RCasFISH: CRISPR/dCas9-mediated *In Situ* imaging of mRNA transcripts in fixed cells and tissues

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Supporting Information

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Supplemental Experimental Section

Collection of patient samples

The study materials consisted of FFPE samples from 82 patients, newly diagnosed with breast cancer between 2018 and 2019 at the Department of Molecular Diagnostics, Sun Yat-Sen University Cancer Center, China. This 82-patient cohort consisted of 37 positive, 33 negative, and 12 equivocal samples based on FISH test. All FISH-equivocal cases were tested reflexively by IHC (6 scored as positive, 4 scored as negative, and 2 remained equivocal). We sliced 3 sections (4-5 μm thick), each for IHC, FISH, and RCasFISH from the same block. The study was conducted under ethical permission granted by the Ethics Committee of Sun Yat-Sen University Cancer Center. A summary of patient clinicopathological characteristics is shown in Table S5.

Plasmid construction and PAMmer synthesis

The pET302-6His-dCas9-Halo vector containing an N-terminal hexa-histidine affinity tag for purification and a C-terminal Halo-tag for synthetic dye labeling was a gift from Timothee Lionnet [10], and was used for protein expression and purification *in vitro*. MCP sequence fused to a Halo-tag at the C-terminus was synthesized (Sangon Biotech) and inserted into the pC013-Twinstrep-SUMO-huLwCas13a vector (Addgene # 90097) by digesting with BamHI and NotI to replace the Cas13a fragment.

PAMmer sequences that consist of mixed 2′-O-methyl (2′-OMe) modified RNA and DNA bases with 8-nt long 5′-extension were synthesized and purified by Sangon. The sequences of the primers and oligo templates used are listed in Table S6.

Protein expression and purification

Protein expression and purification were performed as described previously with some modification. In brief, dCas9 and MCP were expressed in Rosetta™ 2(DE3) pLysS Singles™ competent cells (Merck Millipore 71401), which were cultured in Terrific Broth at 37 °C at 250 rotations per minute (rpm). IPTG (Amresco, USA) was added to a final concentration of 1 mM to induce protein expression as soon as the OD600 reached 0.6, and cells were subsequently cultured overnight at 16 °C at 250 rpm for protein expression. Cells were then harvested by centrifugation at 8,000 rpm

for 15 min at 4 °C and washed 3 times with autoclaved 1× PBS. Cell pellets were stored at -80 °C for subsequent lysis. The cell pellet was lysed in lysis buffer containing 50 mM sodium phosphate (pH 7.0), 300 mM NaCl, 5% glycerol, freshly added protease inhibitor (Roche Switzerland), lysozyme (Solarbio L8120), 1 mM TCEP, and 1 mM PMSF, followed by sonication. Clarified lysate was obtained by centrifugation at 12,000 rpm, 4 °C for 1 h and subsequent filtration with 0.22 μm filter (Merck Millipore SLGP033RB).

For dCas9, the lysate was dialyzed into lysis buffer with additional 20 mM imidazole, and then applied onto a 1 mL HisTALON^{TM} Superflow column (Clontech 635649). Proteins were eluted in 50 mM sodium phosphate, 300 mM NaCl, 150 mM imidazole (pH 7.0) and were exchanged into cation exchange chromatography start buffer containing 50 mM Hepes (pH 7.5), 100 mM KCl, 5% glycerol, and 1 mM TCEP. Eluted proteins were loaded onto 1 mL HiTrapTM SP HP (GE Healthcare 17-1151-01) and eluted with a linear KCl gradient (0.1–1.0 M). After SDS-PAGE analysis, the eluted fraction was dialyzed into gel filtration buffer containing 50 mM Hepes (pH 7.5), 150 mM KCl, 5% glycerol, and 1 mM TCEP, and was then applied onto the size-exclusion chromatography column Superdex® 200 Increase 10/300 GL (GE Healthcare 28990944). The protein fractions were verified by SDS-PAGE. Purified protein was aliquoted and stored at -80 °C with additional 10% glycerol.

For MCP-Halotag, the lysate was mixed well with StrepTactin Sepharose (GE Healthcare 28935599) and incubated for 1 h at 4°C with rotation. The sepharose was then washed 3 times with lysis buffer and incubated with SUMO protease (ThermoFisher 12588018) for 16 h at 4 °C in a digestion buffer comprising 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 5% glycerol, and freshly added 1 mM DTT, 0.15% NP-40. The supernatant, containing the MCP-Halotag protein, was collected by centrifugation and verified by SDS-PAGE. Finally, the supernatant was dialyzed into gel filtration buffer containing 50 mM Hepes (pH 7.5), 150 mM KCl, 5% glycerol and 1 mM TCEP, and was applied to the size-exclusion chromatography column Superdex® 200 Increase 10/300 GL (GE Healthcare 28990944). Purified MCP-Halotag protein was stored at -80 °C with additional 10% glycerol.

Protein labeling with fluorescent dyes

MCP-Halo and dCas9-Halo were labeled with HaloTag® Alexa Fluor® 488

Ligand (Promega G1001) and Janelia Fluor 646 Ligand according to the following protocol: protein was incubated with fluorescent dyes (1 μM) at a molar ratio of 1:4 in buffer containing 50 mM Hepes (pH 7.5), 150 mM KCl, 1 mM TCEP, 10% glycerol at room temperature for 30 min followed by incubation at 4 °C overnight, away from light. Excess fluorescent dyes were removed using 40K MWCO Zeba spin desalting columns (ThermoFisher 87766). Fluorescently labeled proteins were aliquoted and stored at -80 °C.

***in vitro* sgRNA transcription**

sgRNA-conventional, sgRNA-2×-MS2, 8×-MS2, 16×-MS2 were transcribed using HiScribe T7 Quick High Yield RNA Synthesis Kit (NEB E2050S) with the templates containing a T7 promoter region upstream of the sequence followed by a sgRNA target sequence and an sgRNA backbone. The transcription templates of sgRNA-conventional, sgRNA-2×MS2, -8×MS2, -16×MS2 were produced by PCR using PrimeSTAR® HS DNA Polymerase (Takara R010A) or PrimeSTAR® GXL DNA Polymerase (Takara R050A). Quality of the transcribed RNAs was confirmed from a denaturing polyacrylamide gel and visualizing RNA bands by staining with ethidium bromide. Related sequences of PCR templates and sgRNAs primers are provided in Table S7.

EMSA assay

EMSA was performed using Electrophoretic Mobility Shift Assay Kit (Invitrogen). In brief, equal amounts of MCPs (4.2 μM) were incubated with increasing amounts of modified sgRNAs (0, 32.8, 65.6, 131.2, 262.5, 525, 1050, and 2100 nM, respectively) at 37℃ for 15 minutes in blocking buffer. Then, reaction mixtures were supplemented with 10% glycerol and applied to electrophoresis of 4.5% native TBE-PAGE in 0.5 × TBE buffer. The gel was visualized using Bio-Rad Gel Doc™ XR+ System.

RNA FISH, DNA FISH, AND IHC ASSAY

Stellaris FISH probes labeled with Quasar 670 recognising human *HER2* mRNA

were purchased from Biosearch Technologies (#VSMF-2103-5, USA). Hybridization was performed according to the manufacturer's instructions. Image acquisition was performed using Nikon Eclipse Ti-E laser-scanning confocal microscope. For colocalization experiment, RCasFISH is performed according to the manufacturer's instructions followed by Stellaris FISH procedure after washing by PBS for 2min.

FISH assays were performed using PathVysion HER-2 DNA Probe Kit (Abbott, USA) following the manufacturer's instructions. After counting a minimum of 20 tumor cell nuclei, gene amplification was determined according to the guidelines released by ASCO-CAP in 2018.

IHC staining was performed using anti-HER2/ErbB2 (29D8) rabbit monoclonal antibody (Cell Signaling Technology, USA). All related steps have been described in our previous study[22]. HER2 protein expressed on the membrane of tumor cells was scored as 0+, 1+, 2+, or 3+ according to the guideline.

RNAscope assay

RNA in situ hybridization was also performed by Advanced Cell Diagnostics (ACD, Cat No. 310081-C2) using an RNAscope multiplex fluorescence assay to detect the *HER2* mRNA in fixed mammalian cell lines. Positive quality control was performed on all samples using Cyclophilin B (PPIB) probes. Bacterial dapB is a non-specific probe that will generate no background signal on properly fixed tissue (negative control). Hybridization was performed according to the manufacturer's instructions.

Preparation of FFPE xenograft tumor samples

For establishing tumor xenografts, cells of all the four breast cancer cell lines described above were injected intradermally (1×10^7 cells per mouse in 0.1 ml PBS) into the backs of female severe combined immunodeficient (SCID) mice (Vital River Laboratories) between 21 and 28 days of age. The procedure for generating tumor xenografts has been described in detail in our previous study and all surgery was performed under ether anesthesia[22]. The animal experiments were performed in compliance with the guidelines specified by the Institute for Experimental Animals, Beijing Hospital.

Imprecision study

We used 15 mouse xenograft tumor samples (5 samples each of low, medium, and high *HER2* expression) to evaluate the imprecision of the RCasFISH assay. We assessed intra-assay (within-run) variation by analyzing 5 control samples on the same day and evaluated inter-assay (between-run) variation by measuring mean mRNA dots per cell over a 5-day period. We then calculated within- and between-run CVs. CVs of <20% were considered to indicate acceptable performance for both within-run imprecision and between- run imprecision.

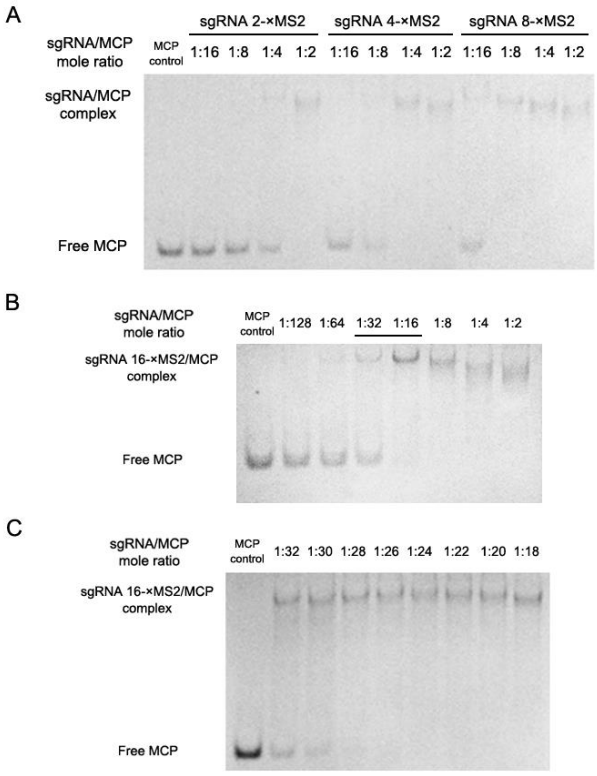

Figure S1. EMSA evaluating MCP-binding ability of sgRNA-MS2 *in vitro*. (A) sgRNA 2×, 4×, 8×-MS2 were incubated with equal amounts of MCPs in different molar ratios. Free MCP bands were undetected at the molecular molar ratio of 1:2, 1:4, 1:8, respectively. (B) Increasing amounts of sgRNA 16×-MS2 were incubated with equal amounts of MCPs at molar ratio of 1:128, 1:64, 1:32, 1:16, 1:8, 1:4 and 1:2. Free MCP bands were undetected at the molecular molar ratio of 1:16. (C) Increasing amounts of detailed sgRNA 16×-MS2 were incubated with equal amounts of MCPs from a molar ratio of 1:32 to 1:18. Free MCP bands were disappeared at the molecular molar ratio of 1:28.

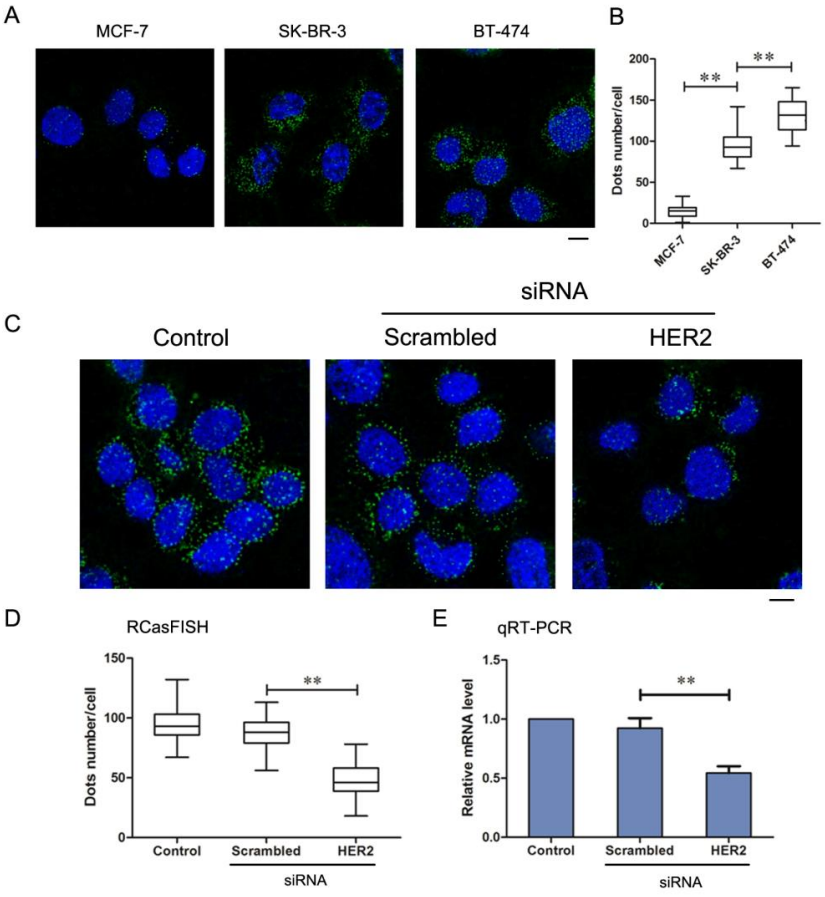

Figure S2. RCasFISH imaging of *HER2* transcripts in fixed cells. (A) Representative images of different breast cancer cell lines after delivering three sgRNA 16×-MS2 probes targeting *HER2* transcripts. Scale bar=10 μm. (B) The number of dots per cell detected by RCasFISH in three different breast cancer cell lines with discrepant *HER2* expression profiles. Asterisks indicate: $p<0.01$ (**). n=200 cells for each group. (C) RCasFISH analysis of *HER2* transcripts in SK-BR-3 cell line. Representative images show cultured cells with or without treatment by siRNA to knock down *HER2* expression, or using a scrambled siRNA sequence. Scale bar=10 μm. (D-E) Single cell RCasFISH counts are shown in comparison with transcript measurements by qRT-PCR for SK-BR-3 cell. Significantly reduced mRNA levels are observed for siRNA treatment, with similar magnitude between RCasFISH and qRT-PCR. The PCR data were obtained from three independent experiments. The error bars represent SD. n=200 cells for each group. Asterisks indicate: $p<0.01$ (**). Relative sequences are shown in Table S6.

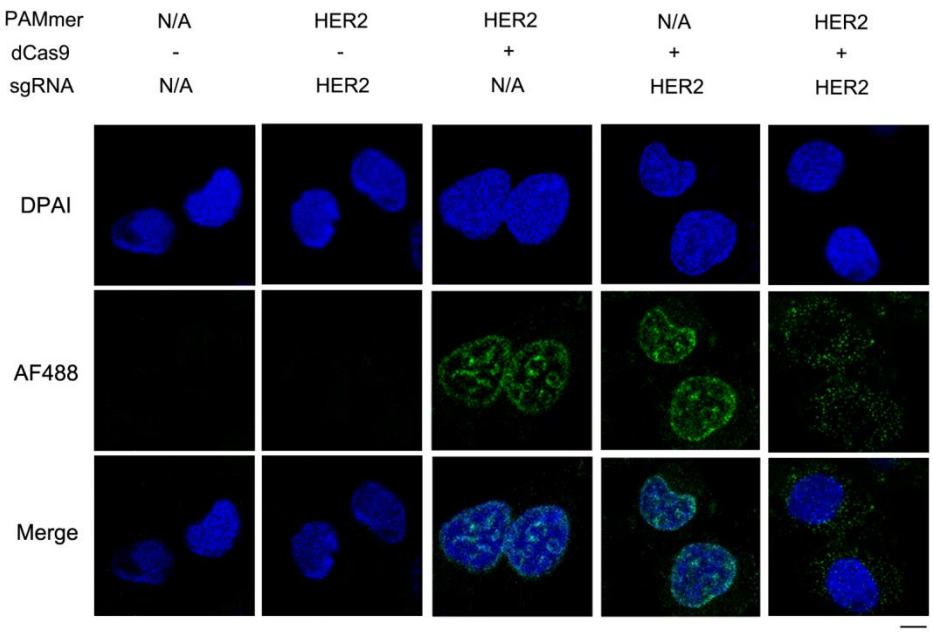

Figure S3. The role of each component on *HER2* transcripts targeting in SK-BR-3 cells. "N/A" represented sgRNA or PAMmer originate from λ bacteriophage that should not exist in human cells. Scale bar=4 μm.

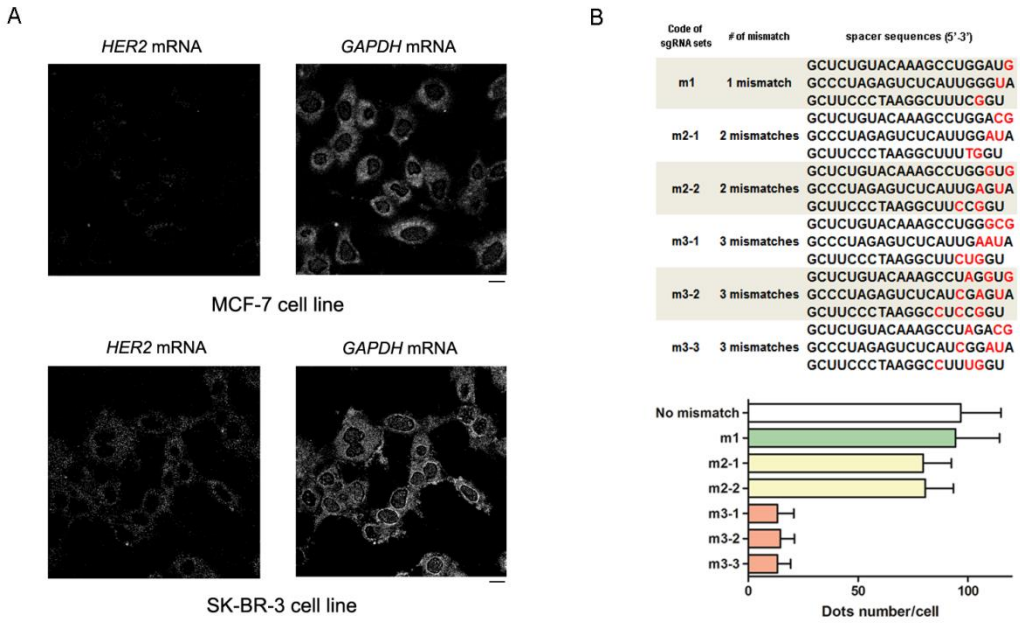

Figure S4. Specificity of RCasFISH probe to target RNA. (A) *HER2* signal could not be detected in *HER2*-negative MCF-7 cell line but was discernible in SK-BR-3 cell line. Scale bar=10 μm. (B) Sequences of three sgRNAs with synthetic mismatches (red highlight) complementary to the 3'UTR region of *HER2* mRNA at the top right. The mean numbers of RNA dots detected in different mismatch groups were shown at the bottom right (n=250 cells).

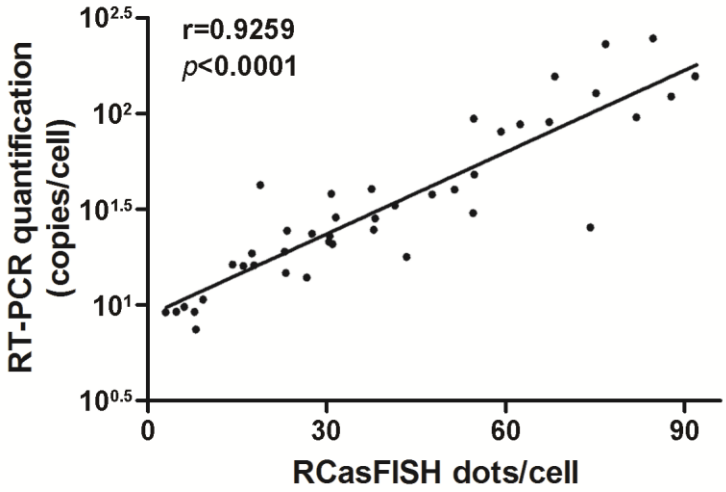

Figure S5 Single RNA molecule detection. *HER2* mRNA in 293T, MCF-7 and SK-BR-3 cells were detected using RCasFISH assay and RT-qPCR analysis (n=42). Pairwise scatterplot between RCasFISH and qPCR shows a good linear relationship. *HER2* mRNA copies per cell determined by qPCR using a standard curve from *in vitro* transcribed RNA.

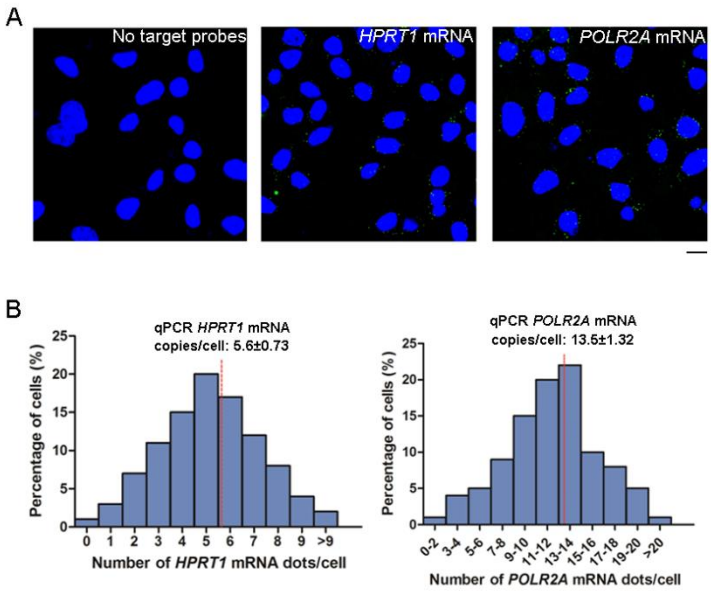

Figure S6 RCasFISH detection of low-copy transcripts in fixed cells. (A) Representative RCasFISH images of fixed SK-BR-3 cells targeting *HPRT1* and *POLR2A* mRNA. Scale bar=10 μm. (B) Histograms of corresponding mRNA dots count by RCasFISH (n=200 cells). The vertical red dashed lines denote the mean RNA copies per cell determined by qPCR.

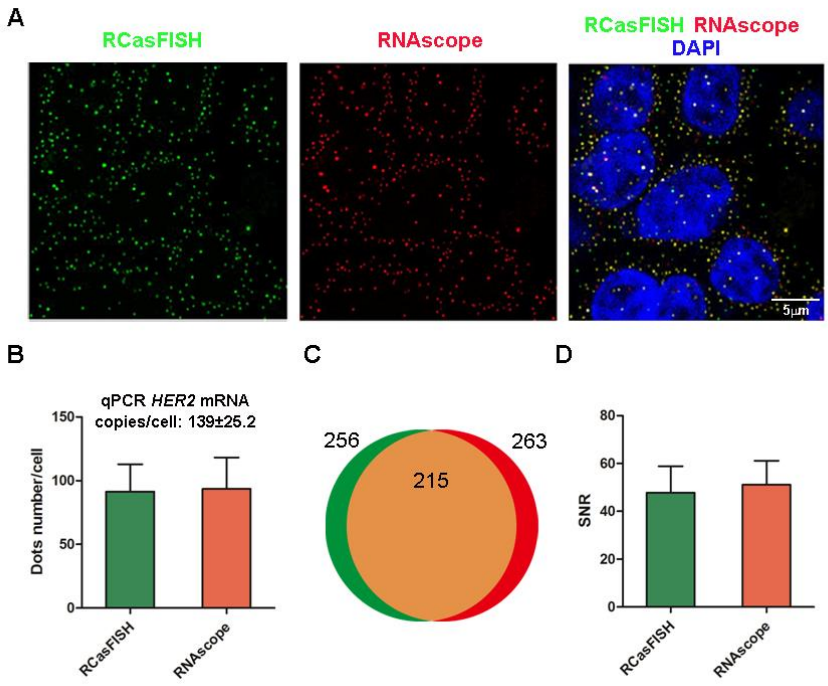

Figure S7. Simultaneous detection of *HER2* transcripts by RCasFISH and RNAscope in fixed SK-BR-3 cells. (A) Representative images showing colocalization of the *HER2* transcripts using RCasFISH (green) and RNAscope (red) (Scale bar= 5μm). (B) Comparison of number of mRNA particles obtained from the two methods and real-time RT-PCR. (C) Quantification of the number of *HER2* mRNA dots stained by RCasFISH and RNAscope as in A. Dots labeled by both probes are colored orange. (D) Comparison of signal-to-noise ratios (SNR) between the two methods. n=250 detected dots from 125 cells each method.

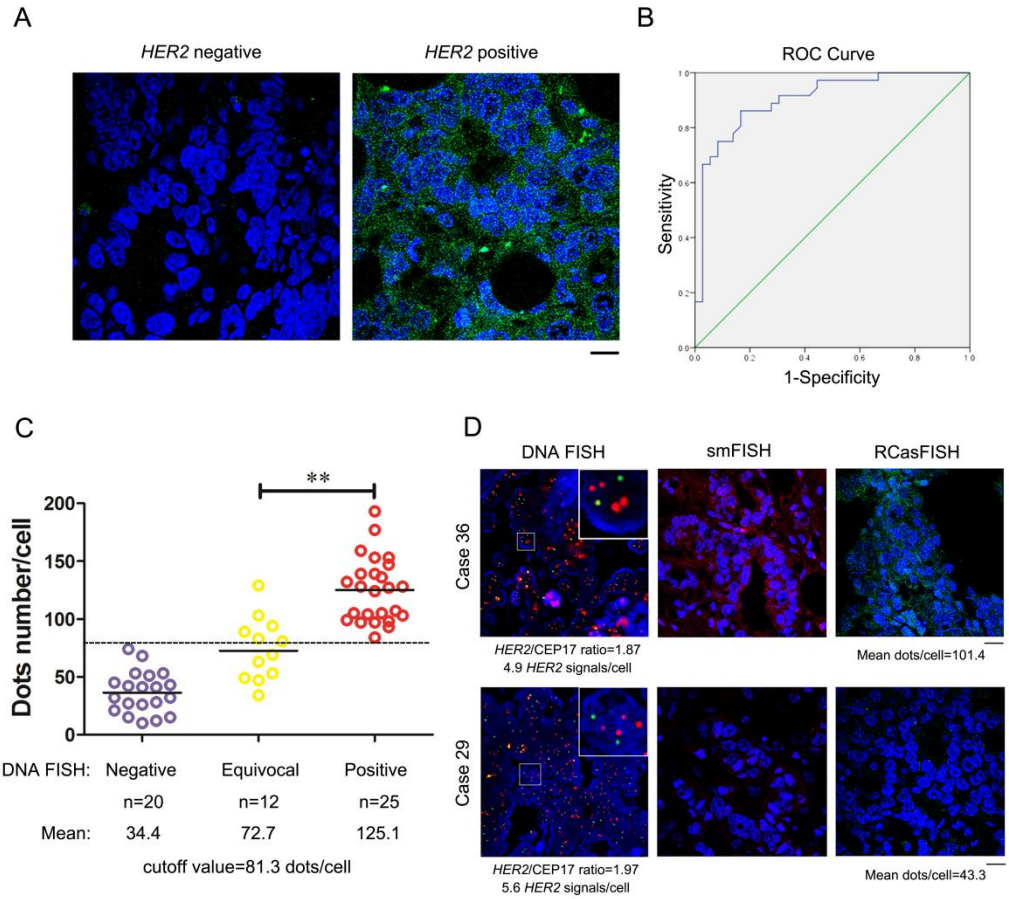

Figure S8. RCasFISH detection of *HER2* mRNA in patients with breast cancer. (A) Representative RCasFISH images of *HER2*-negative and -positive breast cancer FFPE tissue. Scale bar=15 μm. (B) ROC curve for RCasFISH assay (n=25), with conventional FISH and IHC as comparative methods. (C) The number of dots per cell in different samples which are grouped based on *HER2* DNA FISH. Each dot corresponds to the mean dot number/cell in one of 57 analyzed cases. Horizontal black bars represent the mean of all cases in the corresponding group. The dashed line indicates the cutoff value set by ROC curve. (D) Representative figures of the double-equivocal cases with discrepant *HER2* DNA and RNA expression. First column, FISH (original magnification 1000×). Second column, smFISH (1000×). Third column, RCasFISH (1000×). Scale bar= 15 μm.

Table S1 Imprecision study of RCasFISH assay

	Control 1 (n=5)	Control 2 (n=5)	Control 3 (n=5)
Mean dot number/cell	45.3	269.1	450
SD	7.2	36.7	61.2
Inter-assay CV, %	16.0	13.6	10.6
Intra-assay CV, %	4.3	6.1	5.6

Results were collected and analyzed each day over a 5-day period

Table S2 Concordance of RCasFISH assay with conventional IHC, FISH *HER2* assays in breast cancer FFPE tissues (n=55)

RCasFISH	Combination of FISH and IHC		Total	Concordance	*P*[a]
	Positive	Negative			
Positive	29	1	30		
Negative	2	23	25	94.5%	<0.001
Total	31	24	55		

[a] Fisher exact test

Table S3 Summary of discordant cases

Case no.	FISH	IHC	Mean RCasFISH dots/cell	RCasFISH	Heterogeneity of amplification[a]
19	Equivocal	3+	47.8	Negative	No
24	Equivocal	3+	58.4	Negative	No
45	Equivocal	1+	92.6	Positive	No
29	Equivocal	2+	43.3	Negative	No
36	Equivocal	2+	101.4	Positive	No

[a]Heterogeneity of amplification: when 20 cells are counted for evaluation of *HER2*/neu amplification, a single 3:1 *HER2*/CEP17 ratio cell characterizes the sample as heterogeneous.

Table S4 Comparison of RCasFISH with other methods in determining *HER2* status

	DNA FISH	IHC	RNA FISH	qRT-PCR	RCasFISH
Probe	Nucleic acid probe	Specific antibody	Nucleic acid probe	Specific primer	Nucleic acid probe
Experiment duration[a]	1-2 days	1-2 days	~10 hours	4-6 hours	4 hours
DNA denaturation	Yes	No	No	Yes	No
Fixed cell or tissue imaging	Yes	Yes	Yes	No	Yes
Live cell imaging	No	No	No	No	Yes[30]
Identify heterogeneity	Yes	Yes	Yes	No	Yes
Cost[b]	$180/test	$0.31/test	$4.9/test	$5.2/test	$0.97/test
Limitations	Restricted by chromosome 17 polysomy; labor intensive	Relative high false positive and negative rate	Instability of mRNA	Affected by admixture of non-neoplastic cells	Instability of mRNA, long probe

a. Experiment duration means the time spent from sample fixation to imaging.

b. The cost of commercial kits are calculated based on the official purchase price (may include other costs such as service fees, advertising fees, taxes, etc.) divided by the number of available test; The cost of RCasFISH is calculated by dividing the total cost of production by the number of available test.

Table S5 Summary of patient clinicopathological characteristics (n = 82)

Variable	Value (%)
Age (years)	
Range	34-83
Mean	56.3
Male/female ratio	0:82
TNM stage	
Ⅰ	39 (48)
Ⅱ	22 (27)
Ⅲ	13 (16)
Ⅳ	8 (9)
Histologic type	
IDC	64 (78)
ILC	12 (15)
Others	6 (7)
Histologic grade	
1	18 (22)
2	25 (30)
3	39 (48)
Molecular markers	
ER positive	62 (76)
HER2 amplification (FISH)	37 (45)
Triple negative	4 (5)

Data are given as number (percentage) of patients unless otherwise indicated.

IDC: invasive ductal carcinoma; ILC: invasive lobular carcinoma; ER: estrogen receptor

Table S6 PAMmer and sgRNA sequences used in this article

HER2 3’UTR, PAMmer-1	mCCmUGmGAmUAm**CGG**mACmACmCAmUTmGCmUGmUTmCC
HER2 3’UTR, sgRNA-1 spacer sequence	GCUCUGUACAAAGCCUGGAUA
HER2 3’UTR, PAMmer-2	mATmUGmGGmCA**mUGG**mCCmUCmAGmAAmUCmCAmCAmAA
HER2 3’UTR, sgRNA-2 spacer sequence	GCCCUAGAGUCUCAUUGGGCA
HER2 3’UTR, PAMmer-3	mCTmUTmCAmGT**mUGG**mCAmGGmATmCTmGGmAAmGGmAA
HER2 3’UTR, sgRNA-3 spacer sequence	GCUUCCCTAAGGCUUUCAGU
HPRT1 3’UTR, PAMmer-1	mGAmACmATmUG**mUGG**mATmUTmUAmCTmGGmCGmATmGT
HPRT1 3’UTR, sgRNA-1 spacer sequence	GCCACAGAACUAGAACAUUG
HPRT1 3’UTR, PAMmer-2	mAAmAAmCTmUC**mCGG**mGAmUGmCTmGTmCTmUTmGAmUG
HPRT1 3’UTR, sgRNA-2 spacer sequence	GCCAGGACAGAACAAAACUUC
HPRT1 3’UTR, PAMmer-3	mCTmGAmUAmGT**mUGG**mUAmGGmCTmCAmUAmGTmGCmAA
HPRT1 3’UTR, sgRNA-3 spacer sequence	GCCCAAAGGGAACUGAUAGU

POLR2A 3'UTR, PAMmer-1	mAAmGGmCAmUT**mCGG**mGTmACmAAmCGmGAmGCmUGmGG
POLR2A 3'UTR, sgRNA-1 spacer sequence	GAGCUCUGCCACAAGGCAUU
POLR2A 3'UTR, PAMmer-2	mCCmGAmGGmAT**mUGG**mCTmGTmAAmCCmACmUCmACmAG
POLR2A 3'UTR, sgRNA-2 spacer sequence	GCUUUGUUCUUCCCGAGGAU
POLR2A, PAMmer-3	mACmUGmUCmAT**mCCG**mGGmCTmGAmUAmGCmCGmGGmCT
POLR2A, sgRNA-3 spacer sequence	GUUCUCCUCGUCACUGUCAU
λ2 (Negative control), PAMmer	mATmGCmCAmUGm**UGG**mGCmUGmUCmAAmAAmUTmGAmGC
λ2 (Negative control), sgRNA spacer sequence	GUGAUAAGUGGAAUGCCAUG
GAPDH 3'UTR, PAMmer	mAGmUGmAGmGGm**CGG**mCTmCTmCTmUCmCTmCTmUGmUG
GAPDH 3'UTR, sgRNA spacer sequence	GGACUCCCCAGCAGUGAGGG
U6 promoter-sgRNA 16×-MS2 scaffold	GTTTAAGAGCTATGCTGGGCCAACATGAGGATCACCCATGTCTGCAGGGCCCAGCATAGCAAGTTTAAATAAGGCTAGTCCGTTATCAACTTGGCCAACATGAGGATCACCCATGTCTGCAGGGCCAAGTGGCACCGAGTCGGTGCGGATCCTAAGGTACCTAATTGCCTAGAAAACATGAGGATCACCCATGTCTGCAGGTCGACTCTAGAAAACATGAGGATCACCCATGTCTGCAGTATTCCCGGGTTCATTAGATCCTAAGG

	TACCTAATTGCCTAGAAAACATGAGGATCACCCATGTCT GCAGGTCGACTCTAGAAAACATGAGGATCACCCATGTC TGCAGTATTCCCGGGTTCATTAGATCCTAAGGTACCTAA TTGCCTAGAAAACATGAGGATCACCCATGTCTGCAGGT CGACTCCAGAAAACATGAGGATCACCCATGTCTGCAGT ATTCCCGGGTTCATTAGATCCTAAGGTACCTAATTGCCT AGAAAACATGAGGATCACCCATGTCTGCAGGTCGACTC CAGAAAACATGAGGATCACCCATGTCTGCAGTATTCCC GGGTTCATTAGATCCTAAGGTACCTAATTGCCTAGAAAA CATGAGGATCACCCATGTCTGCAGGTCGACTCTAGAAA ACATGAGGATCACCCATGTCTGCAGTATTCCCGGGTTCA TTAGATCCTAAGGTACCTAATTGCCTAGAAAACATGAG GATCACCCATGTCTGCAGGTCGACTCTAGAAAACATGA GGATCACCCATGTCTGCAGTATTCCCGGGTTCATTAGAT CCTAAGGTACCTAATTGCCTAGAAAACATGAGGATCAC CCATGTCTGCAGGTCGACTCCAGAAAACATGAGGATCA CCCATGTCTGCAGTATTCCCGGGTTCATTAGATCTGCGC GCAATAGCAAGTTAAAATAATATTAGTCCGTTTTTAGCG CGTGCGCCAATTCTGCAGGGGCGCC

Table S7 PCR primer and siRNA sequences used in this article (T7 promoter sequence has been underlined.)

HER2 forward primer	TGTGACTGCCTGTCCCTACAA
HER2 reverse primer	CCAGACCATAGCACACTCGG
GAPDH forward primer	ACCCAGAAGACTGTGGATGG
GAPDH reverse primer	TCTAGACGGCAGGTCAGGTC
HPRT1 forward primer	CTGGAAAGAATGTCTTGATTGTG
HPRT1 reverse primer	GACCTTGACCATCTTTGGATTA
POLR2A forward primer	GAGAGTCCAGTTCGGAGTC
POLR2A reverse primer	GTCGTCTCTGGGTATTTGATG
HER2 sgRNA 16×-MS2 forward	TAATACGACTCACTATAGCTCTGTACAAAGCCTGGATA GTTTAAGAGCTATGCTGGGCCAAC
HER2 sgRNA 16×-MS2 reverse	GGCGCCCCTGCAGAATTGGCGCACGCGCTAAAAA
siRNA *HER2* sequence	5’-CACGUUUGAGUCCAUGCCCAA(dTdT)-3’