SUPPLEMENTARY MATERIALS

Fluorescence-based co-detection with protein markers reveals distinct cellular compartments for altered microRNA expression in solid tumors

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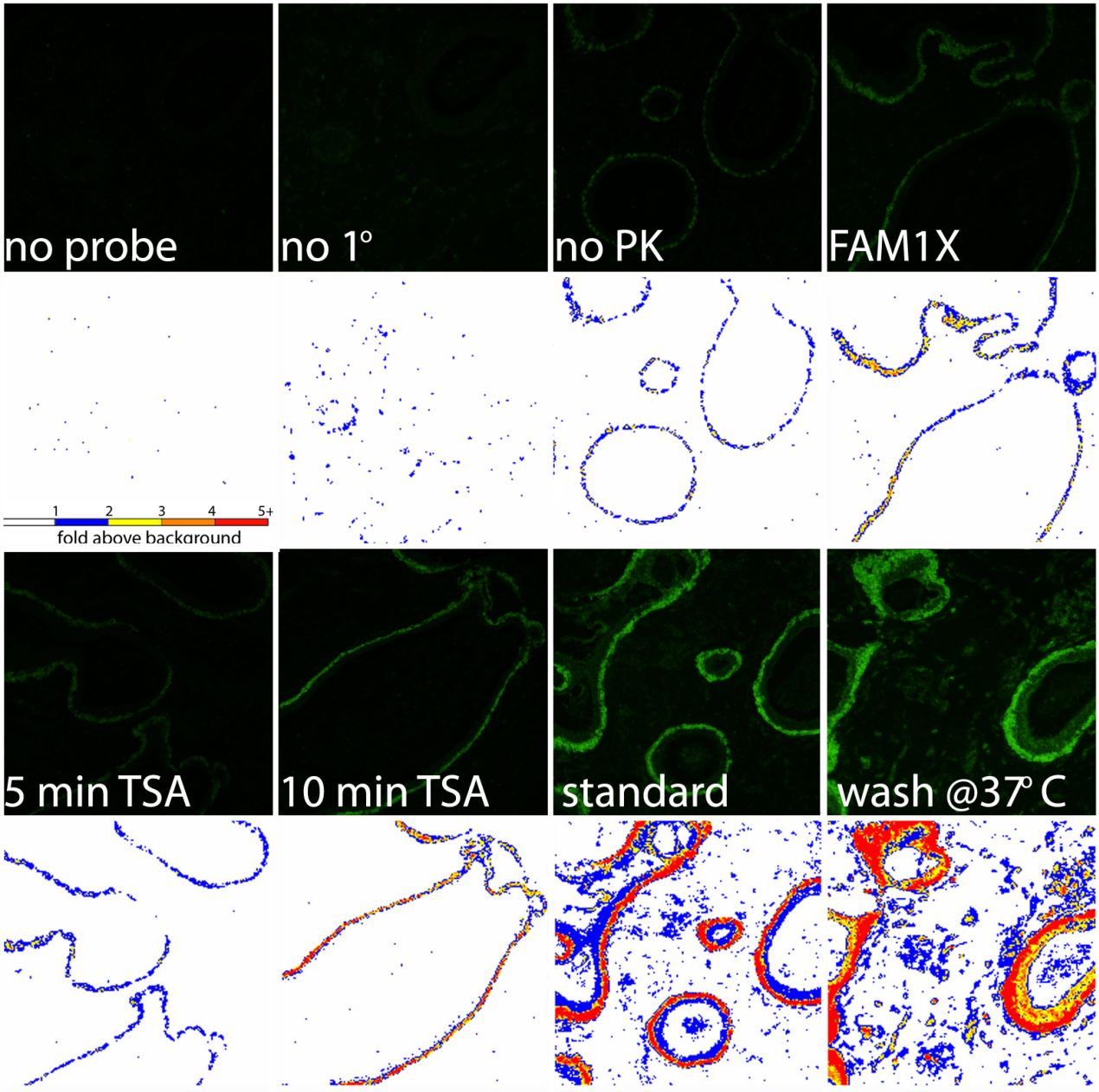
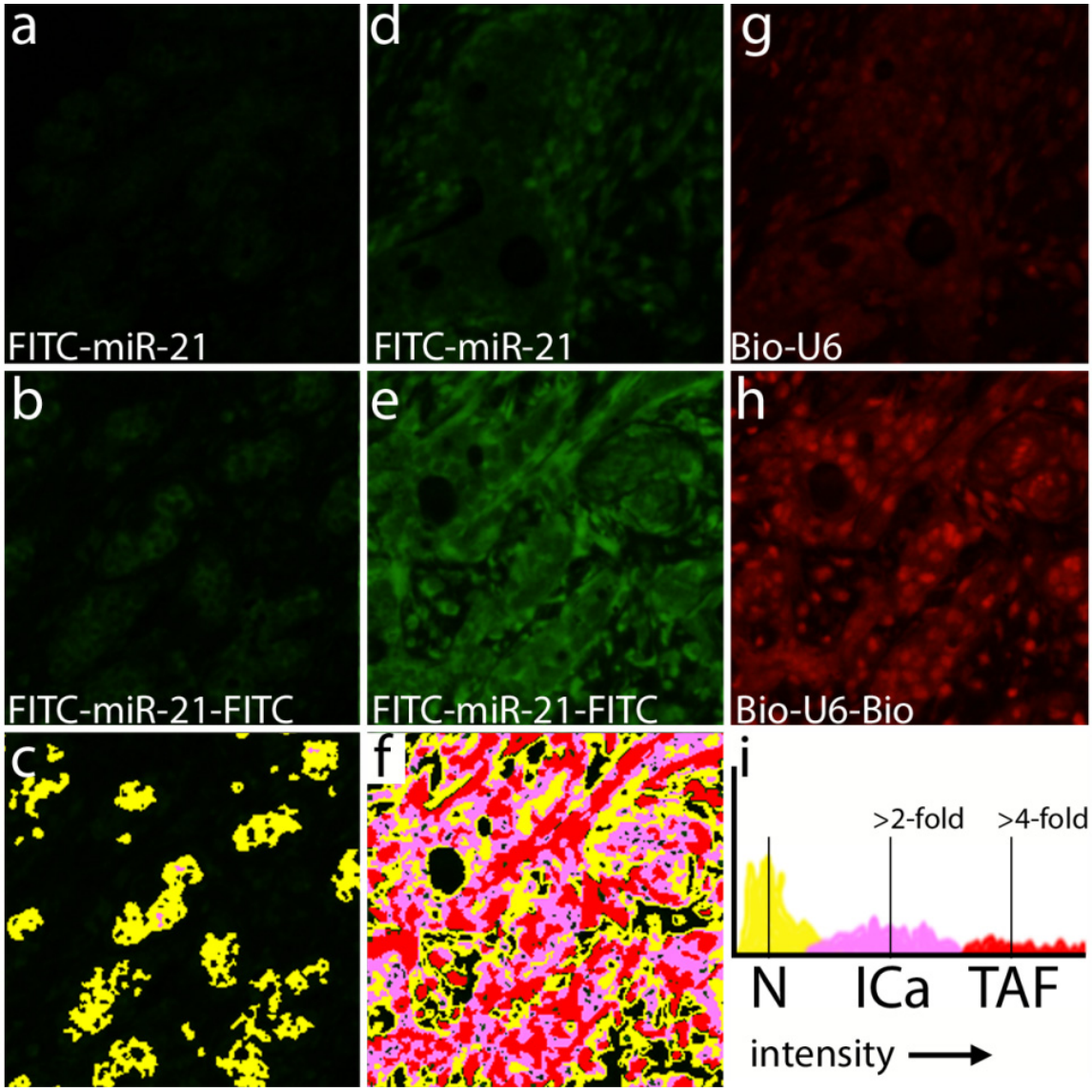
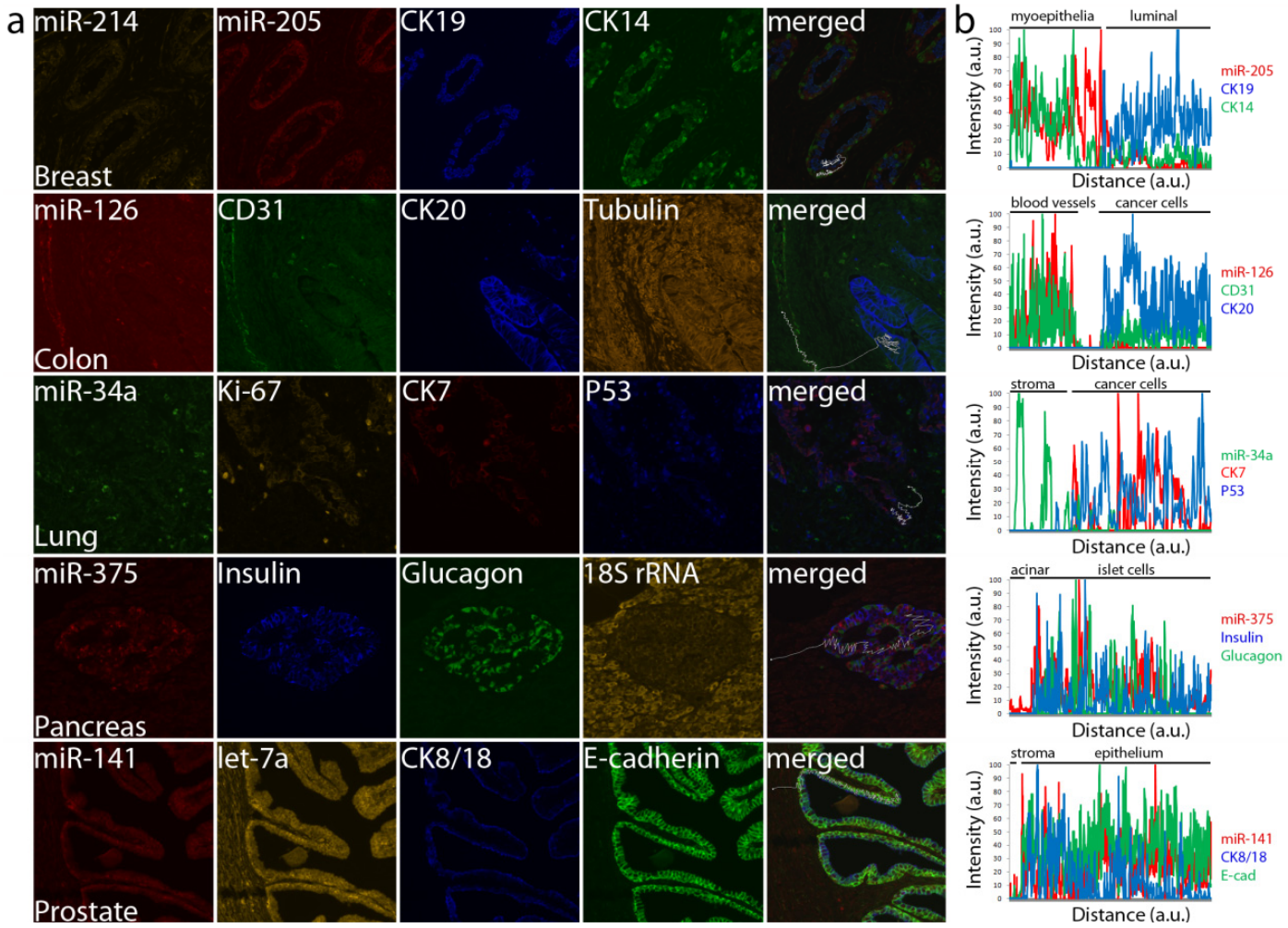


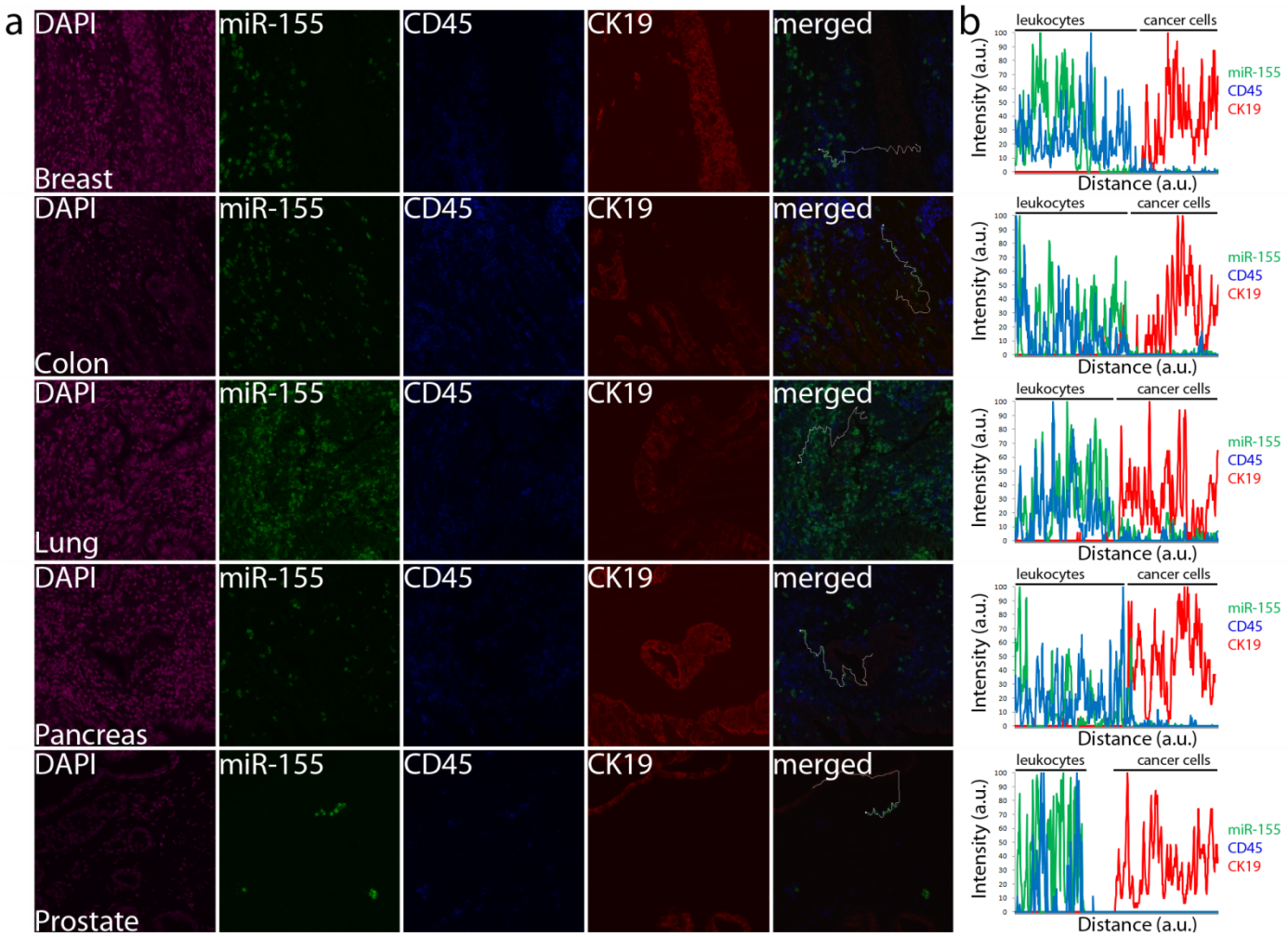
Supplementary Figure 1. Parameter optimization of ISH method. Consecutive 4-μm normal FFPE breast tissue sections were used to detect miR-205 using our standard ISH assay. miR-205 signal was revealed by sequential TSA reactions with FITC-tyramine (green). Deviation from the standard protocol (10 pmol of LNA-modified DNA probe against miR-205, 20 min PK digestion, 1° rabbit anti-Fluorescein/HRP antibody, 2° anti-rabbit/HRP antibody for sandwich amplification, and 15 min TSA reaction) is noted above each image. Upper panel displays raw fluorescent image of normal breast epithelial structures and lower panel displays a heat map generated by Image-Pro Plus software in which each color class highlights pixels within the indicated fold-change range of signal intensity above background (See color scale).



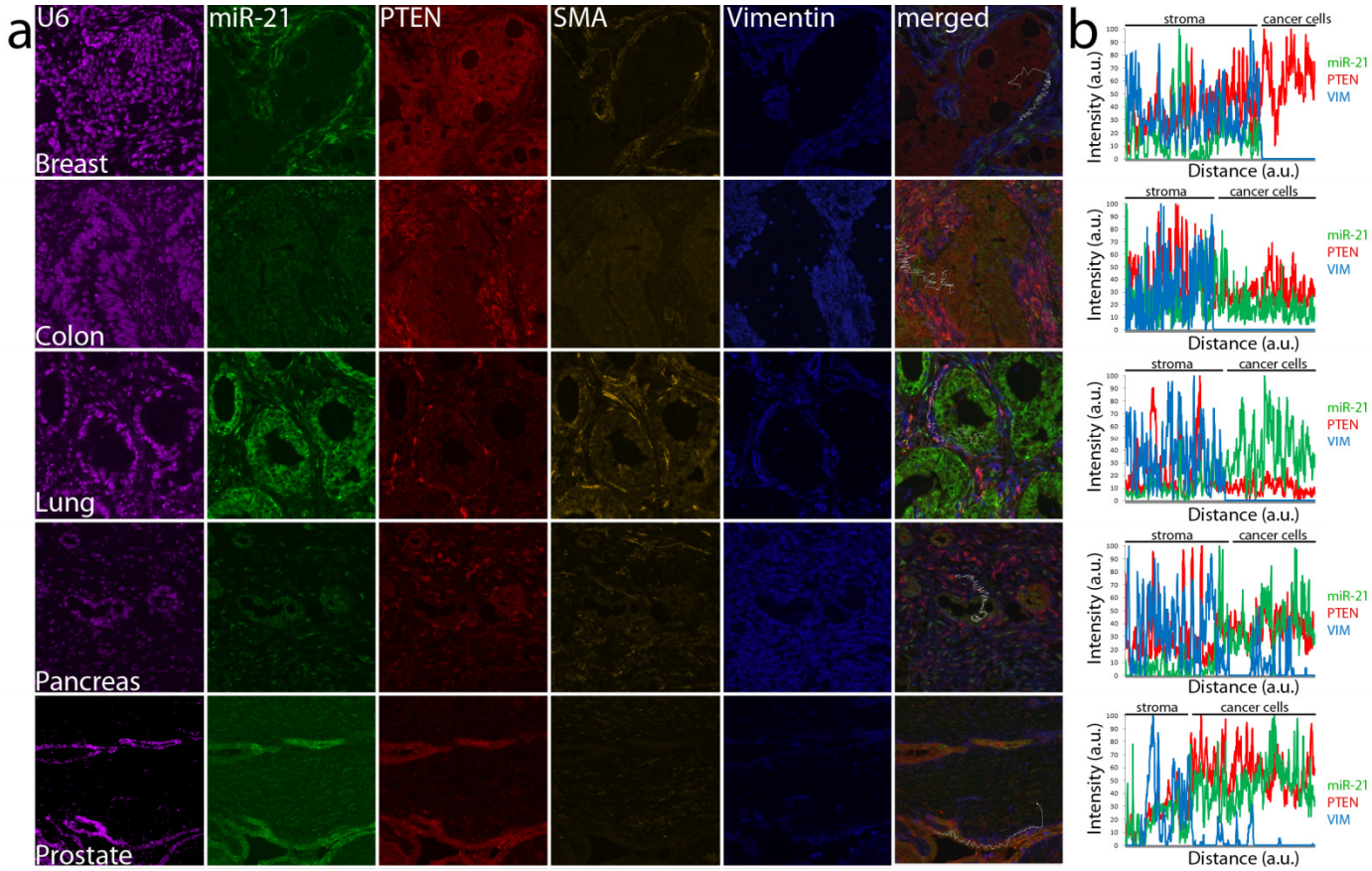
Supplementary Figure 2. Sensitivity of detection is enhanced by incorporation of two terminal hapten moieties. a-h) Probes with one or two hapten moieties were used to co-detect miR-21 and U6 snRNA in consecutive matched normal (**a** & **b**) and tumor (**d** & **e**, **g** & **h**) tissue using our standard ISH assay. A TSA reaction with green fluorochrome substrate (FITC) was carried out to reveal miR-21 expression. Then, HRP was inactivated with H_2O_2 and a second TSA reaction with red fluorochrome substrate (Rhodamine red) was carried out to reveal U6 expression. RNA detection was highly improved by the use of probes 5'- and 3'- terminally tagged with hapten moieties, please compare images of upper and middle panel, which were all captured with the same exposure and gain settings. **c,f)** Signal for miR-21 on images **b** and **e** were quantitated with Image-Pro Plus and displayed as colorized intensity classes on **c** and **f**, respectively. Intensity range of yellow class was set to include all fluorescent objects in normal tissue, pink and red classes encompass fluorescent objects with higher intensity than yellow class. **i)** Histogram displays signal intensity per pixel within a representative area of normal and tumor tissue. Pink class mainly includes signal within cancer cells (ICa) and red class within tumor-associated fibroblasts (TAF).



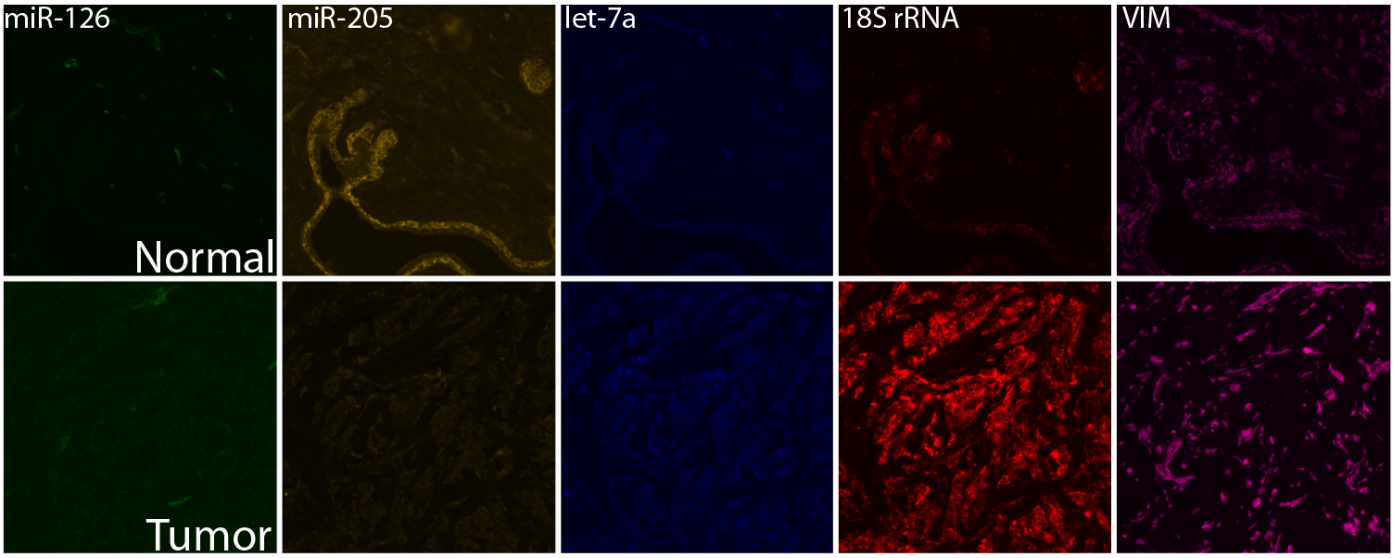
Supplementary Figure 3. Primary data and signal quantification analysis of image panels shown in Figure 2. a) Matching raw fluorescent images of those displayed in Figure 2. Images were captured with an F-view II monochrome camera (Olympus) as grayscale TIF files and later colorized. Color scheme of individual images differs from the one shown in Figure 2; this was needed to conduct expression analysis of indicated miRNAs and proteins. **b)** Green, red and blue images were merged using the combine channels function from cellSens software package and saved as RGB TIF files. Once on Image-Pro software, a free shape line trace (line tool profile analysis) was used to quantitate intensity of indicated miRNA and protein expression. Merged RGB may be shifted with respect to raw images to fully display the line trace. Background intensity was subtracted from the intensity values and intensity values were normalized setting the point with maximum intensity at 100 and calculating other value in relation to this reference. Square dot indicates the beginning of the line profile reading and it corresponds to the most left value of the graph panels.



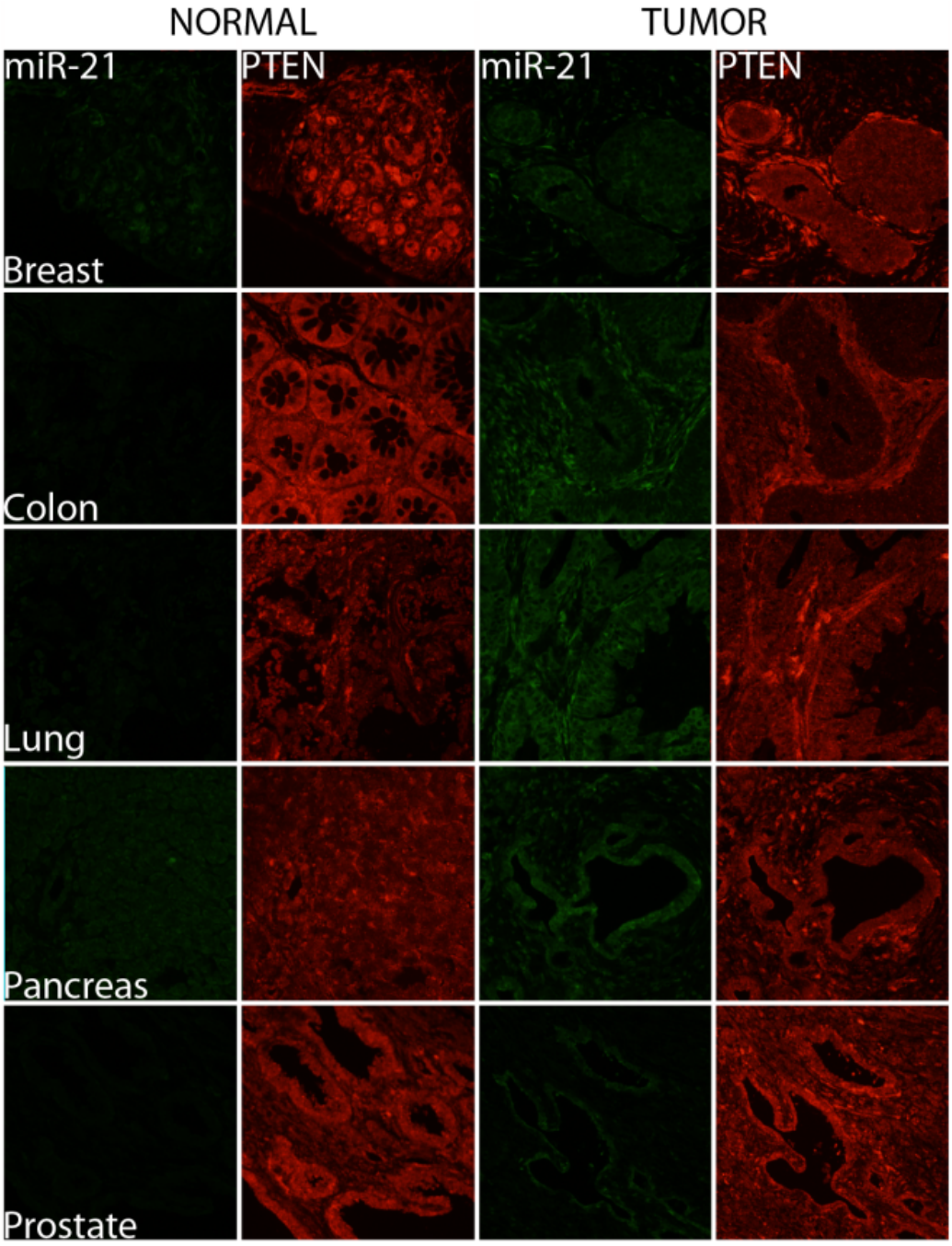
Supplementary Figure 4. Primary data and signal quantization analysis of image panels shown in Figure 3. a) Matching raw fluorescent images of those displayed in Figure 3. Images were captured with an F-view II monochrome camera (Olympus) as grayscale TIFF files and later colorized. Color scheme of individual images differs from the one shown in Figure 3; this was needed to conduct miR-155 (green), CK19 (red) and CD45 (blue) expression analysis with Image Pro-Plus software. **b)** miR-155, CK19 and CD45 images were merged using the combine channels function from cellSens and saved as RGB TIF files. Once on Image Pro, a free shape line trace (line tool profile analysis) was used to quantitate intensity of miR-155, CK19 and CD45 expression. Background intensity was subtracted from the intensity values and intensity values were normalized setting the point with maximum intensity at 100 and calculating other value in relation to this reference. Square dot indicates the beginning of the line profile reading and it corresponds to the most left value of the graph panels.



Supplementary Figure 5. Primary data and signal quantization analysis of image panels shown in Figure 4. a) Matching raw fluorescent images of those displayed in Figure 4. Images were captured with an F-view II monochrome camera (Olympus) as grayscale TIFF files and later colorized. Color scheme of individual images differs from the one shown in Figure 4; this was needed to conduct miR-21 (green), PTEN (red) and Vimentin (blue) expression analysis with Image Pro-Plus software. **b)** miR-21, PTEN and Vimentin (VIM) images were merged using the combine channels function from cellSens and saved as RGB TIF files. Once on Image Pro, a free shape line trace (line tool profile analysis) was used to quantitate intensity of miR-21, PTEN and VIM expression. Merged RGB may be shifted with respect to raw images to fully display the line trace. Background intensity was subtracted from the intensity values and intensity values were normalized setting the point with maximum intensity at 100 and calculating other value in relation to this reference. Square dot indicates the beginning of the line profile reading and it corresponds to the most left value of the graph panels.



Supplementary Figure 6. Co-detection of microRNAs, ribosomal RNA and protein markers in breast tissue. Serial 4-μm FFPE tumor tissue sections of breast tissue were to standard ISH assay using FAM2X-tagged probe against miR-126, BrdU2X-tagged probe against miR-205, DIG2X-tagged against let-7a and Bio2X-tagged probes against 18S rRNA. miR-126, miR-205, let-7a and 18S rRNA signal was revealed by sequential TSA reactions with FITC-tyramine (green), Rhodamine-tyramine (orange), AMCA-tyramine (blue) and Dylight594 (red) substrates. After a HIER with citrate, Vimentin (VIM) expression was revealed by TSA reaction with Dylight680 (magenta). Matched normal and tumor raw fluorescent images for indicated RNA and protein markers were captured with the same exposure and gain settings.



Supplementary Figure 7. miR-21 and PTEN expression in matched normal and tumor tissues. a) Matched normal and tumor tissue of indicated organs were subject to our standard ISH assay to detect miR-21 expression using a FAM2X-tagged probe. A TSA reaction with green fluorochrome substrate (FITC-tyramine) was carried out to reveal miR-21 expression. After HIER with citrate, PTEN expression was revealed with red fluorochrome substrate (Rhodamine-tyramine). Matched normal and tumor raw fluorescent images for miR-21 and PTEN were captured with the same exposure and gain settings for each specific organ. These are an independent set of specimens that those shown in other figures.

RNA	T_m	Probe Sequence	°C[a]	SSC[b]	Expression[c]
let-7a	78.3	AA**+C**TA**+T**A**+C**AA**+C**C**+T**A**+C**TA**+C**CT**+C**A	50	0.5X	various
miR-21	73.8	T**+C**AA**+C**AT**+C**A**+G**T**+C**TG**+A**TA**+A**G**+C**TA	45	0.5X	TAFs, cancer cells
miR-24	80	CT**+G**TT**+C**CT**+G**CT**+G**AA**+C**TG**+A**GC**+C**A	37	0.5X	various
mir-34a	80	A**+C**AA**+C**CA**+G**CT**+A**AG**+A**CA**+C**TG**+C**CA	50	0.1X	epithelia, others
miR-34c-5p	79	GCA**+A**TC**+A**GC**+T**AA**+C**TA**+C**A**+C**TG**+C**CT	37	0.5X	epithelia
miR-125b	77.2	TC**+A**CA**+A**GT**+T**AG**+G**GT**+C**TC**+A**GG**+G**A	45	0.5X	fibroblasts, epithelia
miR-126	73.4	CG**+C**AT**+T**AT**+T**AC**+T**CA**+C**GG**+T**AC**+G**A	45	0.5X	endothelia
miR-141	75	C**+C**AT**+C**TT**+T**AC**+C**A**+G**A**+C**A**+G**TG**+T**TA	37	0.5X	epithelia
miR-143	73.4	GA**+G**CT**+A**CA**+G**TG**+C**TT**+C**AT**+C**T**+C**A	50	0.5X	various
miR-145	79	AAG**+G**GA**+T**TC**+C**TG**+G**GA**+A**AA**+C**TG**+G**AC	50	0.1X	smooth muscle cells
miR-155	74	T**+T**A**+A**T**+G**CT**+A**AT**+C**GT**+G**AT**+A**G**+G**G**+G**T	50	0.5X	leukocytes
miR-196	76.8	CC**+C**AA**+C**AA**+C**AT**+G**A**+A**A**+C**T**+A**C**+C**TA	37	0.5X	cancer cells
miR-200b	73.7	GT**+C**AT**+C**AT**+T**A**+C**CA**+G**G**+C**A**+G**TA**+T**TA	37	0.5X	various
miR-205	75.6	CA**+G**AC**+T**CC**+G**GT**+G**GA**+A**TG**+A**AG**+G**A	50	0.1X	myo/basal epithelia
miR-214	80.4	CT**+G**CC**+T**GT**+C**TG**+T**GC**+C**TG**+C**TGT	50	0.1X	various
miR-221	80.8	GA**+A**A**+C**C**+C**AG**+C**AGA**+C**AA**+T**GT**+A**G**+C**T	37	0.5X	various
miR-375	75	TC**+A**CG**+C**GA**+G**CC**+G**AA**+C**GA**+A**CA**+A**A	45	0.5X	endocrine cells
miR-451	76.7	AAA**+C**T**+C**AG**+T**A**+A**T**+G**G**+T**AA**+C**G**+G**T**+T**T	45	0.5X	erythrocytes
U6 snRNA	75	CGTGTCATCCTTGCGCAGGGGCCATGCTAATCTTCTCTGT	50	0.5X	ubiquitous
18S rRNA	79.3	GGGCAGACGTTCGAATGGGTCGTCGCCGCCACGGG	50	0.5X	ubiquitous

Table S1. miRNA probe sequences and hybridization conditions. Sequences of LNA-modified DNA probes against indicated miRNA or other non-coding RNA species. "**+N**" denotes LNA-modified nucleotide. [a]Incubation temperature of hybridization and washing steps. [b]Concentration of SSC in washing steps. [c]Summary of predominant cell type(s) of miRNA expression in normal and tumor tissues from breast, colon, lung, pancreas and/or prostate. "Various" indicates a complex pattern of expression as regards to the cell type(s) in which the miRNA is expressed and/or the direction of miRNA expression changes within cancer cells.

Epitope	Source	Dilution	Retrieval	Clone name	Cat#	Company
Amylase	Mouse	1:200	AR1	G-10	sc-46657	Santa Cruz Biotechnology
CK5/6	Mouse	1:50	AR1+		CM105C	Biocare
CK7	Mouse	1:100	0	OV-TL12/30	MU255-UC	Biogenex
CK8/18	Mouse	1:50	0	5D3	MU131-UC	Biogenex
CK14	Mouse	1:100	AR2		CM185B	Biocare
CK19	Mouse	1:200	0	RCK108	MU246-UC	Biogenex
CK20	Mouse	1:200	0	Ks20.8	M7019	Dako
CD31	Mouse	1:100	AR1	JC70A	M0823	Dako
CD45	Mouse	1:100	AR1	2D1	560274	BD Pharmingen
E-cadherin	Rabbit	1:200	AR1	24E10	3195	Cell signaling
ER	Mouse	1:10	AR1+	1D5	MU272-UCE	Biogenex
Glucagon	Mouse	1:500	AR1	K79bB10	ab10988	Abcam
HER2	Mouse	1:20	AR1+	CB11	MU134-UC	Biogenex
Insulin	Guinea pig	1:500	0		A564	Dako
Ki-67	Mouse	1:200	AR1	MM1	NCL-L-Ki67-MM1	Novocastra
pAKT	Rabbit	1:50	0	736E11	3787	Cell signaling
P53	Mouse	1:200	AR1	DO-1	sc-126	Santa Cruz Biotechnology
PCNA	Mouse	1:300	AR1+	PC10	NCL-L-PCNA	Novocastra
PR	Mouse	1:25	AR1+	PR88	MU328-UC	Biogenex
PTEN	Rabbit	1:100	AR1	138G6	9559	Cell signaling
Smooth muscle actin	Mouse	1:500	0	1A4	MU128-UC	Biogenex
Somatostatin	Rabbit	1:100	0		18-0078	Zymed
Tubulin	Rat	1:1000	0	YL1/2	Ab6160-100	Abcam
Vimentin	Mouse	1:1000	AR1	V9	MU074-UC	Biogenex
Epitope	**Source**	**Dilution**	**Retrieval**	**Name (clone)**	**Cat#**	**Company**
Biotin	N/A	1:7000	0	Streptavidin/HRP	SA100-01	Invitrogen
BrdU	Rat	1:1000	0	Anti-BrdU	NB500-169	Novus Biologicals
DIG	Sheep	1:1000	0	Anti-DIG/HRP	11207733910	Roche
FITC,FAM	Rabbit	1:200	0	Anti-FITC/HRP	P5100	Dako
Guinea pig Fc	Goat	1:500	0	Anti-guinea pig/Cy3	106-165-003	Jackson ImmunoResearch
Goat Fc	Rabbit	1:500	0	Anti-goat/HRP	172-1034	Biorad
Mouse Fc	Goat	1:500	0	Anti-mouse/HRP	170-6516	Biorad
Rat Fc	Goat	1:1000	0	Anti-rat/HRP	401416	Calbiochem
Rabbit Fc	Goat	1:500	0	Anti-rabbit/HRP	170-6515	Biorad
Sheep Fc	Rabbit	1:500	0	Anti-sheep/HRP	402100	Calbiochem

Table S2. Antibody conditions. Protein detection is compatible with previous ISH procedure without further treatment (0) or followed by heat-induced epitope retrieval by 20 min incubation in 95^{o}C water bath with pre-warmed Citrate-based antigen unmasking solution (AR1; Vector Laboratories, H3300) or Reveal (AR2; Biocare Medical, RV1000MMRTU), or by heating in decloaking chamber (Biocare Medical, DC2002-CE) with default program (1 min at 121 ^{o}C) with Citrate-based antigen unmasking solution (AR1+).

Note: Staining with COX-2 antibody (Cell signaling, cat#4842) and Ki-67 (Abcam, ab833) was not compatible with ISH procedure due to intense non-specific background signal.

Substrate	Dilution (miRNA)	Incubation time (miRNA)	Dilution (protein)	Incubation time (protein)
Alexa Fluor 647	Not determined	Not determined	1:100-1:200	15 min (10-30 min)
AMCA	1:300-1:500	15 min (10-30 min)	1:500-1:1000	10 min (5-20 min)
Dylight405	1:50-1:100	15 min (10-30 min)	1:100-1:200	15 min (10-30 min)
Dylight594	1:50-1:100	15 min (10-30 min)	1:100-1:200	15 min (10-30 min)
Dylight649	Not determined	Not determined	1:100-1:200	15 min (10-30 min)
Dylight680	Not determined	Not determined	1:100-1:200	15 min (10-30 min)
FITC	1:100-1:200	15 min (10-30 min)	1:200-1:400	10 min (5-20 min)
Rhodamine	1:500	15 min (10-30 min)	1:1000	10 min (5-20 min)

Reagent	Cat #	Company
Alexa Fluor 647-NHS ester	A-20006	Invitrogen
AMCA-NHS ester	33005	Pierce
Dylight405-NHS ester	46400	Pierce
Dylight594-NHS ester	46408	Pierce
Dylight649-NHS ester	46415	Pierce
Dylight680-NHS ester	46418	Pierce
Fluorescein-NHS ester	46100	Pierce
Rhodamine-NHS ester	46102	Pierce
Tyramine hydrochloride	T2879	Sigma
Dimethyl formamide (DMF)	D4551	Sigma
Triethylamine (TEA)*	T0886	Sigma

Table S3. Preparation of tyramide substrates and conditions for TSA reaction for microRNA and protein detection. Preparation of these reagents is based on Davidson protocol for FITC tyramide substrate (http://www.xenbase.org/other/static/methods/FISH/fluorescein_tyramide.jsp, but see also Vize et al. 2009(1) for additional information. *Please note that for acetylation reaction we used triethanolamine, which is a different compound.

Coupling Reaction:
Work under a chemical hood in a dry environment. If stock reagents are frozen, let equilibrate at room temperature and wipe with a paper towel the tubes to remove any moisture. Use only fresh reagents:

- Dissolve fluorochrome-NHS ester at 10 mg/mL stock in DMF.
- Prepare DMF-TEA solution (100:1 v/v) as needed.
- Dissolve the tyramine at 10 mg/mL in DMF-TEA.
- Add needed volume of tyramide/DMF-TEA into fluorochrome-NHS ester/DMF to achieve a molar ratio of 1:1.
- Incubate in dark at room temperature for 2 hours.
- Add 1 volume of 100% ethanol and store at -20^{o}C. Use as indicated above.

Note: AMCA-NHS ester (blue), Fluorescein-NHS ester (green), Rhodamine-NHS ester (red) provide a convenient and economic fluorochrome choice for a three-color co-staining scheme using conventional fluorescence microscopy. A series of DyLight-NHS (Pierce) or Alexa Fluor-NHS (Invitrogen) fluorescent compounds with distinct excitation/emission spectra allow for of additional colors. In our hand, miRNA detection with commercially available TSA kits was less sensitive than with our reagents and protocol; this could be due to considerable differences in concentration between the fluorescent substrates in Alexa Fluor® TSA™ kit (Invitrogen) and TSA™ Plus System (PerkinElmer) and our in-house synthesized fluorochromes (data not shown). In our hand, miRNA detection with commercially available TSA kits was less sensitive than with our reagents and protocol; this could be due to considerable differences in concentration between the fluorescent substrates in Alexa Fluor® TSA™ kit (Invitrogen) and TSA™ Plus System (PerkinElmer) and our in-house synthesized fluorochromes (data not shown).

Supplementary References

(1) Vize PD, McCoy KE, Zhou X. Multichannel wholemount fluorescent and fluorescent/chromogenic in situ hybridization in Xenopus embryos. Nat Protoc 2009;4(6):975-83.