Supplementary figures and tables
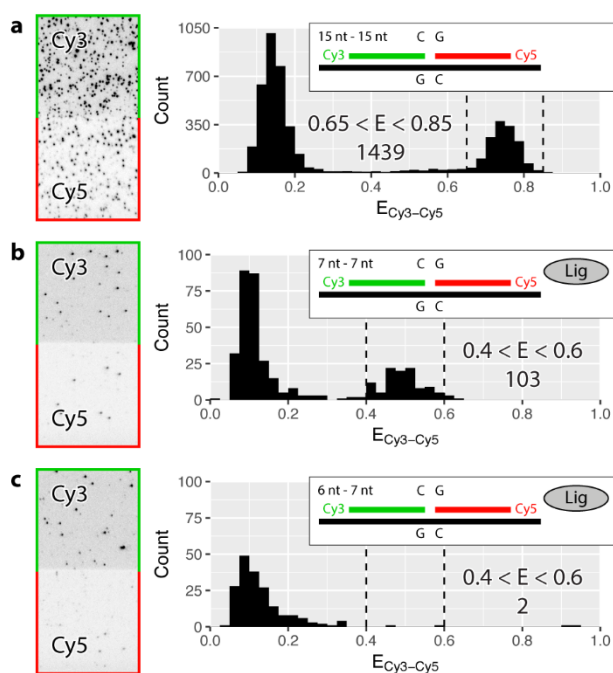

FIGURE S1 | Effect of barcode length on the efficiency of hybridization and ligation.

Detection of DNA target with complementary barcodes of various length: (*a*) two 15-nt barcodes in the absence of ligase; (*b*) two 7-nt barcodes in the presence of ligase; (*c*) 6-nt upstream and 7-nt downstream barcodes in the presence of ligase.

All experiments are based on 15 fields of view. Panels show CCD camera images (*left*) of the Cy3 (green) and Cy5 (red) channels upon Cy3 excitation, and experimental schemes and FRET efficiency (E) histograms (*right*), where the dotted lines indicate the range of E of the target-barcodes complexes (0.65<E<0.85 in (*a*) and 0.4<E<0.6 in (*b-c*)).
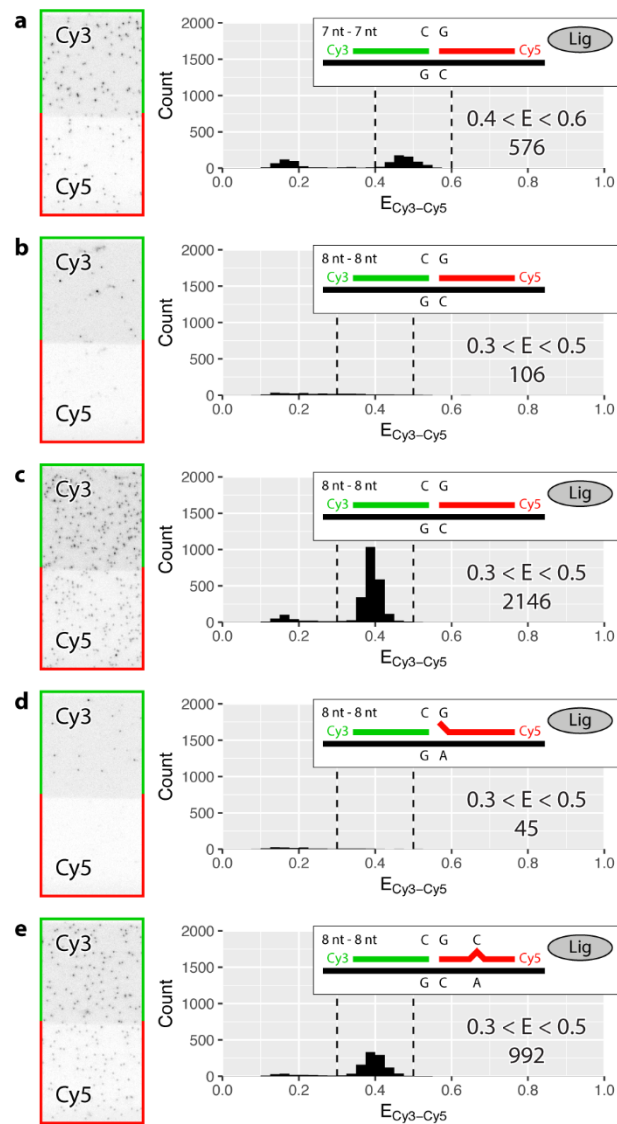
FIGURE S2 | 8-nt barcodes show higher ligation efficiency and lower specificity than 7-nt barcodes.

Detection of DNA target with (*a*) complementary 7-nt barcodes in the presence of ligase; (*b*) complementary 8-nt barcodes in the absence of ligase; (c) complementary 8-nt barcodes in the presence of ligase; (*d*, *e*) complementary 8-nt upstream barcode and mismatching 8-nt downstream barcode (at the 3'-end and center nucleotide, respectively) in the presence of ligase.

All experiments are based on 10 fields of view. Panels show CCD camera images (*left*) of the Cy3 (green) and Cy5 (red) channels upon Cy3 excitation, and experimental schemes and FRET efficiency (E) histograms (*right*), where the dotted lines indicate the range of E of the target-barcodes complexes (0.4<E<0.6 in (*a)* and 0.3<E<0.5 in (*b-e*)).
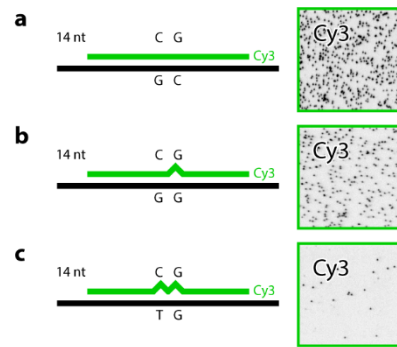
FIGURE S3 | Stable hybridization of mismatched 14-nt barcodes.

Hybridization of DNA target and a single 14-nt barcode with: (*a*) no mismatch (complete complementarity), (*b*) with one mismatch, and (*c*) with two mismatches. Panels show experimental schemes (*left*) and CCD camera images (*right*) of the Cy3 channel (green) upon Cy3 illumination.
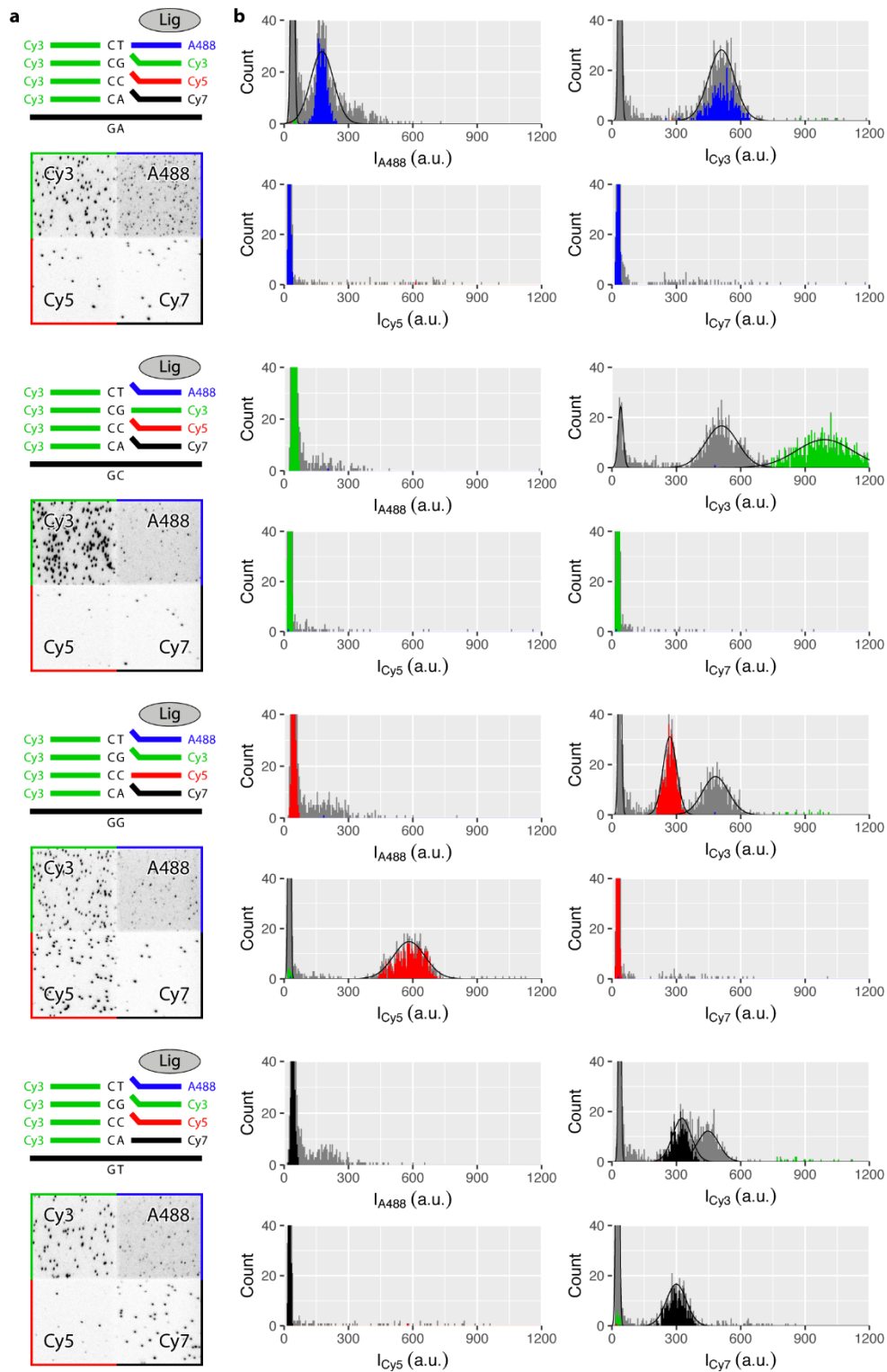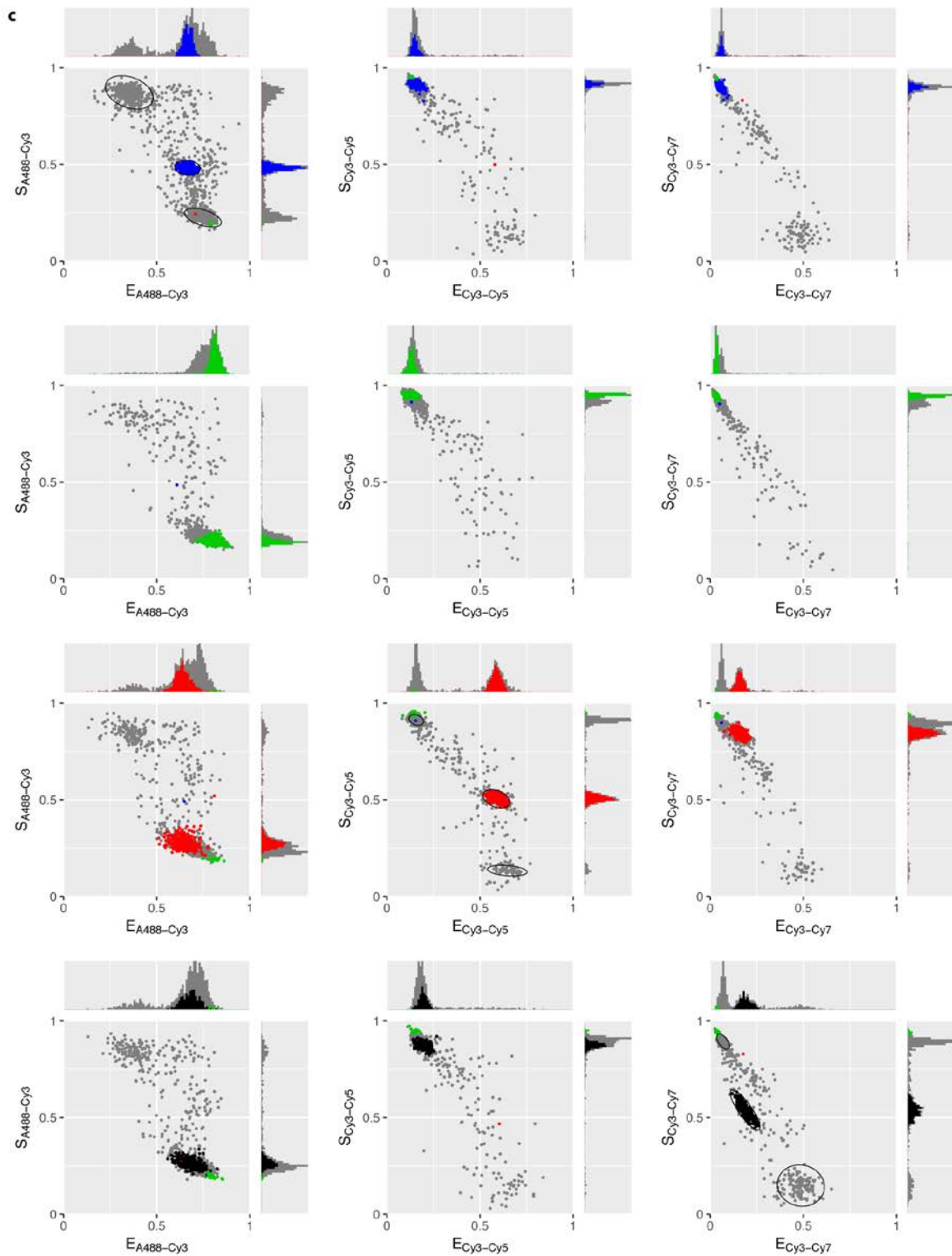
FIGURE S4 | Single target complementary to one of four distinctively labelled barcode pairs.

(*a*) Experimental scheme and CCD camera image of the four channels: A488 (blue), Cy3 (green), Cy5 (red), and Cy7 (black) upon respective direct excitation.

(*b*) Intensity (I) histograms for A488, Cy3, Cy5, and Cy7. Solid lines show fits of univariate Gaussian distributions.

(*c*) E-S scatter plots for the A488-Cy3 (*left*), Cy3-Cy5 (*middle*), and Cy3-Cy7 (*right*) fluorophore pairs. In each plot only relevant molecules are shown, i.e. with one or both of the two fluorophore intensities above background. Ellipses indicate the 95% confidence interval of fitted bivariate Gaussian distributions.

Data based on 20 fields of view. Molecules selected as A488-Cy3, Cy3-Cy3, Cy3-Cy5, and Cy3-Cy7 barcode pairs are indicated with blue, green, red, and black, respectively; unselected molecules are shown in grey. The selection criteria were based on the combination of four experiments, each of them using a different target sequence.
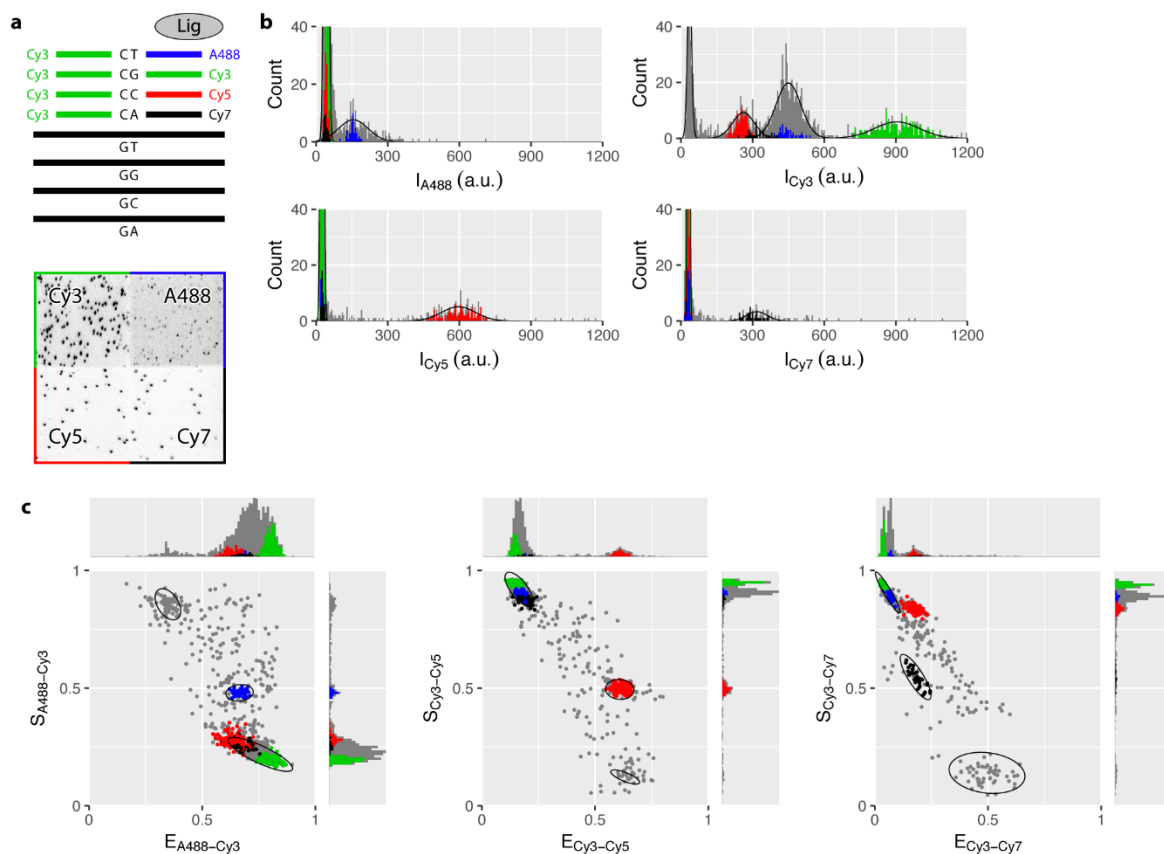
FIGURE S5 | Four targets in mixture identified by four complementary barcode pairs.

(*a*) Experimental scheme and CCD camera image of the four channels: A488 (blue), Cy3 (green), Cy5 (red), and Cy7 (black) upon respective direct excitation.

(*b*) Intensity (I) histograms for A488, Cy3, Cy5, and Cy7. Solid lines show fits of univariate Gaussian distributions.

(*c*) E-S scatter plots for the A488-Cy3 (*left*), Cy3-Cy5 (*middle*), and Cy3-Cy7 (*right*) fluorophore pairs. In each plot only relevant molecules are shown, i.e. with one or both of the two fluorophore intensities above background. Ellipses indicate the 95% confidence interval of fitted bivariate Gaussian distributions.

Data based on 20 fields of view. Molecules selected as A488-Cy3, Cy3-Cy3, Cy3-Cy5, and Cy3-Cy7 barcode pairs are indicated with blue, green, red, and black, respectively; unselected molecules are shown in grey. The selection criteria were based on a single experiment with four different target sequences.

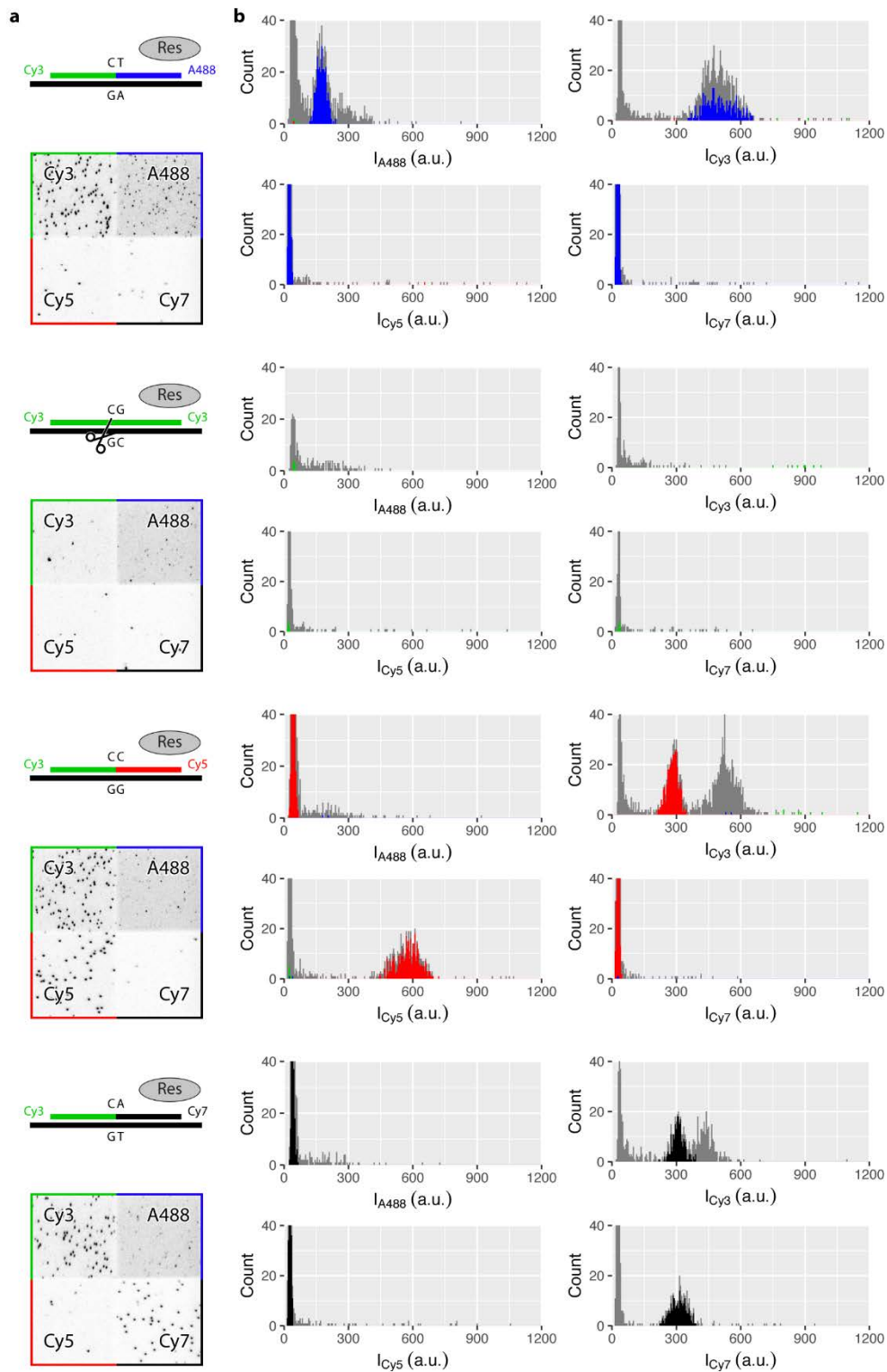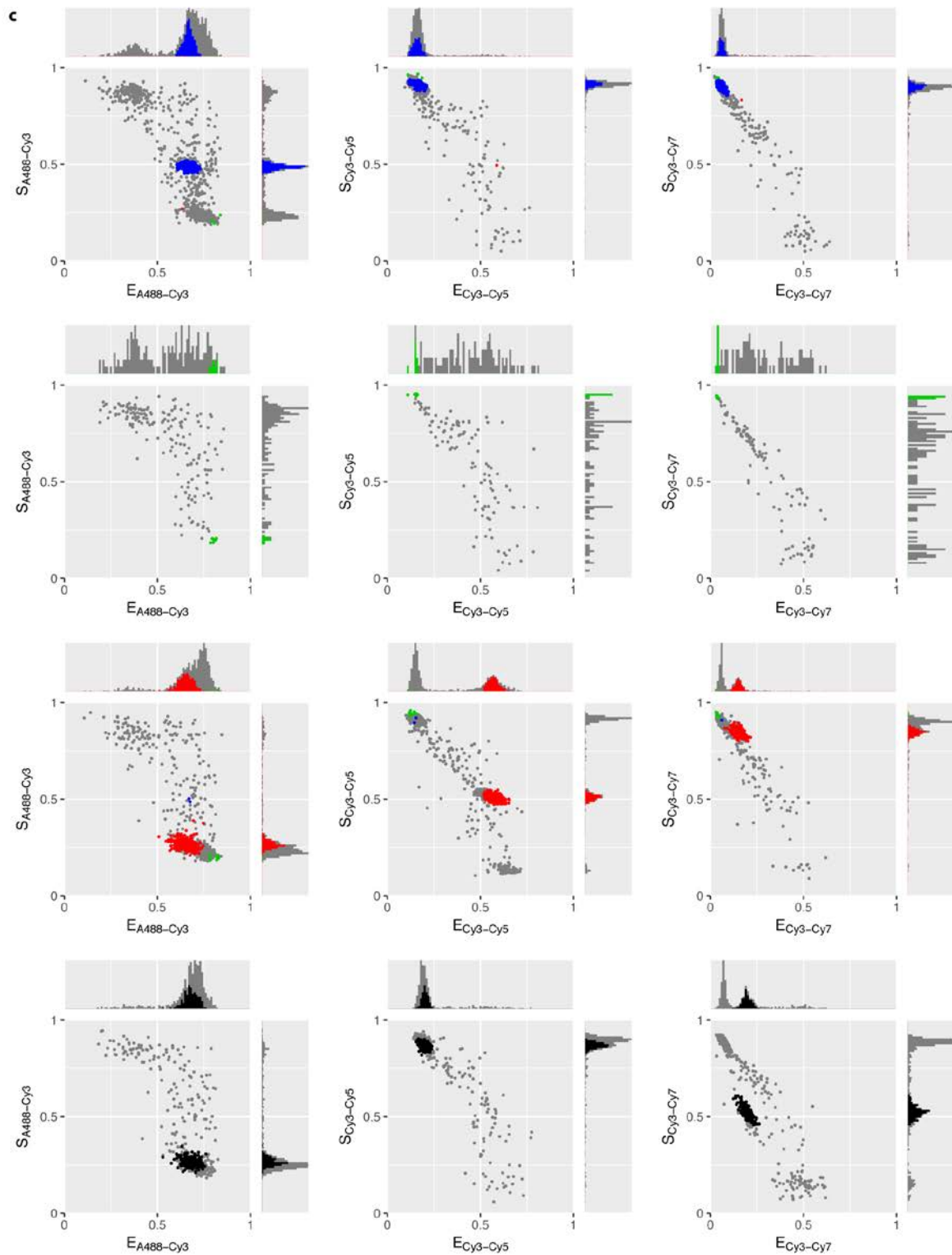**FIGURE S6 | Single target complementary to one of four distinctively labelled barcode pairs - after addition of a restriction enzyme specific to the bound Cy3-Cy3 barcode pair.**

(*a*) Experimental scheme and CCD camera image of the four channels: A488 (blue), Cy3 (green), Cy5 (red), and Cy7 (black) upon respective direct excitation.

(*b*) Intensity (I) histograms for A488, Cy3, Cy5, and Cy7.

(*c*) E-S scatter plots for the A488-Cy3 (*left*), Cy3-Cy5 (*middle*), and Cy3-Cy7 (*right*) fluorophore pairs. In each plot only relevant molecules are shown, i.e. with one or both of the two fluorophore intensities above background.

Data based on 20 fields of view. Molecules selected as A488-Cy3, Cy3-Cy3, Cy3-Cy5, and Cy3-Cy7 barcode pairs are indicated with blue, green, red, and black, respectively; unselected molecules are shown in grey. The selection criteria were based on the combination of four experiments, each of them using a different target sequence.
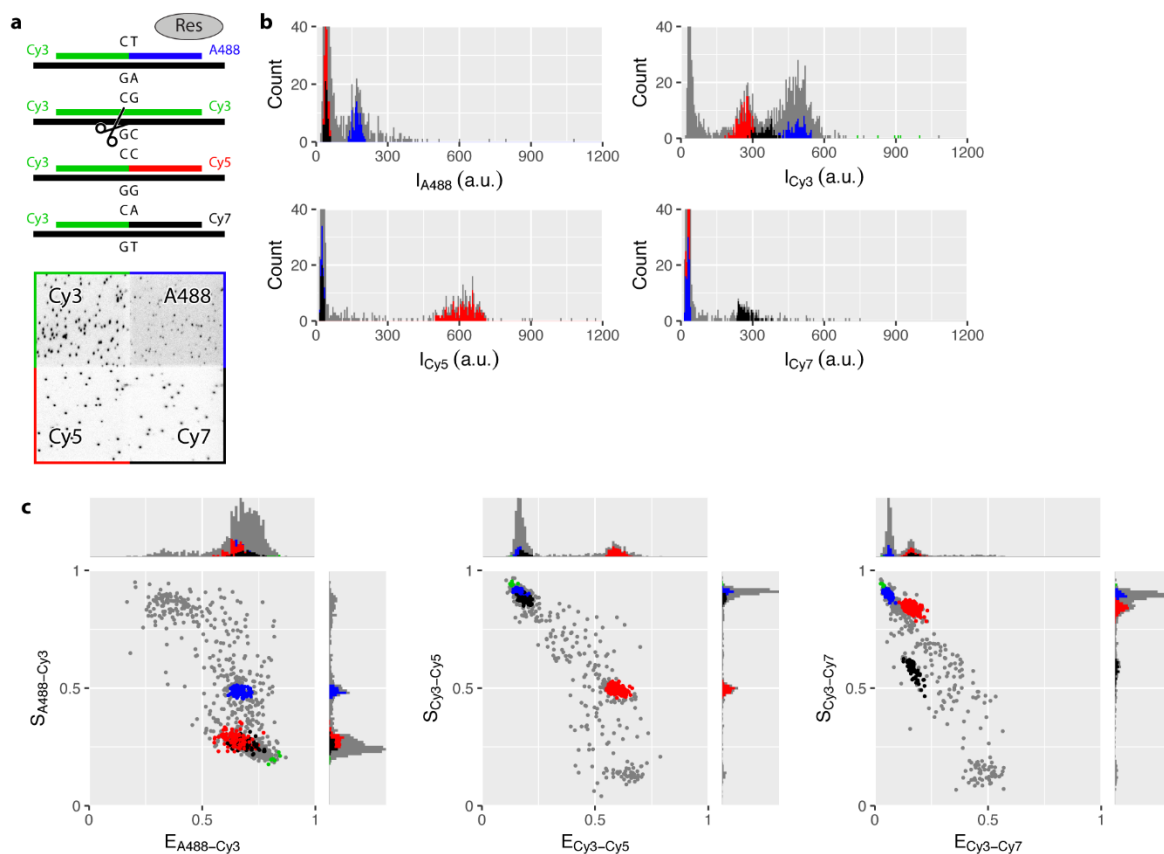
FIGURE S7 | Four targets in mixture identified by four complementary barcode pairs - after addition of a restriction enzyme specific to the bound Cy3-Cy3 barcode pair.

(*a*) Experimental scheme and CCD camera image of the four channels: A488 (blue), Cy3 (green), Cy5 (red), and Cy7 (black) upon respective direct excitation.

(*b*) Intensity (I) histograms for A488, Cy3, Cy5, and Cy7.

(*c*) E-S scatter plots for the A488-Cy3 (*left*), Cy3-Cy5 (*middle*), and Cy3-Cy7 (*right*) fluorophore pairs. In each plot only relevant molecules are shown, i.e. with one or both of the two fluorophore intensities above background.

Data based on 20 fields of view. Molecules selected as A488-Cy3, Cy3-Cy3, Cy3-Cy5, and Cy3-Cy7 barcode pairs are indicated with blue, green, red, and black, respectively; unselected molecules are shown in grey. The selection criteria were based on a single experiment with four different target sequences.
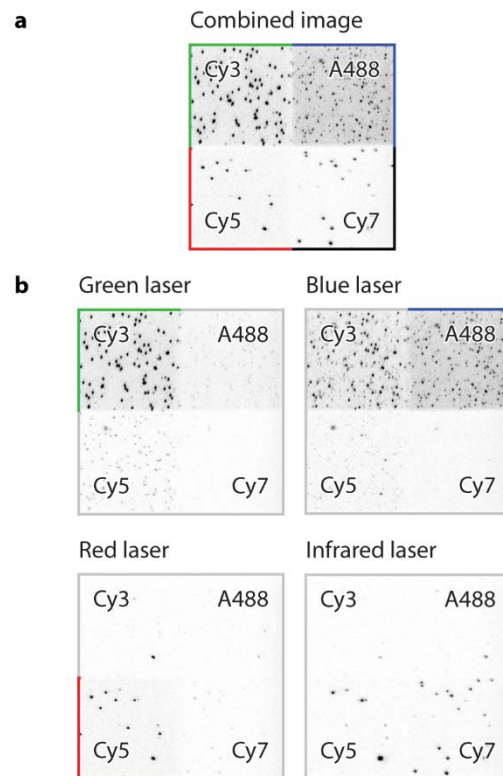
FIGURE S8 | CCD camera images used to create a CCD camera image in Fig. 3b.

(*a*) Combined CCD camera image as shown in Fig. 3b.

(*b*) CCD camera images upon direct excitation of (clockwise): Cy3, A488, Cy7, and Cy5, respectively.

TABLE S1 | Sequences of target and barcode DNA strands.

Targets	Sequences 5' to 3'		
60 nt - Target GC "Wild type"	TGG CGA CGG CAG CGA GGC TTT TTT TTT CAC TAA GAC AGA TGG CCT ATA CAG ATC GAG TTT		
60 nt - Target XY "Mutated ligation site"	TGG CGA CGG CAG CGA GGC TTT TTT TTT CAC TAA GAC AGA TG**X Y**CT ATA CAG ATC GAG TTT		
18 nt - Biotinylated DNA Complementary to target	GCC TCG CTG CCG TCG CCA /Biotin/		
Barcodes	**Sequences 5' to 3'**	**Label**	**Figure**
14 nt barcode control	*GTA TAG GCC ATC TG	Cy3	S3
Downstream			
7 nt - Label 6th nt	GT*A TAG G	Cy5	2, S1, S2
7 nt - Label 6th nt - G1T	GT*A TAG **T**	Cy5	2
7 nt - Label 6th nt - T4C	GT*A **C**AG G	Cy5	2
7 nt - Label 6th nt - G7A	**A**T*A TAG G	Cy5	2
7 nt - Label 5'-end	*GTA TAG G	Cy3	3, 4, S4-7
7 nt - Label 5'-end - G1C	*GTA TAG **C**	Cy5	3, 4, S4-7
7 nt - Label 5'-end - G1A	*GTA TAG **A**	Cy7	3, 4, S4-7
7 nt - Label 5'-end - G1T	*GTA TAG **T**	A488	3, 4, S4-8
8 nt - Label 5'-end	*TGT ATA GG	Cy5	S2
8 nt - Label 5'-end - T4C	*TGT A**C**A GG	Cy5	S2
15 nt - Label 6th nt	CTC GAT CTG T*AT AGG	Cy5	S1
Upstream			
6 nt - Label 4th nt	/Phosphate/ CCA T*CT	Cy3	S1
7 nt - Label 4th nt	/Phosphate/ CCA T*CT G	Cy3	2, S1
7 nt - Label 4th nt - G1A	/Phosphate/ **A**CA T*CT G	Cy3	2
7 nt - Label 4th nt - A3G	/Phosphate/ CC**G** T*CT G	Cy3	2
7 nt - Label 4th nt - G7A	/Phosphate/ CCA T*CT **A**	Cy3	2
7 nt - Label 3'-end	/Phosphate/ CCA TCT G*	Cy3	3, 4, S2, S4-8
8 nt - Label 3'-end	/Phosphate/ CCA TCT GT*	Cy3	S2
15 nt - Label 4th nt	/Phosphate/ CCA T*CT GTC TTA GTG	Cy3	S1

X and Y = A, C, G, or T. * at either end (5' or 3') indicates the labeling position, T* indicates an internally labeled amino-modified thymine. Mutations are indicated in **bold**.

TABLE S2 | Number of detected barcode pairs in four-color single-target and four-target experiments (without restriction).

Target		GA	GC	GG	GT	All
Barcode pair	A488-Cy3	344	1	1	0	47
	Cy3-Cy3	4	681	15	20	226
	Cy3-Cy5	1	0	385	1	106
	Cy3-Cy7	0	0	0	259	35
Total		349	682	401	280	414

Single-target and four-target experiments are indicated with the sequence at the ligation site ("GA", "GC", "GG", and "GT") and with "All", respectively.

TABLE S3 | Number of detected barcode pairs in four-color single-target and four-target experiments - after addition of a restriction enzyme specific to the bound Cy3-Cy3 barcode pair.

Target		GA	GC	GG	GT	All
Barcode pair	A488-Cy3	292	0	2	0	91
	Cy3-Cy3	3	7	9	0	6
	Cy3-Cy5	1	0	322	0	149
	Cy3-Cy7	0	0	0	198	63
Total		296	7	333	198	309

Single-target and four-target experiments are indicated with the sequence at the ligation site ("GA", "GC", "GG", and "GT") and with "All", respectively.