1290.44 (5.36 %)	6399413.57 ± 325245.31 (5.08 %) 8581432.90 ± 310469.40 (3.62 %)	14452.78 ± 1557.929 (10.78 %) 144773.14 ± 2615.30 (1.81 %)	59554.48 ± 2728.60 (4.58 %) 24554.81 ± 3038.29 (12.37 %)

Table S2. Averaged summed intensities for reporters and reporter+probes. Average was from three trials with three frames each (N = 9).

	740 nm Excitation	**945 nm Excitation**
Reporter Type	**670 nm Emission % ΔS_Q ± SD (RSD %)**	**670 nm Emission % ΔS_Q ± SD (RSD %)**
IAbRQ|Cy5 Pair 0 Spacers	5800.14 ± 68.71 (1.18 %)	3313.04 ± 1041.62 (31.44 %)
IAbRQ|Cy5 Pair 18 Spacers	1492.60 ± 34.44 (2.31 %)	1326.94 ± 176.22 (13.28 %)

Table S3. Percent change in signal for quenching, ΔS_Q, (Equation 1) from the IAbRQ|Cy5 reporters. Standard deviation and percent relative standard deviation (RSD %) are shown with average percent change in signal (N = 3). Excitation was at 740 and 945 nm. Only the 670 peak emission wavelength range was used for the Cy5 dye.

The percent change in signal for quenching of a Cy5 dye on reporters with IAbRQ|Cy5 as a quencher-dye pair is presented in Table S3. Representative emission spectra of the IAbRQ|Cy5 reporters

are in the Figure S1. The purpose of a reporter that used a quencher-dye pair was to compare the percent change in signal and types of detection limits (next section) from a purely quenching sensor to a sensor not designed for pure quenching (Cy3|Cy5). Since the Cy3 on the Cy3|Cy5 reporter was excited at 945 nm for FRET enhancement of Cy5, the IAbRQ|Cy5 reporter was also excited at 945 nm to compare how Cy5 behaved in a purely quenching mode.

At 740 nm excitation, addition of 18 spacers caused a significant decrease ($p < 0.05$) in quenching of Cy5 (670 nm emission) by a factor of ~ 4 compared to the 0 spacers. At 945 nm excitation, there was no significant ($p < 0.05$) difference in the percent change in signal for quenching between reporters with 0 and 18 spacers. The statistical similarity in the percent change in signal at 945 nm excitation was due to the poor excitation of Cy5 and subsequent weak emission, as well as poor signal to noise ratio (S/N). Figure S1 shows the difference in S/N for the IAbRQ|Cy5 reporters with 0 and 18 spacers excited at 740 and 945 nm.

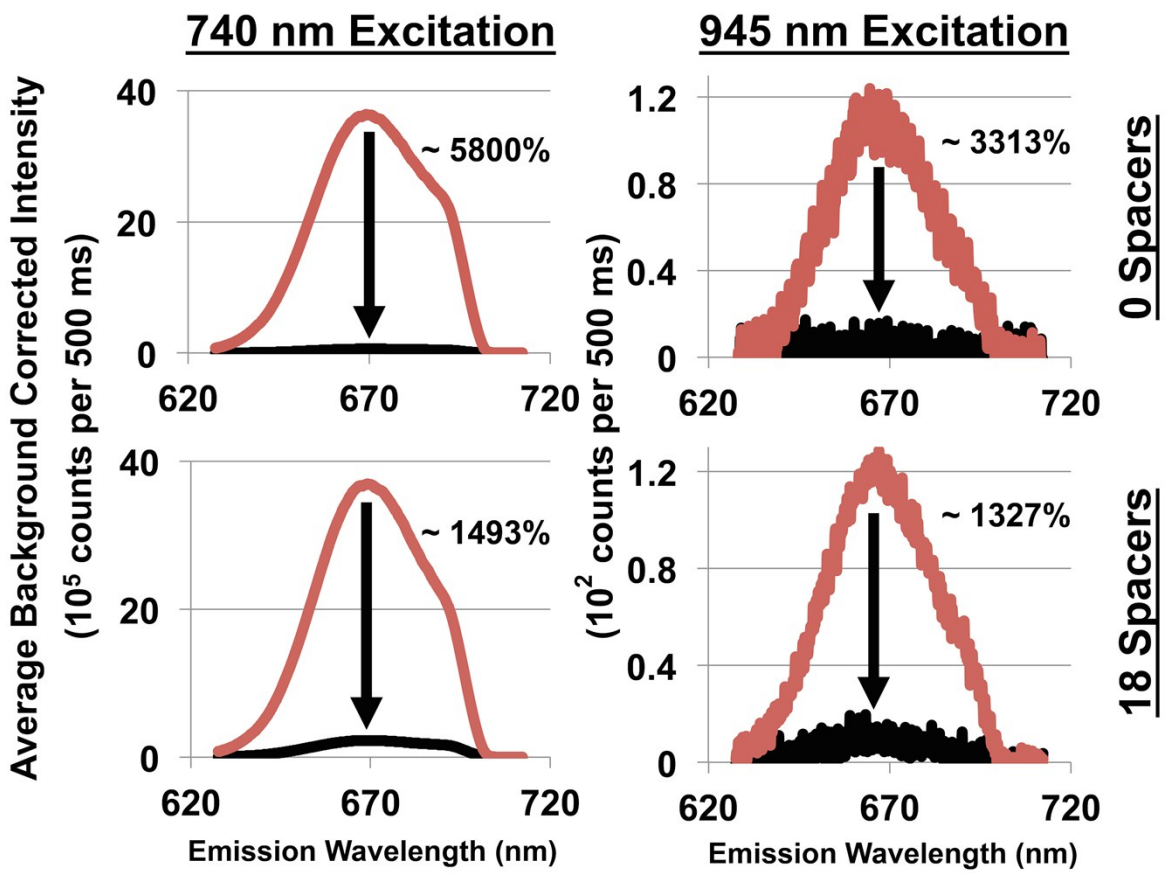

Figure S1. Emission Spectra of 100 nM IAbRQ|Cy5 reporters with and without probe excited at 740 (left) and 945 nm (Right). Top: 0 and Bottom: 18 spacers. Emission collected with the grating centered at 670 nm for the Cy5 dye. Black emission corresponds to reporter-hairpin, and red for reporter+probe. Arrows show direction of signal change from the reporter+probe to the reporter-hairpin. Percent signal quenching is estimated from data summarized in Table 1 using Eqn 1.

	740 nm Excitation		945 nm Excitation		
Reporter Type	565 nm Emission % ΔS_Q ± SD (RSD %)	670 nm Emission % ΔS_Q ± SD (RSD %)	565 nm Emission % ΔS_Q ± SD (RSD %)	670 nm Emission % ΔS_Q ± SD (RSD %)	670 nm Emission % ΔS_E ± SD (RSD %)
Cy3|Cy5 Pair 0 Spacers	616.28 ± 73.97 (12.00 %)	174.10 ± 4.22 (2.42 %)	1098.22 ± 84.59 (7.70 %)	-13.97 ± 2.28 (16.32 %)	16.29 ± 3.07 (18.85 %)
Cy3|Cy5 Pair 18 Spacers	156.47 ± 19.51 (12.47 %)	76.28 ± 17.90 (23.47 %)	308.89 ± 17.42 (5.64 %)	-32.03 ± 2.80 (8.74 %)	47.28 ± 5.93 (12.54 %)

Table S4. Percent change in signal for quenching, ΔS_Q, (Equation 1) and percent change in signal for enhancement, ΔS_E, (Equation 2) from the Cy3|Cy5 reporters. Standard deviation and percent relative standard deviation (RSD %) are shown with average percent change in signal (N = 3). Emission centers at 565 and 670 nm were used for Cy3 and Cy5, respectively. Both dyes were excited at 740 or 945 nm.

Next we investigated the effect of spacers on the quenching and enhancement ability of reporters with the Cy3|Cy5 dye pair used in previous studies.[32,34] The values in Table S4 indicate that addition of 18 spacers influenced the Cy3|Cy5 dye pair's percent change in signal at 740 and 945 nm excitation. When excited at 740 nm, both Cy3 (565 nm emission) and Cy5 (670 nm emission) showed a reduction in quenching ($p < 0.05$) by a factor of ~ 4 and ~2, respectively, after 18 spacers were added. At 945 nm excitation, the extent that Cy3 was quenched decreased ($p < 0.05$) by a factor of ~ 3.6 upon addition of 18 spacers. However, the Cy5 showed a statistically significant ($p<0.05$) signal enhancement by a factor of ~ 3 when 18 spacers were added.

	740 nm excitation		935 nm excitation		
Reporter Type	**520 nm Emission % ΔS_Q ± SD (RSD %)**	**660 nm Emission % ΔS_Q ± SD (RSD %)**	**520 nm Emission % ΔS_Q ± SD (RSD %)**	**660 nm Emission % ΔS_Q ± SD (RSD %)**	**660 nm Emission % ΔS_E ± SD (RSD %)**
6-FAM|ATTO 633 Pair 0 Spacers	947.42 ± 76.14 (8.04 %)	197.66 ± 3.29 (1.66 %)	1479.93 ± 151.06 (10.21 %)	-19.79 ± 2.17 (10.97 %)	24.73 ± 3.38 (13.67 %)
6-FAM|ATTO 633 Pair 18 Spacers	702.29 ± 96.28 (13.71 %)	34.19 ± 2.39 (6.99 %)	912.09 ± 129.68 (14.22 %)	-58.89 ± 3.52 (5.98 %)	144.38 ± 20.33 (14.08 %)

Table S5. Percent change in signal for quenching, ΔS_Q, (Equation 1) and percent change in signal for enhancement, ΔS_E, (Equation 2) from the 6-FAM|ATTO 633 reporters. Standard deviation and percent relative standard deviation (RSD %) are shown with average percent change in signal (N = 3). Emission centers at 520 and 660 nm were used for 6-FAM and ATTO 633, respectively. Both dyes were excited at either 740 or 935 nm.

From unpublished work screening various potential FRET pairs for two-photon imaging, we found the 6-FAM|ATTO 633 pair exhibited FRET enhancement when the 6-FAM was excited at 935 nm. Here reporters with 18 spacers excited at 935 nm showed a statistically significant ($p < 0.05$) increase in the percent enhancement of ATTO 633 (660 nm emission) by a factor of ~ 6 compared to reporters without spacers. When the 6-FAM was excited at 935 nm it showed a statistical ($p < 0.05$) decrease in the quenching of 6-FAM (520 nm emission) by a factor of ~ 1.6 when 18 spacers were added. Excitation of both the 6-FAM and ATTO 633 at 740 nm demonstrated reductions ($p < 0.05$) in the percent change in signal for quenching of the 6-FAM|ATTO 633 reporters with 18 spacers compared to those reporters without spacers.

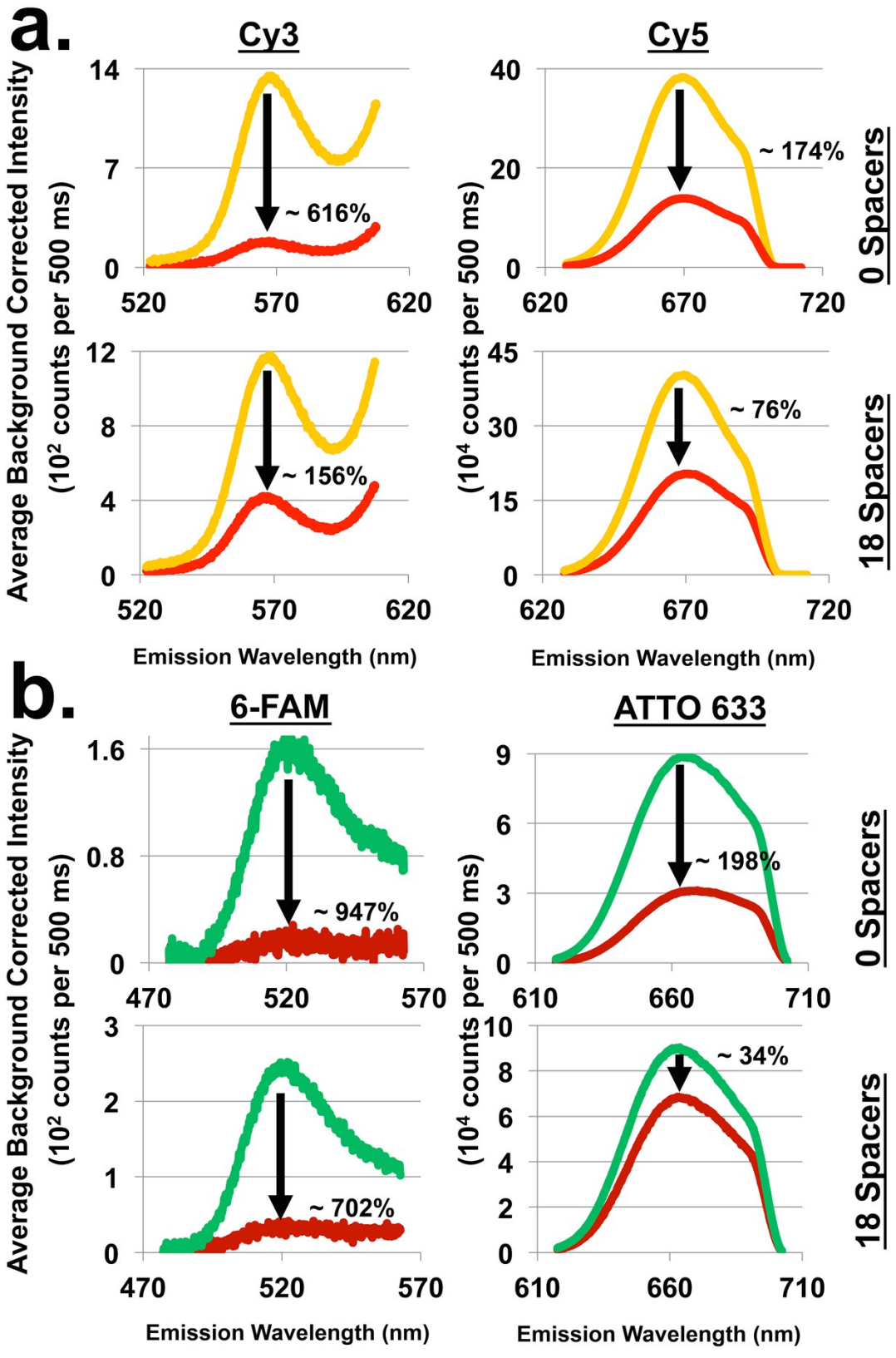

Figure S2. Donor and acceptor emission spectra from 100 nM reporters with and without probe when excited at 740 nm. (a.) Cy3|Cy5 reporters with 0 or 18 spacers (top and bottom). The red and yellow lines correspond to reporter-

hairpin and reporter+probe, respectively. (b.) 6-FAM|ATTO 633 reporters with 0 and 18 spacers (top and bottom). The maroon and green lines correspond to reporter-hairpin and reporter+probe, respectively. Arrows show direction of signal change from the reporter+probe to the reporter-hairpin. Percent signal quenching is estimated from data summarized in Tables 2 and 3 using Eqn 1.

Section S3 Detection Limits:

There was no statistical difference in the 5 nM LODs for signal-off when excited at 945 nm and there was no statistical difference in 3 to 7 nM LODs for signal-off when excited at 740 nm. To test for a difference in the signal-off LODs based on excitation wavelength, the LODs at 945 nm were averaged and compared to the average LODs from 740 nm excitation. From this comparison there was no statistical difference ($p < 0.05$) observed from signal-off LOD based on excitation wavelength.

To see if there was a statistical difference between the signal-on (18 spacers only) and signal-off reporters (0 and 18 spacers), a similar approach as described above was used. In this case there was no statistical difference ($p < 0.05$) between signal-on and –off LODs. Even comparison of just the Cy5 signal-off to signal-on from the reporters with 18 spacers did not show a statistical difference in LOD. In general, neither the use of spacers, excitation wavelength, nor direction of signal change influenced LODs for the Cy3|Cy5 reporters.

The same approach to compare the influence of spacers on LOD was applied to compare the sensitivity (nM^{-1}) of the Cy3 and Cy5 dyes. This analysis revealed statistical differences ($p < 0.05$) in sensitivity after the spacers were added to the reporters. For Cy5 signal-on, the sensitivity increased by more than a factor of three due to adding spacers to reporter. In the case of signal-off under 740 and 945 nm excitation, addition of the spacers showed a decrease in sensitivity for both Cy3 and Cy5. Upon comparison of Cy3 to Cy5 for signal-off, the Cy3 reporters with 0 spacers were the most sensitive (both about $– 7.5 \times 10^{-3}$ nM^{-1}, $p < 0.05$) when excited at either wavelength. For Cy3, the addition of spacers had the least effect on sensitivity (reduced by $\sim 1.5 \times 10^{-3}$ nM^{-1}) when excited at 945 nm compared to

excitation at 740 nm (reduced by ~ 2.6 x 10^{-3} nM^{-1}). These results show that reporters with 18 spacers excited at 945 nm will give optimal FOM to measure over- or under-expression of miRNA.

For signal-off there was no statistical difference in the 20 to 30 nM LODs when excited at 740 nm excitation and there was no statistical difference in ~ 13 nM LODs when excited at 935 nm. As before, to test for a difference in signal-off LOD based on excitation wavelength the averaged LODs at each excitation wavelength were compared. From this comparison there was no statistical difference ($p < 0.05$) in signal-off LOD based on excitation wavelength.

There was no statistical difference ($p < 0.05$) in the signal-on LODs upon addition of spacers for the ATTO 633 because the error in the calculated LOD was very large for the reporter with 0 spacers. To test if there was statistical difference between the signal-on and signal-off, a similar analysis as described for Cy3|Cy5 was used. For the 6-FAM|ATTO 633 reporters there was no statistical difference ($p < 0.05$) between signal-on and –off over the 13 to 30 nM range. Much like the Cy3|Cy5 reporters, the general trend was that the 6-FAM|ATTO 633 reporters had LODs that were not influenced by addition of spacers, excitation wavelength, or direction of signal change.

The spacer's influence on the dyes' sensitivities (nM^{-1}) was determined following statistical comparisons previously outlined for LODs. For ATTO 633 signal-on when excited at 935 nm, addition of spacers showed a statistical ($p < 0.05$) increase in the sensitivity by almost a factor of 10. Signal-off sensitivity was only seen to decrease from addition of spacers to reporters for 6-FAM and ATTO 633 when excited at 740 nm. Spacer addition to the 6-FAM when excited at 935 nm did not show a statistical change ($p < 0.05$) in the signal-off sensitivity. Upon comparison of 6-FAM to ATTO 633 for signal-off, the 6-FAM reporters with 0 spacers were the most sensitive and similar at both excitation wavelengths ($p < 0.05$). For 6-FAM on the reporter with 18 spacers excited at 935 nm, the sensitivity was statistically similar ($p < 0.05$) to the reporter that had 0 spacers (~ 6 x 10^{-3} nM^{-1}). These results establish that the reporter can be excited at just 935 nm to observe two colors without a major loss in the analytical FOM.

Compared to the other spectroscopic conditions and 6-FAM|ATTO 633 reporters with 0 spacers, the sensitivity and tens of nanomolar LODs for signal-off were maintained when the 6-FAM|ATTO 633 biosensor with 18 spacers was excited at 935 nm. The 6-FAM|ATTO 633-18 spacer reporters excited at 935 nm was able to leverage suitable sensitivities and LODs for both signal-on and signal-off. Thus a biosensor with the 6-FAM|ATTO 633-18 spacers will have the best chance to achieve good contrast to visualize the over and under expression of miRNA.

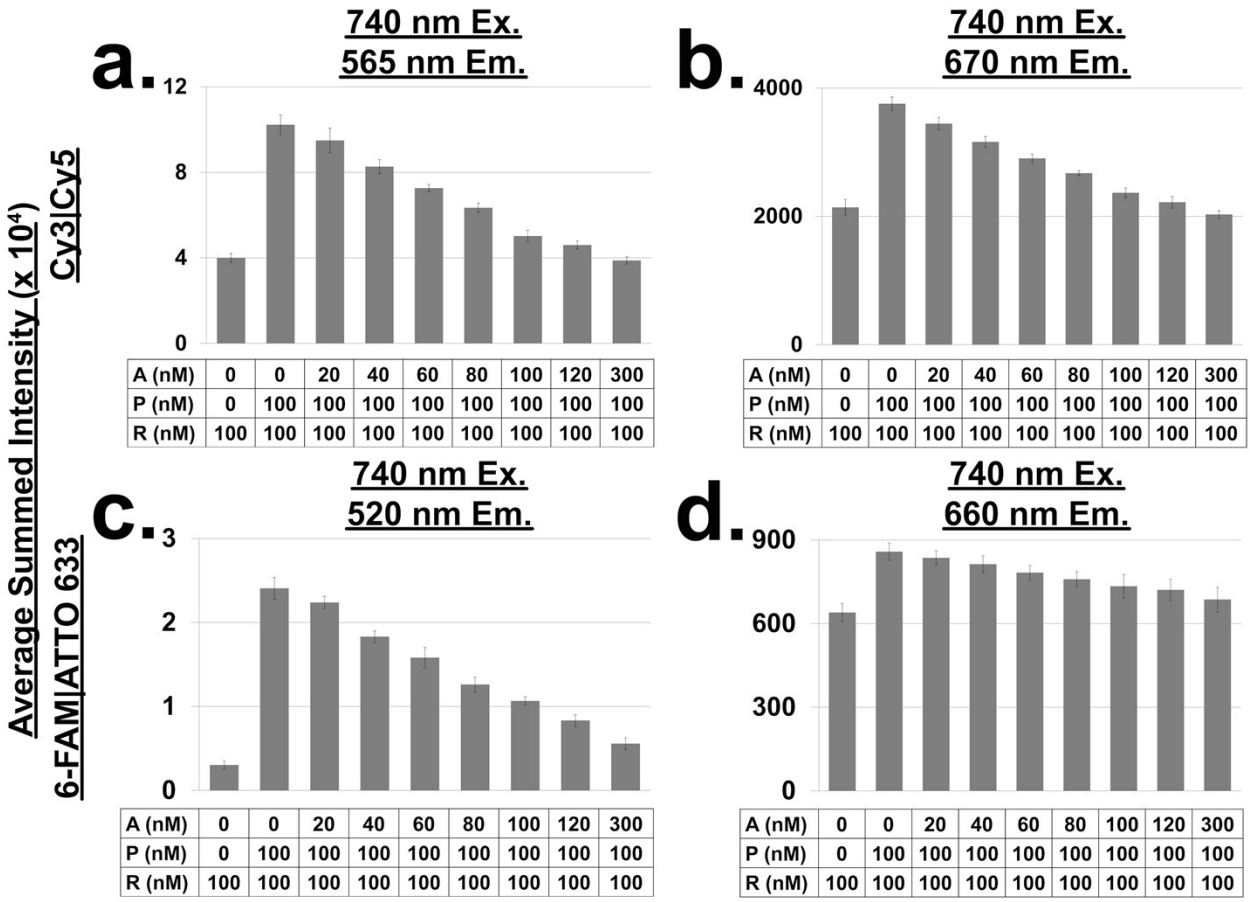

Figure S3. Calibration curves from miRNA analyte additions to 100 nM reporter+probe biosensors with Cy3|Cy5-18 spacers (a-b) and 6-FAM|ATTO 633-18 spacers (c-d). All sensors were excited at 740 nm. (a) Emission collected with the grating centered at 565 nm for the Cy3 dye. (b). Emission collected with the grating centered at 670 nm for the Cy5 dye. (c). Emission collected with the grating centered at 520 nm for the 6-FAM dye. (d). Emission collected with the grating centered at 660 nm for the ATTO 633 dye. N = 9 from three calibration experiments with three frames each collected and averaged.

	740 nm Excitation		935/945 nm Excitation	
Reporter Type	**Average Slope ± RSD (Normalized counts nM^{-1})**	**LOD (nM Analyte) ± STDEV LOQ (nM Analyte) ± STDEV RSD**	**Average Slope ± RSD (Normalized counts nM^{-1})**	**LOD (nM Analyte) ± STDEV LOQ (nM Analyte) ± STDEV RSD**
Cy3|Cy5 Pair 0 Spacer	-4.24E-3 ± 6.10 %	14.25 ± 5.19 47.50 ± 17.31 36.44%	-7.87E-3 ± 1.62 %	10.53 ± 0.53 35.10 ± 1.76 5.01%
Cy3,|Cy5 Pair 18 Spacer	-1.74E-3 ± 27.54 %	19.2 ± 9.99 64.03 ± 33.32 52.04%	-7.32E-3 ± 2.39 %	7.74 ± 2.33 25.81 ± 7.78 30.16%
6-FAM|ATTO 633 Pair 0 Spacer	-2.78E-3 ± 9.51 %	48.22 ± 25.29 160.74 ± 84.30 52.45%	-7.03E-3 ± 1.55 %	23.34 ± 5.81 77.79 ± 19.37 24.90%
6-FAM|ATTO 633 Pair 18 Spacer	-4.82E-3 ± 8.91 %	37.33 ± 9.21 124.43 ± 30.70 24.67%	-9.01E-3 ± 3.72 %	21.08 ± 7.58 70.26 ± 25.26 35.96%

Table S6. Biosensor figures of merit (FOM) from the ratio of 'donor to acceptor' emission centers for both Cy3|Cy5 and 6-FAM|ATTO 633 dye pairs with either 0 or 18 spacers. Cy3|Cy5 reporters excited at 740 and 945 nm, and 6-FAM|ATTO 633 reporters excited at 740 and 935 nm. Data obtained from normalized calibration curves using 'donor to acceptor' ratios over the 0-100 nM miR-26a range in 20 nM steps. N=3 for each FOM.

For each FRET pair on each reporter excited at 740, 935, or 945 nm, the LODs from the D/A ratio did not show statistical differences ($p < 0.05$) based on number of spacers. In general, when excited at 740 nm the ~17 nM LODs from Cy3|Cy5 reporters were lower than the ~ 37 nM LODs from the 6-FAM|ATTO 633 analogs ($p < 0.05$). Reporters excited at 935 or 945 nm, showed a general trend of LODs near 8 nM for Cy3|Cy5 reporters and larger LODs near 23 nM from the 6-FAM|ATTO 633 reporters ($p < 0.05$). When 6-FAM|ATTO 633-18 spacers were excited at 935 nm instead of 740 nm, the LOD was lowered by ~ 20 nM ($p < 0.05$). All the other reporters did not show any difference in LOD based on the wavelength that excited the reporter.

The excitation wavelength did not influence how precise the LOD measurements were at a given spacer length except for the Cy3|Cy5-0 spacers that was more precise when excited at 945 nm than when excited at 740 nm. There was no difference in precision based on spacer lengths for either the Cy3|Cy5 or the 6-FAM|ATTO 633 reporters except for the Cy3|Cy5-0 spacers excited at 945 nm. In the case of 945 nm excitation, the Cy3|Cy5-0 spacers were more precise than the Cy3|Cy5-18 spacers. At 935/945 nm excitation, only the Cy3|Cy5-0 spacers had precisions that were statistically smaller than each of the other reporters. The 6-FAM|ATTO 633-0 spacers excited at 740 nm were not very precise with a standard deviation of ~ 25 nM (50 % RSD). However, only the reporter with Cy3|Cy5-0 spacers was statistically more precise than the 6-FAM|ATTO 633-0 spacers. All other reporters were equally as precise with standard deviations in the 2 to 25 nM range when excited at 740, 935, and 945 nm. The small sample size was the expected reason why the 6-FAM|ATTO 633-0 spacers' 25 nM precision was not statistically different from the 2 to 10 nM precisions of the other reporters. Recall that much of the precision was dependent on the sample preparation rather than instrumental error. Since a majority of the precision was equal among the reporters, we suspect that the instances of improved precision were just instances of good sample preparation.

Regardless of the wavelength to excite the reporters the following trend in sensitivity was observed: Cy3|Cy5-0 spacers were more sensitive than 18 spacers and 6-FAM|ATTO 633-18 spacers were more sensitive than the 0 spacers. The reporters demonstrated the greatest sensitivity when excited at either 935 or 945 nm, depending on the donor dye excited. At 935 nm excitation, the 6-FAM|ATTO 633-18 spacers was the most sensitive of all the reporters (~ 9×10^{-3} nM^{-1}, $p < 0.05$). The remainder of reporters decreased in sensitivity in the order: Cy3|Cy5-0 spacers, Cy3|Cy5-18 spacers, and 6-FAM|ATTO 633-0 spacers (excited at 935 or 945 nm). At 740 nm excitation, the sensitivity of the Cy3|Cy5-0 spacers and the 6-FAM|ATTO 633-18 spacers were statistically similar (~ 4×10^{-3} nM^{-1}). The 6-FAM|ATTO 633-0 spacers were the next most sensitive followed by the Cy3|Cy5-18 spacers (740 nm excitation). Excitation at 935 and 945 nm was statistically more sensitive than the corresponding 740 nm

excitation for each type of reporter. However, the precision was not statistically influenced by the excitation wavelength.

Section S4 MicroRNA-induced reporter+probe displacement efficiency:

	miRNA added (nM)	Reporter+Probe remaining (nM)	Reporter-hairpin formed (nM)	Reporter-hairpin + Reporter+Probe (nM)
Trial 1	100	35.20 ± 8.44	66.50 ± 2.80	101.69 ± 8.90
	300	11.25 ± 15.71	90.84 ± 4.86	102.10 ± 16.44
Trial 2	100	36.46 ± 10.71	67.14 ± 4.54	103.60 ± 11.63
	300	10.82 ± 15.38	94.16 ± 6.03	104.98 ± 16.52
Trial 3	100	33.21 ± 8.58	70.11 ± 2.62	103.32 ± 8.98
	300	9.71 ± 8.92	94.06 ± 2.95	103.77 ± 9.39

Table S7. Experimentally determined molar concentrations of reporter-hairpin and reporter+probe upon addition of either 100 or 300 nM miR-26a analyte. Calculations performed by comparison of 6-FAM|ATTO 633 dyes for 100 nM reporter-hairpin and reporter+probe. N =3.

Section S5 reporter+probe formation analysis:

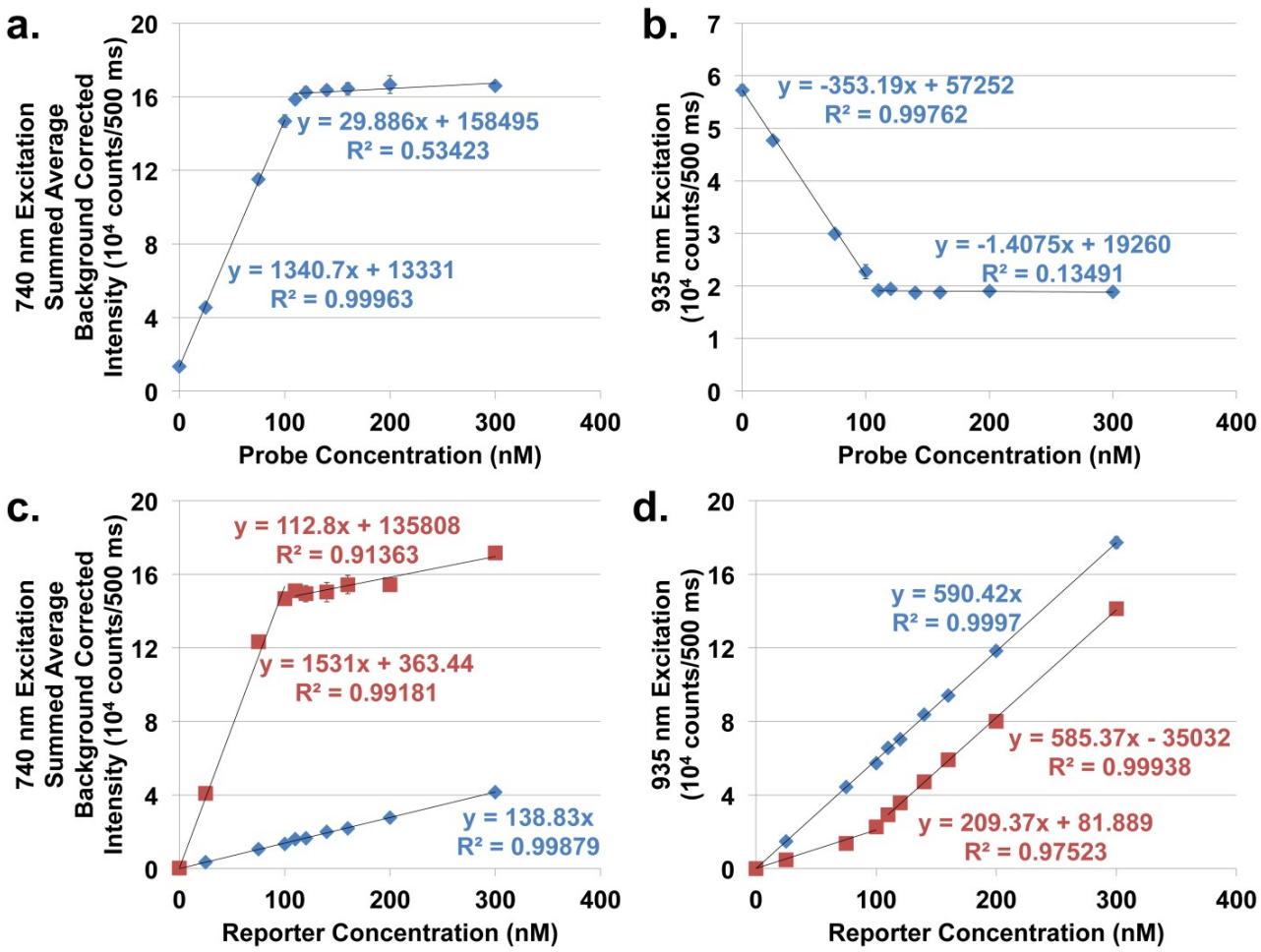

Figure S4. Extent of reporter+probe formation by analysis of changes in reporter 6-FAM|ATTO 633-18 spacers emission. Emission was taken from the ATTO 633 signal (660 nm emission center). Increasing concentration of probe to a constant 100 nM solution of reporter excited at 740 nm (a) and 935 nm (b). Increasing concentration of reporter to a constant 100 nM solution of probe excited at 740 nm (c) and 935 nm (d). Lines were fit from 0-100 nM and 110-300 nM. Additional controls for the reporter-hairpin in the absence of probe for (c) and (d) were fit from 0-300 nM reporter. Analysis of the changes in the signals slope aided in determination of the extent of reporter+probe formation.

In Figure S4a (740 nm excitation) and S4b (935 nm excitation), excess probe was added to saturate the reporter and cause the signal to plateau to a static-state. The line of fit for the dynamic and static signals were extrapolated to estimate the amount of reporter+probe formed. For 100 nM reporter the signal was expected to stabilize at 100 nM probe. The extrapolation analysis revealed the signal stopped changing at about 110 nM probe addition. This result suggested that the reporter concentration before probe addition was closer to 110 nM.

Next to further validate the extent of reporter+probe formed, the amount of reporter was increased with respect to a constant probe concentration (Figures S4c and S4d). After the reporter and probe was fully complexed, addition of more reporter was expected to cause the ATTO 633 signal to increase but at a different slope. The reason for a different slope is due to the dependence of the ATTO 633 signal intensity on the FRET distance from the 6-FAM dye. The FRET distance between the dyes was controlled by the absence or presence of probe. To determine the slope of the ATTO 633 signal from uncomplexed reporter-hairpin, a control experiment was performed where the reporter concentration was increased in the absence of probe. The results in Figures S4c (740 nm excitation) and S4d (935 nm excitation) show how the ATTO 633 signal changed slope as the reporter concentration was increased. The red data were for reporter additions to a constant probe concentration. The blue data were the control reporter-hairpin additions in the absence of probe.

The slope of the reporter-hairpin control was compared to the slope after all the probe was complexed. This approach helped guide which data points to fit in order to find the slopes before and after all the reporter and the probe was complexed. Finally, the data was interpreted by the intersection of the extrapolated slopes from reporter signal before and after all the probe was complexed. The amount of reporter that corresponded to where the two slopes intersected was taken as the amount of probe in solution.

Based on the slope extrapolation method there was about 100 nM probe in solution. The reporter-hairpin control slopes when excited at 740 and 935 nm were within 22 and 1 %, respectively, of the resultant slopes after all the reporter and probe was complexed. This confirmed that excess reporter-hairpin was in the solution with complexed reporter+probe. The discrepancy in slopes when excited at 740 nm was due to the weak signal from the quenched state of ATTO 633. The results from these titrations validated that the amount of reporter+probe formed was ~ 100 nM with an excess of about 10 nM reporter.