Supporting Information for

High-Fidelity Single Molecule Quantification in a Flow Cytometer Using Multiparametric Optical Analysis

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1. Supporting Figures

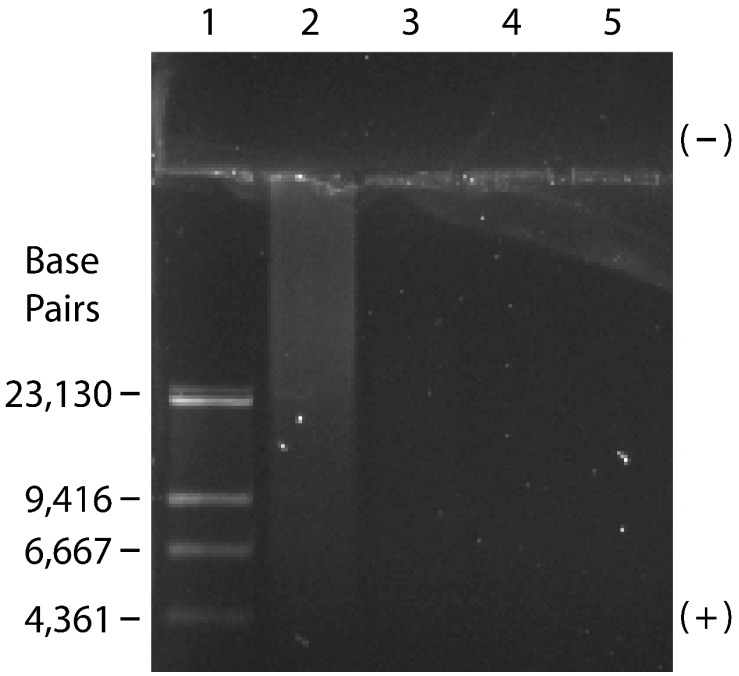

Figure S1. Agarose gel electrophoresis of microRNA amplicons after rolling circle amplification. Lanes contain: (1) molecular weight ladder or (2-5) RCA reactions containing miR-375 at the following concentrations: (2) 1,000 pM, (3) 100 pM, (4) 10 pM, and (5) 0 pM.

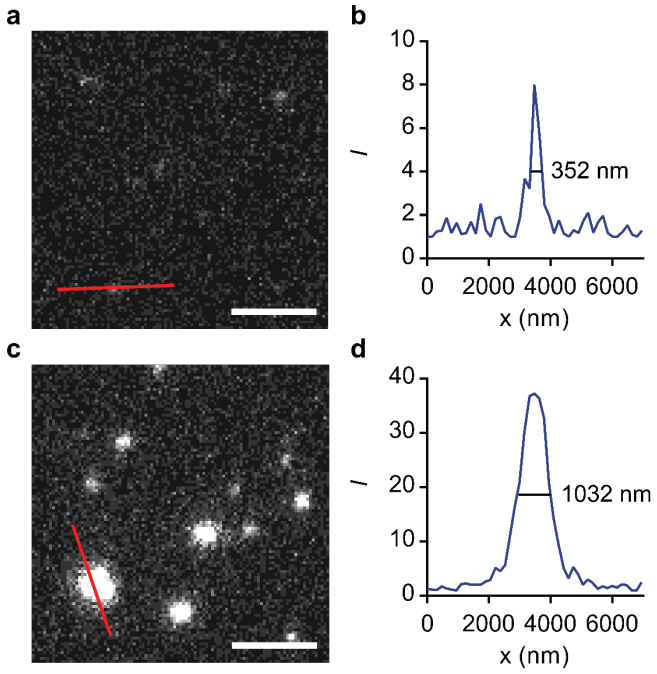

Figure S2. Single molecule fluorescence imaging of labeled microRNA amplicons. (a,c) Fluorescence micrographs of dilute (a) dye-DNA and (c) labeled miR amplicons, each captured on glass coverslips through DNA-directed immobilization. Scale bars represent 5 μm. **(b,d)** Fluorescence intensity line scans corresponding to red lines in panels (a) and (c).

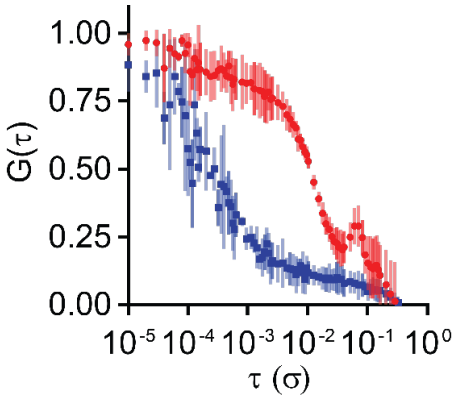

Figure S3. Fluorescence correlation spectroscopy (FCS) of fluorescent labels and labeled microRNA amplicons. FCS autocorrelation curves show dye-DNA probes alone (blue) and miR amplicons (red) labeled with the same dye-DNA probes. Error bars indicate standard deviation for 3 replicates.

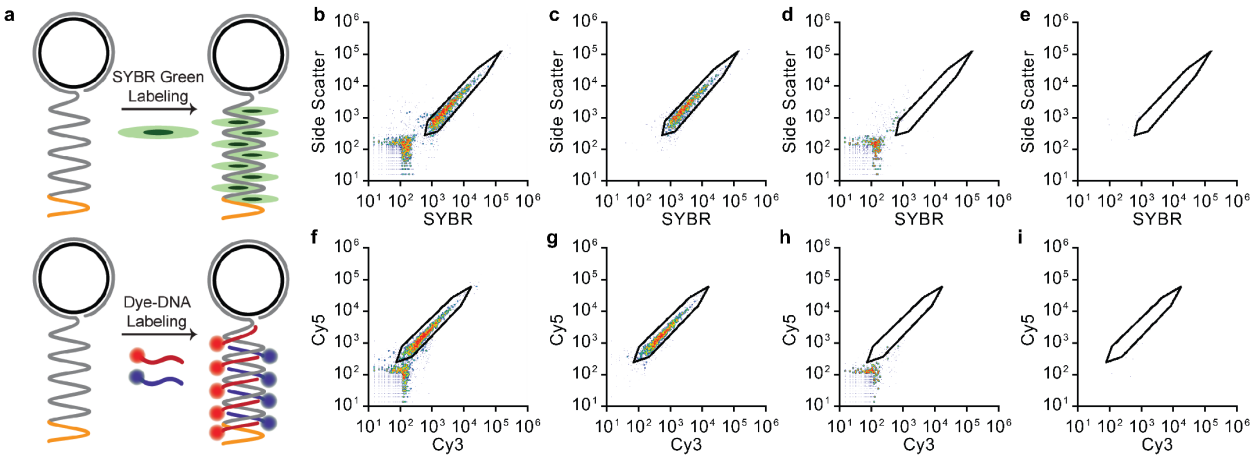

Figure S4. Flow cytometry gating of fluorescently labeled microRNA amplicons eliminates spurious off-target events. (a) Schematic shows labeling with SYBR Green intercalating dye and dye-DNA hybridization probes. **(b)** Scatter plot of side scattering and SYBR intensity for labeled amplicons from 1 nM miR-375 solutions. Black lines indicate gate to designate events corresponding to miR amplicons. **(c)** Plot shows events and gate from (b) with events eliminated outside the Cy3 and Cy5 gate (see panel f). Note that few events remain outside of the scatter-SYBR gate. **(d)** Scatter plot of side scattering and SYBR intensity for labeled amplicons from 0 nM miR-375 solutions. Black lines indicate the same gate region generated in panel (b). **(e)** Data show events and gate from (d) with events eliminated based on Cy3-Cy5 gating (see panel h). Note that few events are detected in this negative control. (**f)** Scatter plot of Cy3 and Cy5 intensity for labeled amplicons from 1 nM miR-375 solutions. Black lines indicate gate to designate events corresponding to miR amplicons. **(g)** Data show events and gate from (f) with events eliminated based on side scatter and SYBR gating (see panel b). Note that few events remain outside of the Cy3-Cy5 gate. **(h)** Scatter plot of Cy3 and Cy5 intensity for labeled amplicons from 0 nM miR-375 solutions. **(i)** Plot shows events from (h) with points eliminated based on side scatter and SYBR gate (see panel d). Note that few points are detected in this negative control.

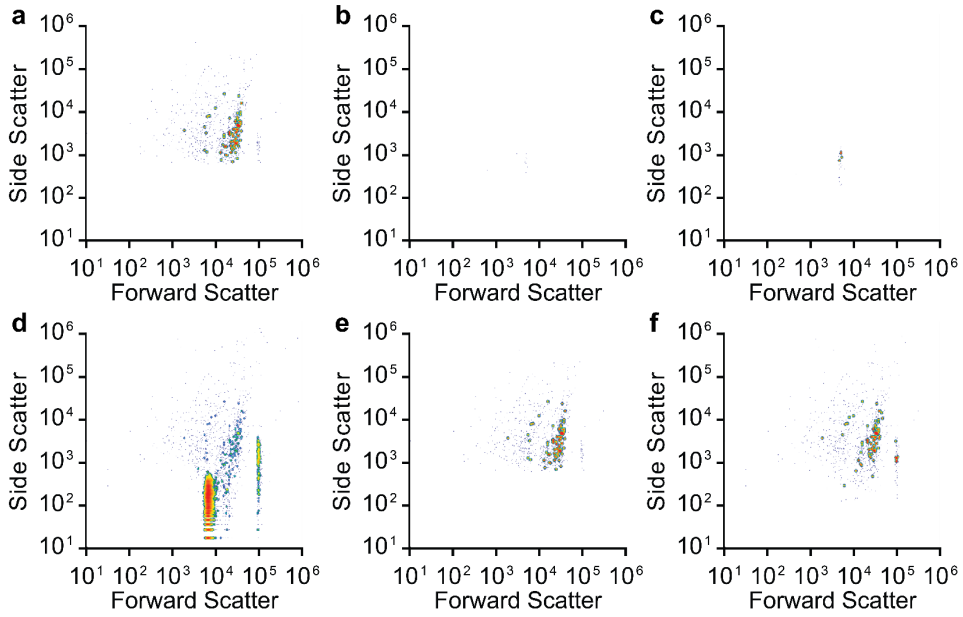

Figure S5. Side and forward scattering signal for labeled microRNA products of rolling circle amplification (RCA). (a) Side scattering and forward scattering measured for products of RCA reactions with 0 nM miR. **(b)** Same as (a) but with only events present within the side scattering and SYBR gate. **(c)** Same as (a) but with only events present within the Cy3 and Cy5 gate. **(d)** Side scattering and forward scattering for RCA reactions with 1 nM miR present. **(e)** Same as (d) but with only present within the side scattering and SYBR gate (same experiment for Figure 3a). (**f)** Same as (d) but with only present within the Cy3 and Cy5 gate (same experiment for Figure 3b).

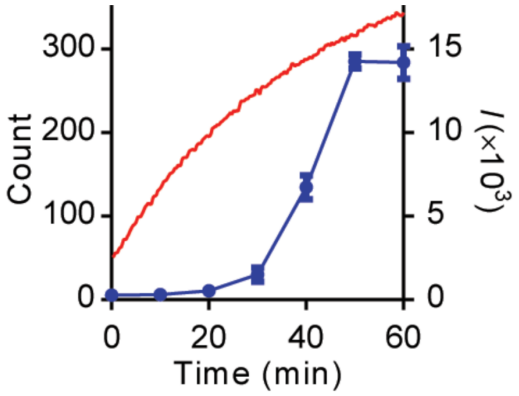

Figure S6. Impact of rolling circle amplification reaction time on microRNA count and fluorescence intensity. Flow cytometry amplicon counts are shown in blue, corresponding to the left y-axis. The ensemble fluorescence intensity from SYBR Gold enhancement resulting from synthesis of ssDNA is shown in red, corresponding to the right y-axis.

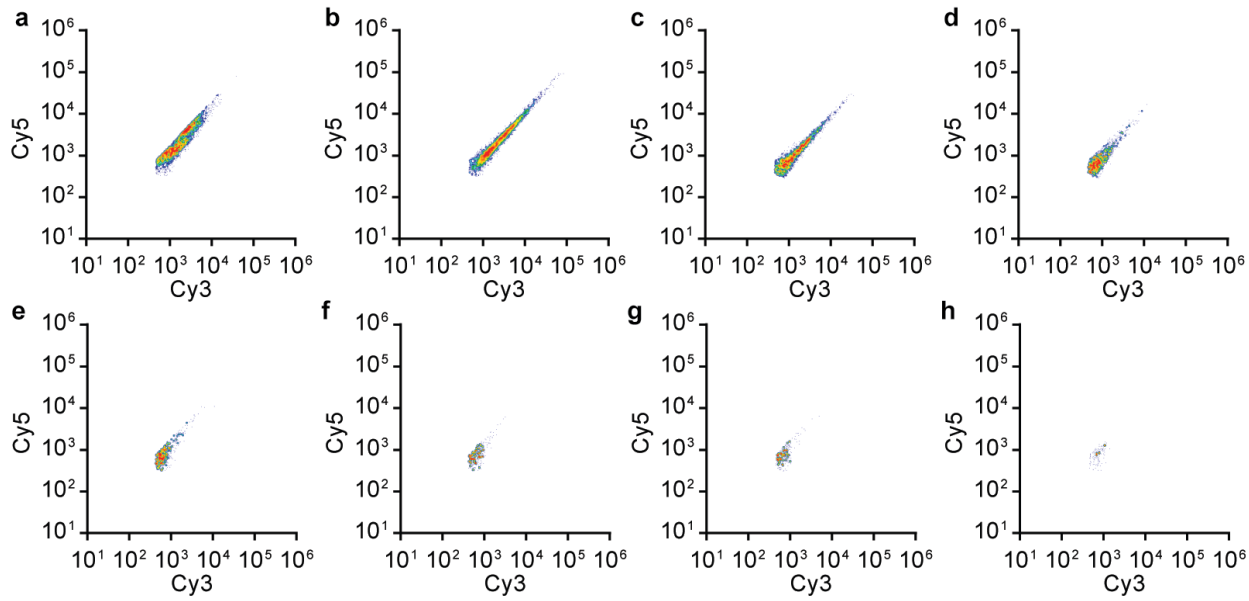

Figure S7. Quantification of microRNA by flow cytometry. Scatter plots show flow cytometry-based fluorescence intensity of events deriving from miR amplicons labeled with Cy3 and Cy5 dye-DNA at the following miR concentrations: **(a)**10,000 pM, **(b)** 1000 pM, **(c)** 100 pM, **(d)** 10 pM, **(e)** 1 pM, **(f)** 0.1 pM, **(g)** 0.01 pM, and **(h)** 0 pM. SYBR Green fluorescence and side scatter were used for gating all data sets.

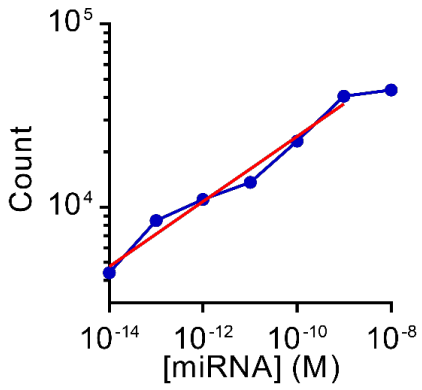

Figure S8. Detection of fluorescently labeled microRNA amplicons in the presence of RNA extract from human plasma in a flow cytometer. Counts are shown at each miR-375 concentration. Error bars represent the standard deviation from 3 independent experiments. The red line represents a power law fit of data points with R^2 of 0.975.

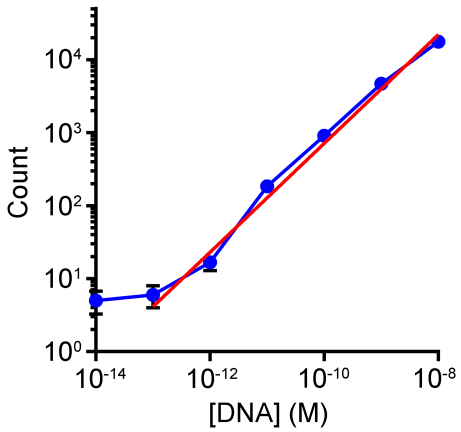

Figure S9. Detection of fluorescently labeled amplicons derived from DNA targets in a flow cytometer. Counts of amplicons are shown at indicated concentrations of DNA in the initial reaction solution. Error bars represent the standard deviation from 3 independent experiments. The red line represents a power law fit of data points with R^2 of 0.992.

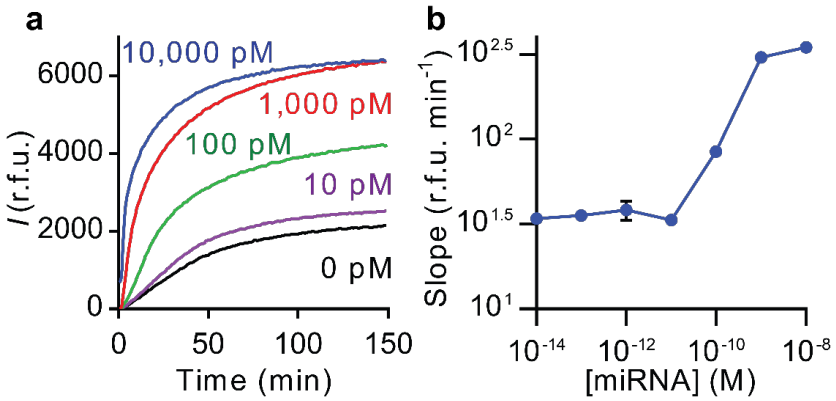

Figure S10. Monitoring of microRNA amplification through ensemble fluorescence intensity. (a) SYBR Gold fluorescence intensity as a function of time during rolling circle amplification with indicated miR concentrations. r.f.u. = relative fluorescence units. **(b)** Dependence of SYBR Gold fluorescence slope on miR concentration during the amplification reactions in (a).

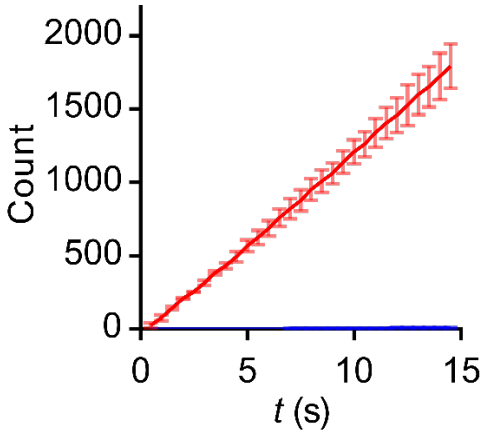

Figure S11. Counts of labeled microRNA amplicons as a function of flow cytometry acquisition time. Red line indicates labeled miR amplicons deriving from 1 nM miR-375 solutions. Blue line shows the product of a reaction containing no miR-375. Data points represent mean counts for 3 replicates acquired at indicated time points.

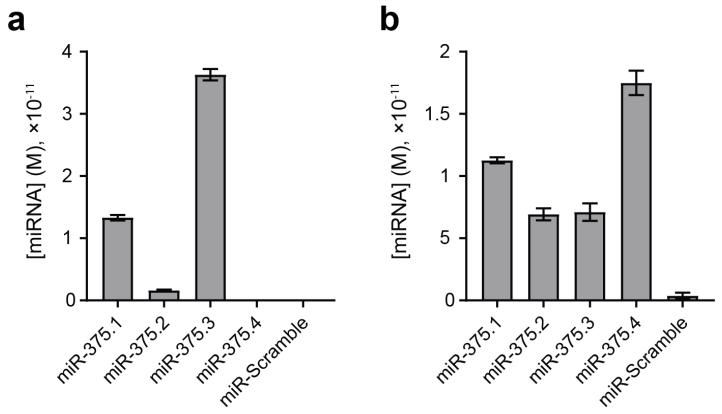

Figure S12. Characterization of sequence specificity of qRT-PCR and RCA for miR-375 isomiRs. The four most prevalent miR-375 isoforms were analyzed at a concentration of 10 pM using (**a**) qRT-PCR or (**b**) RCA. Detected effective concentrations were calculated using a standard curve generated using the most prevalent isoform, miR-375.1. Bar height and error bars represent the mean and standard deviation for 3 independent experiments, respectively. Sequences are provided in Table S1.

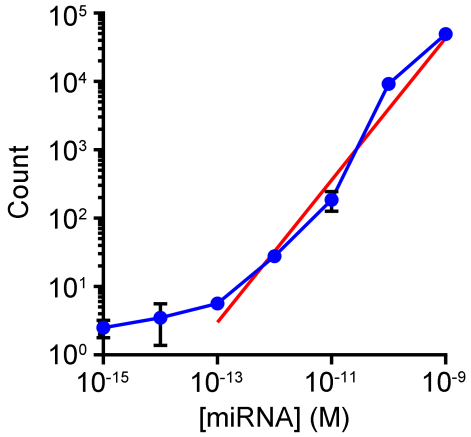

Figure S13. Detection of fluorescently labeled miR amplicons generated from padlock probes using a flow cytometer. Circular DNA templates were prepared using padlock probes circularized through miR-375 binding and ligation with SplintR Ligase. Counts are shown at each miR-375 concentration. Error bars represent the standard deviation from 3 independent experiments. The red line represents a power law fit of data points with R^2 of 0.975.

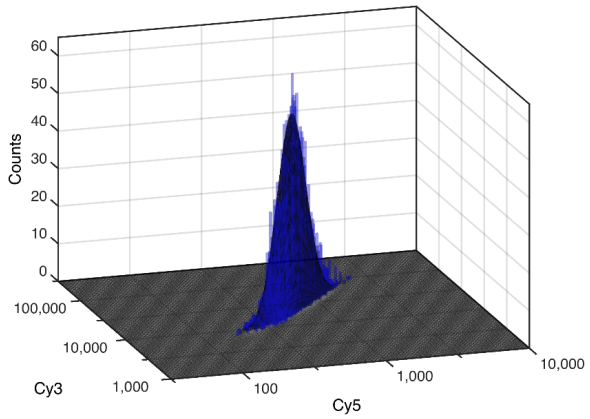

Figure S14. Example fit of binned flow cytometry data from miR amplicons labeled with both Cy3 and Cy5. Blue bar graph shows binned event histogram overlaid on a black two-dimensional elliptical Gaussian function.

2. Supporting Tables

Table S1. Oligonucleotide sequences used for miR amplification and labeling

Name	Sequence
miR-375.1	rUrUrU rGrUrU rCrGrU rUrCrG rGrCrU rCrGrC rGrUrG rA
miR-375.2	rUrUrU rGrUrU rCrGrU rUrCrG rGrCrU rCrGrC rGrUrG
miR-375.3	rUrUrU rGrUrU rCrGrU rUrCrG rGrCrU rCrGrC rGrUrG rU
miR-375.4	rUrUrU rGrUrU rCrGrU rUrCrG rGrCrU rCrGrC rGrU
Scramble miR	rUrGrC rUrArA rGrGrU rCrCrG rUrGrU rCrCrA rUrArU rC
miR-30a-5p	rUrGrU rArArAr CrArUrCrCrU rCrGrA rCrUrG rGrArArG
DNA-375.1	TTT GTT CGT TCG GCT CGC GTG A
miR-375 RCA Template	/5Phos/ CAA CAA CCA ACA AAC ACA GAA TGC TCA CGC GAG CCG AAC GAA CAA ACC TCA GCA ACA CCA AAC AAC AAA C
miR-30a-5p RCA Template	/5Phos/GGA TGT TTA CAC CTC AGC GTT CTA TCT CCG CTC TCA ATT TCG GTT CTA TCT CCG CTC TCA ATT TCG CTT CCA GTC GA
miR-375 Padlock Probe	/5Phos/CGA ACG AAC AAA CCT CAG CAA CAC CAC ACA GAA TGC TTC ACG CGA GC
Amplicon Capture DNA	/5DBCOTEG/ ACT TTA CTT TAC TTT ACT TTA CTT TAC TTT ACT TTA CTT TAC TTT ACT TTA CTT TAC TTT ACT TTA CTC ACG CGA GCC GAA CGA ACA AA
A430-DNA	/5Alex430N/ CCT CAG CAA CAC C
Cy3-DNA	/5Cy3/ CCT CAG CAA CAC C
A594-DNA	/5Alex594N/ CCT CAG CAA CAC C
Cy5-DNA	/5Cy5/ CCT CAG CAA CAC C
Cy5-DNA T2	/5Cy5/ ACA CAG AAT GCT
A700-DNA	/5Alex700N/ CCT CAG CAA CAC C
A430-DNA T3	/5Alex430N/GTT CTA TCT CCG
A594-DNA T4	/5Alex594N/CTC TCA ATT TCG

/5Phos/ indicates 5’ phosphate group modification.
/5DBCOTEG/ indicates 5’-dibenzocyclooctyne (DBCO) modification.
/5Alex430N/, /5Cy3/, /5Alex594N/, /5Cy5/, and /5Alex700N/ indicate 5’ modifications with the dyes Alexa Fluor 430, Cy3, Alexa Fluor 594, Cy5, and Alexa Fluor 700, respectively.

Table S2. Fluorophore pairs used in Figure 6a

Code	Fluorophore Pair
1	Alexa Fluor 430 + Alexa Fluor 594
2	Alexa Fluor 430 + Cy3
3	Alexa Fluor 430 + Cy5
4	Cy5 + Alexa Fluor 700
5	Alexa Fluor 430 + Alexa Fluor 700
6	Alexa Fluor 594 + Alexa Fluor 700
7	Alexa Fluor 594 / Cy5
8	Cy3 + Alexa Fluor 594
9	Cy3 + Alexa Fluor 700
10	Cy3 + Cy5

Table S3. Cy3/Cy5 fluorophore ratios used in Figure 6b

Code	Ratio (Cy3:Cy5)
1	47 : 1
2	16 : 1
3	5 : 1
4	7 : 4
5	4 : 7
6	1 : 5
7	1 : 16
8	1 : 47

Table S4. Flow cytometer lasers and optical filters

Target	Laser Wavelength (nm)	Longpass Dichroic Filter (nm)	Bandpass Emission Filter (nm)
Alexa Fluor 430	403	505	525/30
Side Scatter	488	N.A.	488/10
SYBR Green	488	505	530/30
Cy3	561	N.A.	582/15
Alexa Fluor 594	561	595	610/20
Cy5	640	N.A.	670/30
Alexa Fluor 700	640	685	730/45