ADVANCED MATERIALS

Supporting Information

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Experimental Section

Chemicals. All oligos purified by HPLC were ordered from IDT (Integrated DNA Technologies, Inc., Coralville, IA) in separate tubes. Bst LF polymerase was purchased from New England Biolabs® Inc (Ipswich, MA). dNTP Set, PCR purification kit, GeneRuler 100 bp plus DNA ladder, sulfo-SMCC, 8% TBE PAGE gel, Novex™ Hi-density TBE sample buffer (5X), tris(2-carboxyethyl)phosphine (TCEP), EDTA, 10× PBS (pH 7.4), paraformaldehyde, Qdot™ streptavidin conjugate (emitting at 525, 565, 585, 605, and 655 nm), 0.25% trypsin-EDTA, Ki-67 polyclonal antibody, micro BCA™ protein assay kit, Novex™ tris-glycine mini gels, Novex™ tris-glycine SDS sample buffer (2×), Novex™ tris-glycine SDS running buffer (10×), Spectra™ multicolor broad range protein ladder, PageBlue™ protein stain solution, sheared salmon sperm DNA, BlockAid™ blocking solution, $BS(PEG)_5$, formamide, UltraPure™ SSC (saline sodium citrate) 20×, F(ab')2-goat anti-rabbit IgG secondary antibody QD 585, F(ab')2-goat anti-mouse IgG secondary antibody QD 585, goat anti-rabbit IgG highly cross-adsorbed secondary antibody

Alexa Fluor 555, and goat anti-mouse IgG highly cross-adsorbed secondary antibody Alexa Fluor 555 were purchased from Thermo Fisher (Waltham, MA). Modified Eagle medium (MEM), fetal bovine serum (FBS), penicillin/streptomycin were purchased from Gibco (Waltham, MA). Gel Green was purchased from Biotum (Fremont, CA). Dodecyltrimethylammonium chloride (DTAC), Triton X-100, sodium azide (NaN_3), dextran sulfate (MW>500,000), monoclonal anti-β-tubulin antibody produced in mouse, anti-Lamin A (C-terminal) antibody produced in rabbit, anti-calnexin (C-terminal) antibody produced in rabbit, and anti-HSP90AB1 (Ab-254) antibody produced in rabbit were purchased from Sigma-Aldrich (St. Louis, MO). Tween-20 was ordered from Acros Organics. Fisher BioReagents™ bovine serum albumin heat shock treated (BSA), tris buffered saline 10× solution (10× TBS, pH 7.4), and ammonium chloride (NH_4Cl) were purchased from Fisher Scientific (Hampton, NH).

Instruments. An Olympus IX-71 inverted fluorescence microscope (Center Valley, PA) equipped with a hyperspectral imaging (HSI) camera (Nuance, 420–720 nm spectral range, CRI, now Advanced Molecular Vision, Waltham, MA) and an Olympus true-color charge-coupled device (QColor5) was used for cell imaging. A mercury lamp light source combined with Rhodamine LP set cube (band-pass excitation 530-560 nm, long-pass emission 572 nm, Chroma, Bellows Falls, VT) was used for excitation of the Alexa Fluor dyes, while the wide UV filter cube (band-pass excitation 330-385 nm, long-pass emission 420 nm, Olympus) was used for imaging of all QD probes. An Infinite® 200 plate reader (Tecan, Grödig, Austria) was employed to record absorbance.

PER concatemer synthesis, purification, and characterization. Concatemer extensions were prepared as described previously with minor changes.[1-3] 400-µl reactions in PBS containing 10 mM $MgSO_4$, 800 units mL^{-1} of Bst LF polymerase, 600 µM each of dATP, dCTP and dTTP, 100 nM of Clean.G hairpin

(5'CCCCGAAAGTGGCCTCGGGCCTTTTGGCCCGAGGCCACTTTCG-3'), and 0.75 μM-5 μM of hairpin(s) were prepared. The Clean.G hairpin was designed to have a 5'stretch of C nucleotide. Pre-incubation with Clean.G helps to remove any potential dGTP contamination in dATP, dTTP and dCTP.[1-3] The above mixture was incubated for 20 min at 37 °C, followed by addition of 4 μl of 100 μM bridge*-tt-primer and incubated for another 2-3 h at 37 °C. The reaction was stopped by heating to 80 °C for 20 min to deactivate the polymerase. Details of the extension conditions and hairpin sequences are listed in **Supplementary Table S1**. Each complete bridge*-tt-primer chimera consists of three segments: 1) a 42-bp sequence complementary to the bridge, 2) a TT spacer, and 3) the PER primer (see **Supplementary Table S2** for all bridge*-tt-primer sequences). The PER concatemer solutions were purified and concentrated using a PCR purification kit following the manufacturer's instruction. The length of the concatemers was estimated by comparing with DNA standards on 8% TBE PAGE gel (45 min at 200 V). The gel was stained with Gel Green and the image was captured on Lightools imaging system.

Antibody-DNA bioconjugation. Sulfo-SMCC was used to conjugate the free sulfhydryl on reduced antibodies and amine-terminated bridge oligos (see **Supplementary Table S3** for all bridge oligo sequences). Specifically, 100 μL 1 mg mL^{-1} primary antibodies were reduced with 1 mM TCEP in PBS containing 10 mM EDTA at RT for 1h. The antibodies were then purified using Zeba desalting spin columns prewashed with PBS containing 10 mM EDTA for 3 rounds. In parallel, bridge oligos (100 μL, 40 μM) were activated using 2 mM sulfo-SMCC in PBS at RT for 30 min and purified with NAP5 columns. The reduced antibodies were mixed with a 6-fold molar excess of sulfo-SMCC-activated bridge oligos and reacted at RT for 2h or overnight at 4 °C. The reactions were quenched with 10 mM cysteine for 30 min at RT. The bridge oligo-labeled antibodies were then purified by ultrafiltration with Amicon Ultra centrifugal filters (50 kDa

MWCO) for at least 6 times. Micro BCA™ protein assay kits were used to measure the concentration of the antibody-DNA bioconjugates according to the manufacturer's instruction. The bioconjugates were stored in PBS at 4°C for the short term, or frozen at -20°C with added EDTA, BSA, and sodium azide for long term storage.

Characterization of antibody-DNA bioconjugates. PAGE was used for the analysis of antibody-DNA bioconjugates. Antibodies and antibody-DNA bioconjugates were denatured in a tris-glycine SDS sample buffer with 50 mM TCEP at 85 °C for 2 min and left to cool to room temperature. The samples were run on Novex™ tris-glycine mini gels at 225 V for 45 min with a running buffer of tris-glycine SDS. The gels were stained with the PageBlue™ protein staining solution according to the manufacturer's manual.

Preparation of QD-imager probes. QD streptavidin conjugates (emission peaks center at 525, 565, 585, 605, and 655 nm) and biotinylated imager oligos were used for the preparation of QD-imager probes. The biotinylated imager sequences follow the general structure of 5-biotin-tt-primer*-t-primer*-t-inverted dT-3' (see **Supplementary Table S4** for imager oligo sequences). For each QD-imager probe, biotinylated ssDNA (0.6 μL, 10 μM) and QD-streptavidin (6 μL, 1 μM) were incubated in a 0.2 mL PCR tube for at least 30 min at RT. The QD-imager was then blocked with 2 mM biocytin for 20 min. For optimization studies, the molar ratios tested for QD-streptavidin and biotinylated oligos were: 1:1, 1:3, 1:5, and 1:7.

Cell culture and processing. HeLa cells (human cervical cancer cells, ATCC) were grown in MEM culture medium with 10% FBS and 1% penicillin/streptomycin. They were seeded on 24-well plate glass-bottom and cultured in a humidified atmosphere at 37°C with 5% CO_2. When the density of cells reached 80-90%, the cells were rinsed with TBS, fixed with 4% (w/v) paraformaldehyde in TBS for 20 min at 37°C, quenched with 100 mM NH_4Cl in TBS for 20 min,

followed by washing with 1× TBS (2 min x 2). DTAC (2% w/v in TBS) was used to permeabilize cells for 20 min followed by permeabilization with Triton X-100 (0.25% v/v, TBS) for another 5 min. The cells were rinsed five times in TBS for 3 min each. The fixed cells were used immediately for staining or stored in TBS with 0.03% NaN_3 at 4 °C (no loss of antigens for weeks).

Single-cell immuno-staining using SABER. Cells were blocked with a blocking buffer (BlockAid™ blocking solution with 0.05% (w/v) dextran sulfate and 0.2 mg mL^{-1} sheared salmon sperm DNA) for 45 min with gentle rotation. Samples were incubated with the oligo encoded primary antibodies diluted to a final concentration of 2 μg mL^{-1} in 300 μL staining buffer (0.05% dextran sulfate, 0.2 mg mL^{-1} sheared salmon sperm DNA and 4 mM EDTA in BlockAid™ blocking solution) overnight at 4 °C. The cells were brought back to 37 °C for 30 min, washed three times with pre-warmed (37 °C) 1× PBS containing 2% BSA and 0.1% Triton X-100 for 10 min, and with PBS (5 min x 2). The antibodies were fixed onto the samples using $BS(PEG)_5$ in PBS at RT for 30 min. The crosslinking reaction was quenched with 100 mM NH_4Cl in PBS for 5 min and washed with PBS (5 min x 2). The incubation with concatemers (133 nM) was performed in a hybridization buffer (10% dextran sulfate, 20% formamide, 0.2 mg mL^{-1} sheared salmon sperm DNA, 0.1% Tween-20 (v/v in 2×SSC) for 3 h at 37 °C. The samples were washed with 45% formamide in 1× PBS for 5 min at RT, followed by prewarmed 0.1% Triton X-100 in PBS at 37 °C (10 min x 3). The QD imager (20 nM) or organic dye imager (AF546, 1 μM) (see **Supplementary Table S5** for all AF546 imager sequences) was added to the sample in PBS containing 0.1% Triton X-100 for 1 h at RT, followed by rinsing with 0.1% Triton X-100 in PBS (5-min) and PBS (3 min x 2). As needed, an endogenous biotin blocking kit can be applied before incubation with the blocking buffer.

Single-cell immuno-staining using secondary antibodies. After labeling with the primary antibodies as described above, specimens were incubated with either QD585-2'Ab (20 nM) or Alexa Fluor 555-2'Ab (4 μg ml^{-1}) for 1 h.

Imaging and signal analysis. The Nuance image analysis software was used to unmix the acquired spectral images based on the reference spectra of individual QD components along with an additional channel for background autofluorescence.[4, 5] Average signal intensities for each color excluding blank non-stained areas were calculated. True color images were recorded with the Qcolor5 camera. Low-magnification images were obtained with a 40x oil objective and high-magnification with a 100x oil-immersion objective (NA 1.40, Olympus).

Crosstalk characterization for multicolor staining. Crosstalk study was performed by staining Lamin A using two-color QD-imager probes emitting at 525 and 585 nm. The detailed protocol is described above except that immediately before imager hybridization, one fully assembled QD-imager probes were mixed with QD-streptavidin of the other color.

Multicycle imaging with QD-SABER. The general protocol is the same as described above, except that between cycles, 55 % formamide in PBS was used to de-hybridize the QD-imager (37 ^{o}C, 5 min x 3) and the residual trace amount of QDs was quickly quenched with 0.1 M sodium acetate at pH 5.5. The bottom of the wells was marked with a permanent reference point for spatial registration cross different cycles.

QD-SABER application on human prostate cancer FFPE sections. De-identified human prostate FFPE tissue slides were used. Note that according to the published regulations by OHRP (Office for Human Research Protection), the current project did not obtain data through intervention or interaction with living individuals, or identifiable private information, thus did not involve human subjects (45 CFR, part 46 does not apply). The tissue sections were deparaffinized

using fresh xylene. The slides were rehydrated with 100%, 95%, 70%, 50% ethanol 2 times for 3 min each, respectively, and rinsed with deionized water for 5 min 2 times. DAKO® target retrieval solution was used for epitope antigen retrieval. Briefly, the antigen retrieval buffer in a Coplin jar was firstly heated to boiling using a microwave oven. The slides were then placed into the Coplin jar and heated to boiling again. The Coplin jar with slides was transferred to a water bath at 95 °C and maintained for 30 min, followed by cooling for 30 minutes.

For staining, sections were rinsed with 1×PBS for 15 min. A hydrophobic pen (ImmEdge Hydrophobic Barrier PAP Pen; Vector Laboratories, H-4000) was used to draw a circle on the slide around the tissue. The slides were enclosed in a removable chamber. β-tubulin was chosen as a model target. QD-SABER was conducted using a similar protocol to cell staining except that the imager hybridization was extended to 2 h. For comparison, the tissue sections were also stained using the conventional two-step IHC (1'Ab + 2'Ab-dye). A final concentration of 4 μg/mL of Alexa Fluor 555-2'Ab was diluted in the BlockAid™ Blocking Solution and used in the second step. The samples were imaged on an IX-71 inverted fluorescence microscope equipped with a true-color charge-coupled device (QColor5, Olympus), and analyzed by image J.

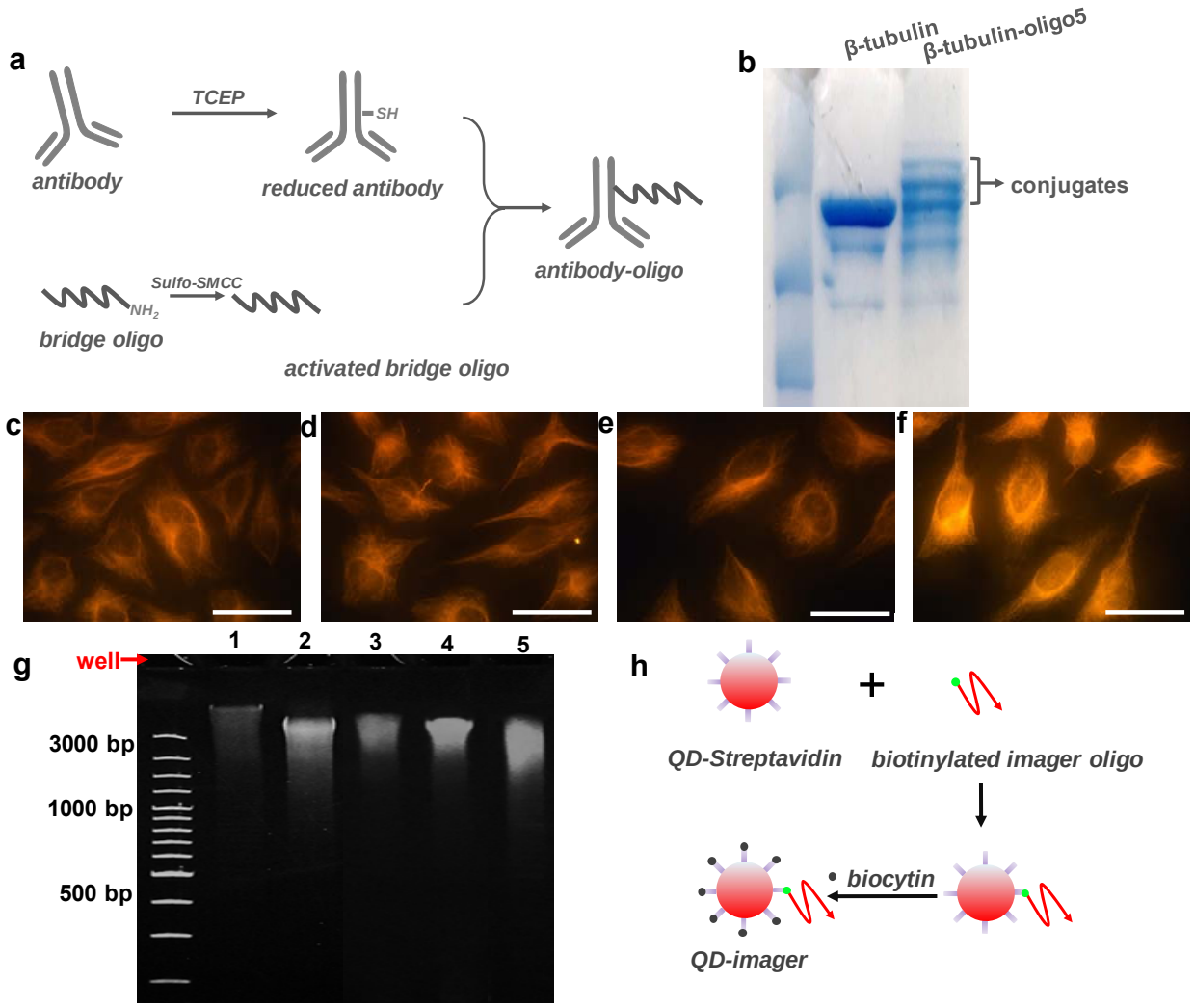

Figure S1. Antibody and QD encoded with oligonucleotides. **a)** TCEP was used to partially reduce primary antibody to expose the sulfhydryl groups for conjugation with amine-functionalized bridge oligos. Bridge oligos with an amine terminal group were activated with sulfo-SMCC. **b)** PAGE analysis of the primary Ab-oligo bioconjugates showing that most antibody molecules have 1-2 copies of bridge oligos. Staining of β-tubulin via incubation first with **c)** mouse anti-β-tubulin 1'Ab, **d)** TCEP-reduced mouse anti-β-tubulin 1'Ab, or **e)** mouse anti-β-tubulin primary antibody-bridge oligo, followed by staining with goat anti-mouse 2'Ab-Alexa Fluor 555, showing no major differences of activity after the chemical treatments. **f)** Specific staining of β-tubulin via SABER (AF546-labelled imager probe). All fluorescent micrographs were obtained with a Qcolor5 camera (100x objective) with consistent exposure times. Scale bar, 50 µm. **g)** PAGE gel proving the formation of concatemers (red arrow shows the position of the loading wells). **h)** Preparation of the QD-imager probe by simple mixing QD-streptavidin and biotinylated imager oligo followed by blocking with biocytin.

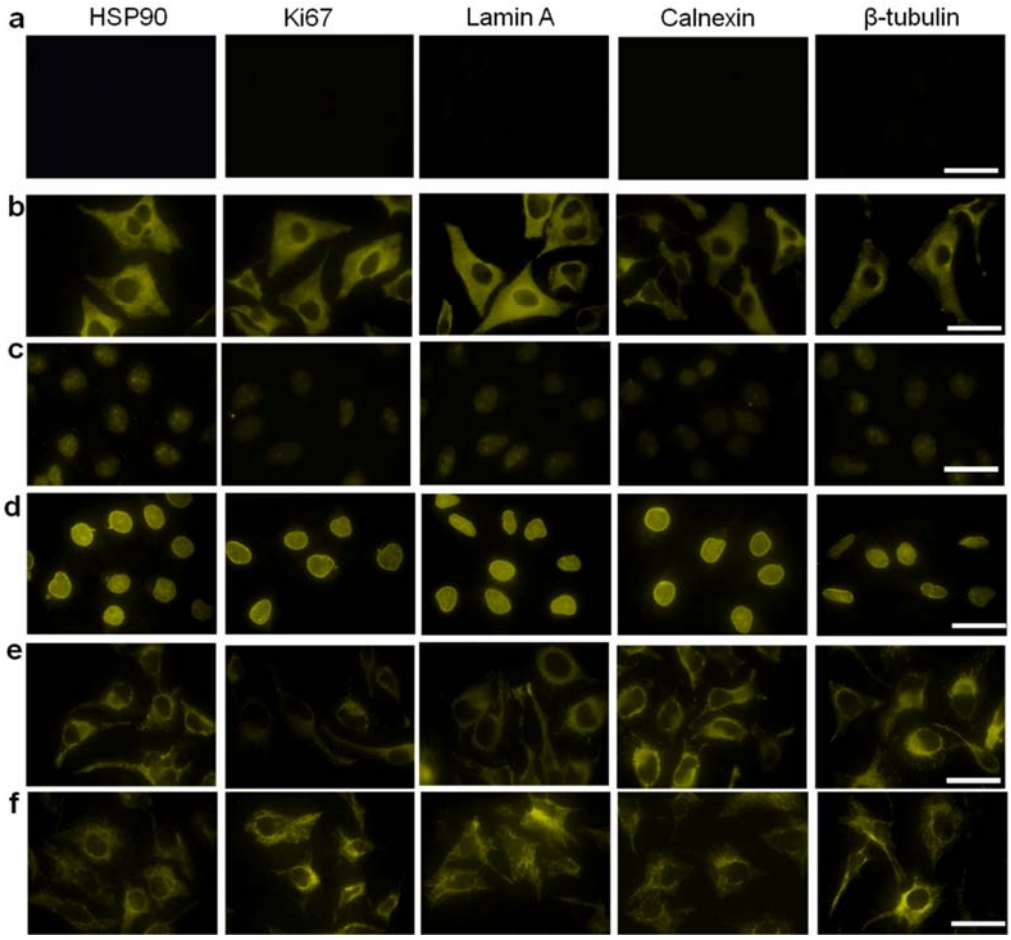

Figure S2. a) Control experiment for QD-SABER staining of HeLa cells. **b-f)** Additional microscopy views of Figure 1d (b-f: HSP90, Ki67, Lamin A, Calnexin, and β-tubulin, respectively) The experiment conditions were the same as Figure 1d bottom panels, except the absence of primary antibody-oligo conjugates for **a**). Scale bar, 50 μm.

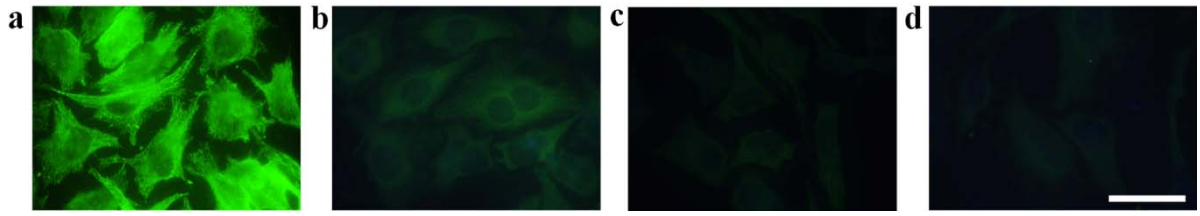

Figure S3. Optimization of the ratio between QD-streptavidin and biotinylated imager oligo by testing ratios of (a) 1:1, (b) 1:3, (c) 1:5 (c), and (d) 1:7. β-tubulin was stained in 4 separate specimens using QD_{565}-streptavidin mixed with the imager oligos. Staining intensity decreases with increasing biotinylated imager oligo concentration due to the existence of free oligos that compete with QD-oligos. Scale bar, 50 μm.

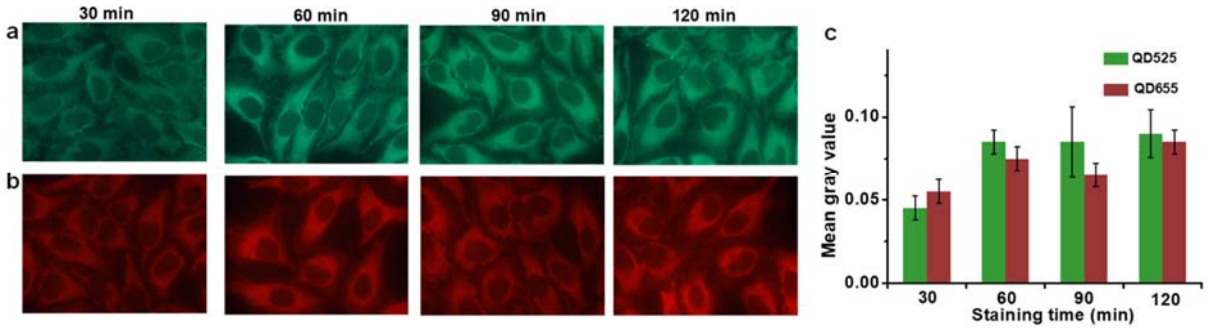

Figure S4. Staining kinetics using QDs of different sizes in Qdot-SABER. HSP90 was stained with either **a)** QD_{525}-imager (the smallest inorganic core) or **b)** QD_{655}-imager (the largest inorganic core) for various incubation times. The imaging exposure time for the green QDs and red QDs was set at 20 ms and 5 ms, respectively, because the red QDs are a brighter probe. Both probes demonstrated a similar trend of signal increase over time, indicating similar binding kinetics. **c)** Image J-based quantitative analysis showed a fast increase in staining intensity in the first hour of incubation and a slow increase over extended incubation.

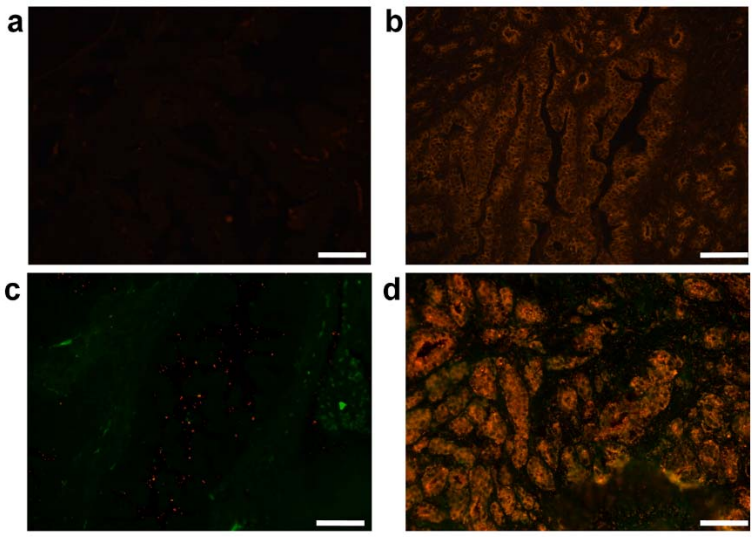

Figure S5. Staining of β-tubulin in FFPE prostate tissue sections using conventional two-step IHC and QD-SABER. Conventional IHC a) control without the 1'Ab, and b) with both the 1'Ab and AF_{555}-modified 2' Ab. c) Control experiment of QD-SABER without the 1'Ab. d) QD-SABER. Under the same exposure time, QD-SABER offers significantly brighter signals for better visualization. Scale bar, 100 μm.

Table S1. Concatemer extension conditions and hairpin sequences, obtained from Ref. [2-3].

ID	Hairpin sequence	Hairpin concentration	Time
hairpin1	AAATACTCTCGGGCCTTTTGGCCCGAGAGTATTTGAGAGTATT/3InvdT/	5 μM	2h
hairpin2	ACCAATAATAGGGCCTTTTGGCCCTATTATTGGTTATTATTGG/3InvdT/	1.5 μM	3h
hairpin 3	AATCCCTATCGGGCCTTTTGGCCCGATAGGGATTGATAGGGAT/3InvdT/	1 μM	3h
hairpin 4	ACAACTTAACGGGCCTTTTGGCCCGTTAAGTTGTGTTAAGTTG/3InvdT/	3 μM	2h
hairpin 5	ACATCATCATGGGCCTTTTGGCCCATGATGATGTATGATGATG/3InvdT/	0.75 μM	3h

Table S2. Sequences of bridge*-tt-primer, obtained from Ref. [2].

ID	Bridge* (5'-3')	spacer	Primer
bridge*-tt-primer1	TAATACAAAGCGCATACGGGAAAATGCGGAAAGAGCGAGGAC	TT	AATACTCTC
bridge*-tt-primer2	GGAGAGGAATAGGATCGTACAGTGGATAAGGCGGCGATAACG	TT	CCAATAATA
bridge*-tt-primer3	TGGTGTGGCTCGTGTCCGAAATGGTGGAGTGTACGGCGTTAC	TT	ATCCCTATC
bridge*-tt-primer4	TACAAGCGGGAACACGAGTTAGACATAGAACGGGCCAAATTC	TT	CAACTTAAC
bridge*-tt-primer5	AACGCAATACCCAGACGAGACAACACGCGAAGACTTAAGATA	TT	CATCATCAT

Table S3. Amine-modified bridge DNA sequences used for Ab conjugation, obtained from Ref. [2]. All bridge ssDNAs have a 3' terminal amine group /5AmMO/ for bioconjugation separated from the pairing sequence by a 3T oligonucleotide spacer for antibody-coupling.

ID	Bridge DNA sequence (5'-3')	Antibody
bridge oligo1	GTCCTCGCTCTTTCCGCATTTTCCCGTATGCGCTTTGTATTATTT/3AmMO/	HSP90
bridge oligo2	CGTTATCGCCGCCTTATCCACTGTACGATCCTATTCCTCTCCTTT/3AmMO/	Ki67
bridge oligo3	GTAACGCCGTACACTCCACCATTTCGGACACGAGCCACACCATTT/3AmMO/	Lamin A
bridge oligo4	GAATTTGGCCCGTTCTATGTCTAACTCGTGTTCCCGCTTGTATTT/3AmMO/	Calnexin
bridge oligo5	TATCTTAAGTCTTCGCGTGTTGTCTCGTCTGGGTATTGCGTTTTT/3AmMO/	β-tubulin

Table S4. List of biotinylated imager oligo sequences. [2,3] All ssDNA imagers have a 5' terminal biotin group /5BiosG/ for bioconjugation separated from the pairing sequence by a 2T oligonucleotide spacer for QD-coupling

ID	biotinylated imager oligo
biotinylated imager oligo1	/5BiosG/TTGAGAGTATTTGAGAGTATTT/3InvdT/
biotinylated imager oligo2	/5BiosG/TTTATTATTGGTTATTATTGGT/3InvdT/
biotinylated imager oligo3	/5BiosG/TTGATAGGGATTGATAGGGATT/3InvdT/
biotinylated imager oligo4	/5BiosG/TTGTTAAGTTGTGTTAAGTTGT/3InvdT/
biotinylated imager oligo5	/5BiosG/TTATGATGATGTATGATGATGT/3InvdT/

Table S5. List of AF546 modified imager oligo sequences.[2,3]

ID	AF546 imager oligo
AF546 imager oligo1	/5Alex546N/TTGAGAGTATTTGAGAGTATTT/3InvdT/
AF546 imager oligo2	/5Alex546N/TTTATTATTGGTTATTATTGGT/3InvdT/
AF546 imager oligo3	/5Alex546N/TTGATAGGGATTGATAGGGATT/3InvdT/
AF546 imager oligo4	/5Alex546N/TTGTTAAGTTGTGTTAAGTTGT/3InvdT/
AF546 imager oligo5	/5Alex546N/TTATGATGATGTATGATGATGT/3InvdT/

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