SUPPORTING INFORMATION FOR:

Fully Automated Protein Proximity Assay in FFPE Tissue using Caged Haptens

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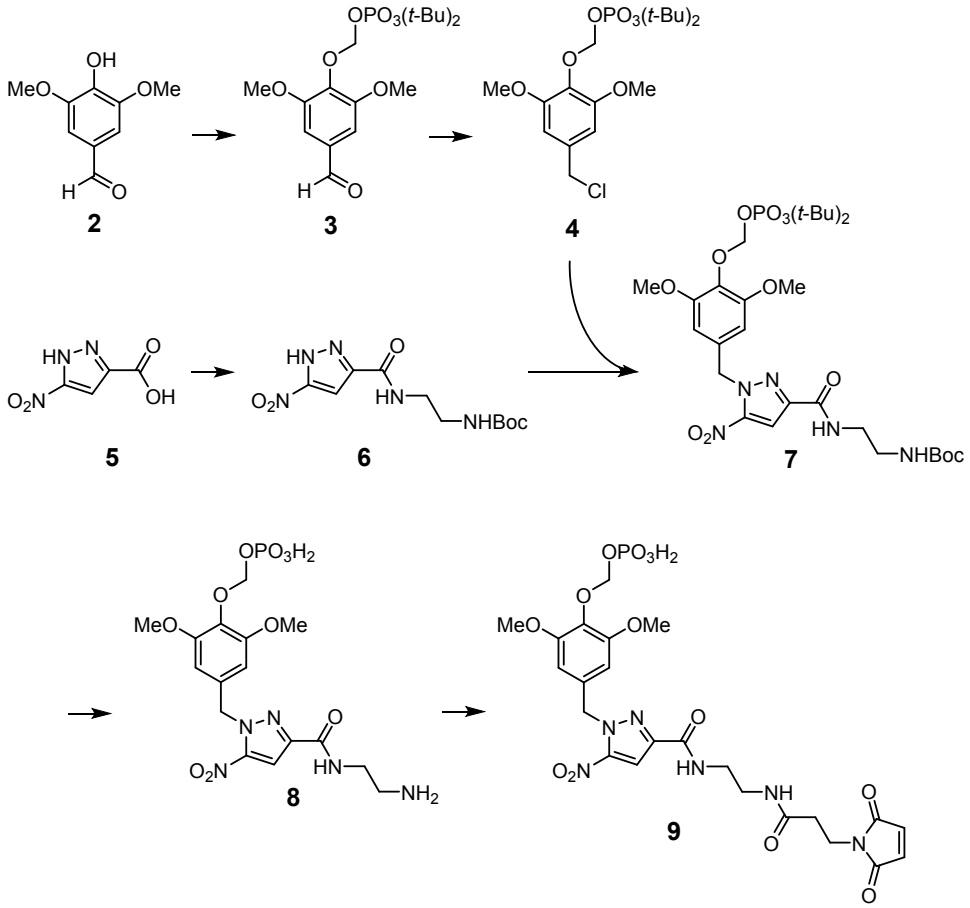
Synthetic Materials and Methods. NMR data was collected on a Bruker 400 MHz Spectrometer running Topspin (Bruker). Chemical shifts were referenced to the deuterated solvent resonance for ^{1}H (7.26 ppm for $CDCl_3$, 2.50 ppm for DMSO-d_6) and ^{13}C (77.0 ppm for $CDCl_3$, 39.51 ppm for DMSO-d_6). MS data was collected on a Waters Acquity QDa (ESI) running Empower 3 (Waters). Analytical HPLC was performed using Waters XBridge columns on a Waters Alliance e2695 running Empower 3 (Waters). Prep HPLC was performed with Waters SunFire columns (Prep C18 OBD 10 µm, 50 mm x 250 mm) on a Waters 2535 running Empower 3. Flash chromatography was performed on a Biotage Isolera One using Biotage SNAP Ultra C18 (reverse-phase) or Biotage SNAP KP-Sil (normal phase). All chemicals were purchased from commercial suppliers and used as received unless otherwise noted.



Scheme S1. Synthesis of caged nitropyrazole

Compound 4. A solution of 3,5-dimethoxy-4-hydroxybenzaldehyde (**2**, 1.0 eq), potassium carbonate (5.0 eq), and di-tert-butyl chloromethyl phosphate (1.5 eq) in DMF (2 mL per mmol **2**) was heated to 60 °C in an oil bath with stirring for 4h (monitored by HPLC to confirm reaction was > 95% complete). The reaction mixture was quenched by addition of 1M HCl and the organic layer was separated and collected. An additional quantity of EtOAc was added and the organics were extracted with 1M HCl, saturated $NaHCO_3$, and brine. The organic layer was dried over $MgSO_4$ and the solvents removed under reduced pressure to give compound **3** as a light brown viscous oil.

The crude **3** was dissolved in THF (5 mL per mmol **1**) followed by addition of $NaBH_4$ (1.5 eq). The reaction was stirred at RT for 4h (monitored by HPLC to confirm the reaction was > 95% complete). The reaction mixture was diluted with EtOAc followed by slow addition of 1M HCl until bubbling ceased. The organic layer was separated, followed by washing with 1M HCl, saturated $NaHCO_3$, and brine. The organic layer was dried over $MgSO_4$ and the solvents removed under reduced pressure to give a light brown viscous oil. The residue was dissolved in CH_2Cl_2 (5 mL per mmol **2**) followed by cooling to 0 °C in an ice bath under an N_2 atmosphere. $SOCl_2$ (2.5 eq) was then slowly added, and the reaction mixture was allowed to warm to RT. The reaction mixture was then stirred at RT for 1h (monitored by HPLC to confirm reaction was > 95% complete) and quenched by addition of saturated $NaHCO_3$. An additional quantity of CH_2Cl_2 was added and the organics were extracted with 1M HCl, saturated $NaHCO_3$, and brine. The organic layer was dried over $MgSO_4$ and the solvents removed under reduced pressure to give compound **4** as a light brown, viscous oil. MS (ESI) m/z $(M+H)^+$ calcd for $C_{18}H_{31}ClO_7P^+$ 425.1, found 425.2. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.40 (s, 18H), 3.85 (s, 6H), 4.53 (s, 2H), 5.56 (d, 2H, J = 8.0 Hz), 6.60 (s, 2H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 29.67, 29.71, 29.82, 29.86, 29.93, 46.7, 56.1, 82.56, 82.63, 91.8, 91.9, 105.6, 134.0, 134.1, 153.2.

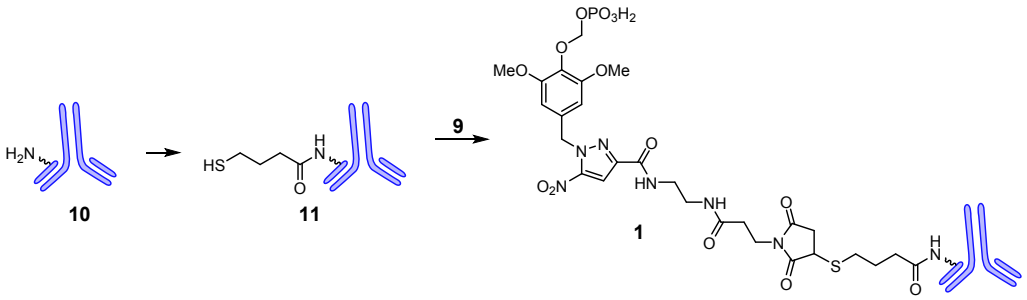
Compound 6. To a stirred soln. of 5-nitro-3-pyrazolecarboxylic acid (**5**, 1.0 eq) in DMF (16 mL per mmol **5**) was added TEA (1.5 eq) and DMAP (1.5 eq), followed by N,N'-disuccinimidyl carbonate (DSC, 1.5 eq). The formation of the NHS ester was followed by HPLC and additional 0.1 eq N,N'-disuccinimidyl carbonate was added until the reaction was complete. N-Boc-ethylenediamine (1.5 eq) was then added and the reaction mixture stirred at RT for 15 min. The reaction was monitored by HPLC and additional 0.1 eq N-boc-ethylenediamine was added until the reaction was complete. The reaction mixture was diluted with EtOAc (16 mL per mmol **5**) followed by slow addition of 1M HCl (16 mL per mmol **5**). The organic layer was separated, followed by washing with 1M HCl, saturated $NaHCO_3$, and brine. The organic layer was dried over $MgSO_4$ and the solvents removed under reduced pressure to give compound **6** as a white solid. MS (ESI) m/z $(M+H\text{-}Boc)^+$ calcd for $C_6H_{10}N_5O_3^+$ 200.1, found 200.1. ^{1}H NMR (dimethyl sulfoxide-d_6, 400 MHz): δ 1.37 (s, 9H), 3.11 (q, 2H, J = 6.0 Hz), 3.29 (q, 2H, J = 6.0 Hz), 6.94 (t, 1H, J = 6.0 Hz), 7.57 (s, 1H), 8.78 (t, 1H, J = 6.0 Hz), 14.81 (s, 1H). ^{13}C NMR (dimethyl sulfoxide-d_6, 100 MHz): δ 28.7, 31.2, 36.2, 78.2, 101.8, 140.1, 156.2, 158.0, 162.8.

Compound 7. To a solution of compound **6** (1.0 eq), sodium carbonate (5.0 eq), and tetrabutylammonium bromide (1.5 eq) in $CHCl_3$ (2 mL per mmol **6**) was added compound **4** (1.5 eq). The reaction vessel was sealed and the reaction mixture was heated to 80 °C in an oil bath with vigorous stirring for 4h (monitored by HPLC to confirm reaction was >

95% complete). The reaction was removed from the oil bath and was allowed to cool to RT. The reaction mixture was diluted with EtOAc followed by slow addition of 1M HCl until bubbling ceased. The organic layer was separated, followed by washing with 1M HCl and brine. The organic layer was dried over $MgSO_4$ and the solvents removed under reduced pressure to give a light brown viscous oil. The crude oil was purified by flash chromatography (hexanes:EtOAc) to give compound **7** as a light brown viscous oil. MS (ESI) m/z $(M+H)^+$ calcd for $C_{29}H_{47}N_5O_{12}P^+$ 688.3, found 688.5. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 1.38 (s, 18H), 1.44 (s, 9H), 3.40 (m, 2H), 3.57 (m, 2H), 3.82 (s, 6H), 5.04 (s, 1H), 5.54 (d, 2H, J = 8.0 Hz), 5.71 (s, 2H), 6.53 (s, 2H), 7.52 (s, 1H), 7.56 (s, 1H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 28.3, 29.59, 29.64, 40.2, 40.6, 53.4, 56.2, 57.1, 79.8, 82.6, 82.7, 91.7, 91.8, 105.1, 107.2, 130.8, 134.4, 144.8, 146.0, 153.4, 156.8, 160.3.

Compound 8. Compound **7** was dissolved in a 9:1 mixture of CH_2Cl_2:TFA (5 mL per mmol **7**) and the resulting reaction mixture was stirred at RT for 30 min (monitored by HPLC to confirm reaction was >95% complete). MS (ESI) m/z $(M+H)^+$ calcd for C16H23N5O10P$^+$ 476.1, found 476.3. ^{1}H NMR (dimethyl sulfoxide-d_6, 400 MHz): δ 3.20 (t, 2H, J = 5.8 Hz), 3.33 (m, 2H), 3.70 (t, 2H, J = 5.8 Hz), 3.80 (s, 6H), 5.48 (d, 2H, J = 11.3 Hz), 5.78 (s, 2H), 6.63 (s, 2H), 7.58 (s, 1H). ^{13}C NMR (dimethyl sulfoxide-d_6, 100 MHz): δ 36.8, 39.5, 56.4, 56.5, 56.6, 91.57, 91.61, 104.9, 105.2, 106.4, 131.8, 134.1, 144.1, 146.3, 153.2, 162.0.

Compound 9. Compound **8** was diluted with toluene, followed by removal of the solvents under reduced pressure. The residue was suspended in DMF (2 mL per mmol **8**), followed by addition of triethylamine (5 eq) and finally 3-maleimidopropionic acid NHS ester (1.1 eq). The reaction vessel was sealed and the reaction mixture was vigorously stirred at RT for 4h (check HPLC to confirm reaction completion). The reaction mixture was then diluted with MeOH and directly purified by prep RP-HPLC (0.05% TFA in H_2O:MeCN 99:1 to 5:95 over 40 min) to give compound **8** as a light yellow solid. MS (ESI) m/z $(M+H)^+$ calcd for $C_{23}H_{28}N_6O_{13}P^+$ 627.1, found 627.3. ^{1}H NMR (dimethyl sulfoxide-d_6, 400 MHz): δ 2.33 (t, 2H, J = 7.2 Hz), 3.18 (q, 2H, J = 6.0 Hz), 3.28 (q, 2H, J = 6.0 Hz), 3.61 (t, 2H, J = 7.1 Hz), 3.73 (s, 6H), 5.35 (d, 2H, J = 9.3 Hz), 5.72 (s, 2H), 6.57 (s, 2H), 7.00 (s, 2H), 7.61 (s, 1H), 8.09 (t, 2H, J = 5.6 Hz), 8.59 (t, 2H, J = 5.6 Hz). ^{13}C NMR (dimethyl sulfoxide-d_6, 100 MHz): δ 34.49, 34.55, 38.6, 39.0, 56.5, 56.6, 78.0, 91.2, 91.3, 105.3, 106.9, 132.3, 133.9, 135.0, 145.3, 146.6, 153.3, 159.9, 170.2, 171.3.

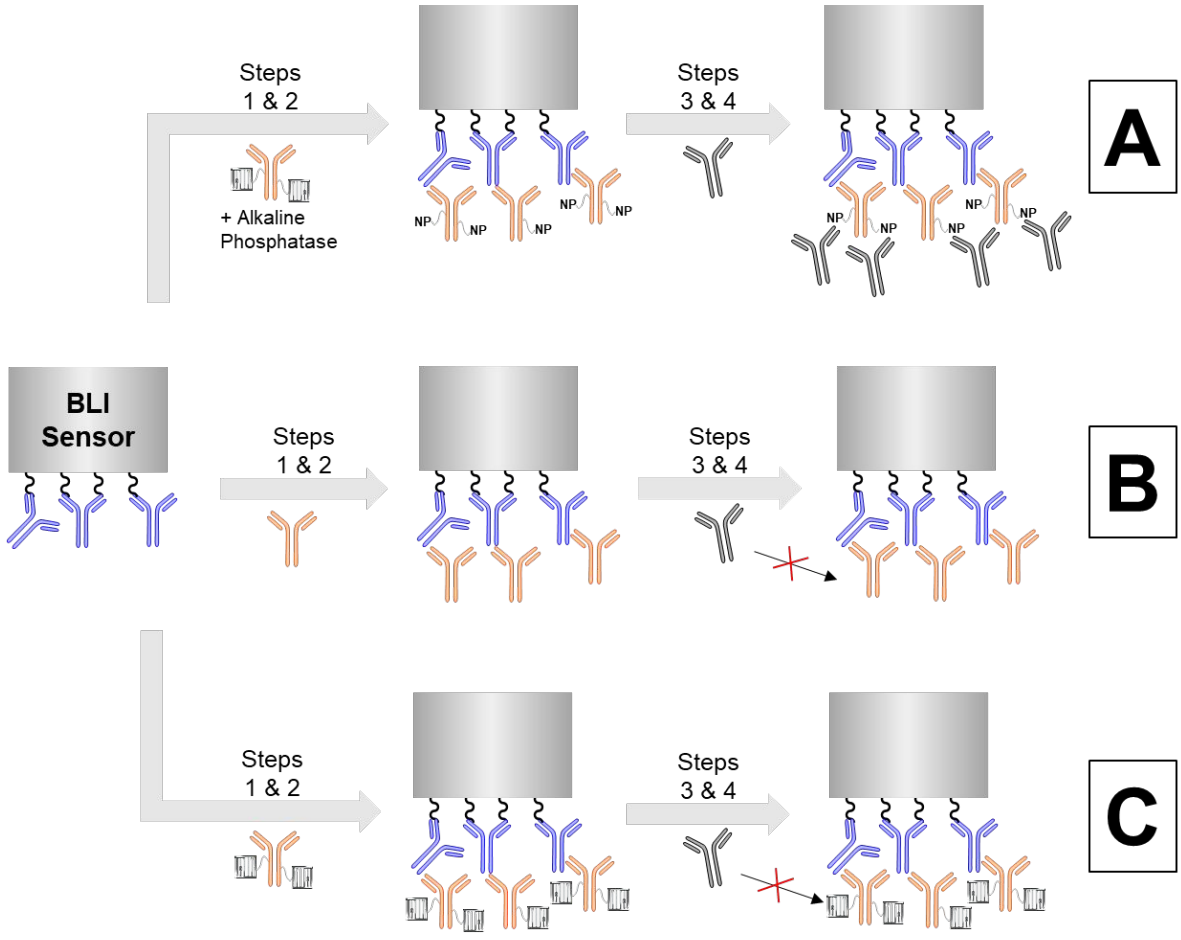


Scheme S2. Conjugation of caged nitropyrazole to antibody (IgG)

Antibody conjugate preparation. To 20 mg of goat-anti-rabbit IgG (**10**) in 2 mL of 1x PBS (pH 7.2) was added EDTA to a final concentration of 10 mM followed by 2 mg of Traut's reagent (2-iminothiolane hydrochloride). The reaction mixture was kept at room temperature for 1h followed by size exclusion chromatography purification (AKTA, Superdex 200 10/300 GL column) with 1x PBS (pH 7.2), containing 10 mM EDTA. To the combined fractions of thiolated antibody (**11**, adjusted to 6 mg/ml) were added 4.2 mg of compound **9** in 0.2 mL of DMF. The reaction mixture was kept at room temperature for 3h followed by size exclusion chromatography purification (AKTA, Superdex 200 10/300 GL column) with 1x PBS (pH 7.2) to give caged nitropyrazole-modified antibody (**1**, 3.7 mg/mL) with approximately 6 haptens per antibody, as calculated based on extinction coefficient of 6,520 L mol^{-1} cm^{-1} at 310 nm.

BLI Experiment

The assay was adapted from previous literature (Kumaraswamy *et al*, *Methods Mol. Biol.* 2015). The experiments were carried out at ambient temperatures. Briefly, the caged hapten goat-anti-rabbit antibody conjugate **1** in the presence of alkaline phosphatase (Experiment A), goat-anti-rabbit antibody (Experiment B), or the caged hapten goat-anti-rabbit antibody conjugate **1** (Experiment C) (25 μg mL^{-1} each) was immobilized on the tip surface of a rabbit antibody-coated sensors in phosphate buffer saline (PBS) for 300 s. Unbound antibodies were removed from the surface of the sensors by incubation in PBS buffer for 120 s. Note that in Experiment A, stickiness of the Alkaline Phosphatase caused an apparent increase in signal in Step 1, relative to controls B & C. This was also observed in control experiments not reported here. Next, the antibody-coated sensors were incubated with 25 μg mL^{-1} of a mouse-anti-NP antibody for 300 s (association). After association step, the sensors were dipped into PBS buffer for 300 s, and the bound mouse-anti-NP antibody was allowed to come off the antibody conjugate **1** (dissociation). The binding response was measured in real time on a sensorgram.



Scheme S3. Illustration of molecular interactions at sensor tip for BLI experimental designs A, B, and C.

General immunohistochemistry (IHC) protocols.

All IHC staining experiments were carried out on a VENTANA DISCOVERY® Ultra automated tissue staining platform. The reagents used in these protocols were from Roche Tissue Diagnostics (Tucson, AZ, USA; "RTD") unless otherwise specified.

Single IHC general procedure

All formalin fixed, paraffin embedded (FFPE) tissue and cell line samples were mounted on Superfrost Plus glass slides (Fisher Scientific, #12-550-15). These were deparaffinized using 1x EZ Prep (RTD, #950-101) and heat induced epitope retrieval (HIER), or antigen retrieval (AR), was performed with Cell Conditioning 1 (CC1, RTD, #950-124). For single IHC control experiments the staining was carried out as described in the package insert for each antibody. The general steps after deparaffinization and AR were: (1) inactivation of endogenous peroxidases with Inhibitor CM (RTD, 760-4307); (2) incubation with the primary antibody (37 °C, time ranging from 8 – 32 minutes depending on the antibody); (3) incubation with a goat-anti-species secondary antibody conjugated to horse radish peroxidase (HRP); (4) detection with 3,3'-diaminobenzidine (DAB), hydrogen peroxide (H_2O_2), and toning with copper; (5) counterstaining with Hematoxylin II (RTD, #790-2208) and Bluing (RTD, #760-2037) to stain the nuclei; (6) dehydration with gradient alcohols and xylenes, followed by coverslipping. The slides were washed with 1x Reaction Buffer (RTD, #950-300) between each of the assay incubation steps.

Table S1: Conditions for single IHC controls.

Antibody	Antibody species	Clone	Product	AR time	Incubation time
PSA	Mouse	ER-PR8	RTD #760-4271	24 mins	16 mins
E-Cadherin	Rabbit	EP700Y	RTD #760-4440	32 mins	16 mins
Beta-Catenin	Mouse	14	RTD #760-4242	60 mins	32 mins
EGFR	Rabbit	5B7	RTD #790-4347	60 mins	16 mins
c_MET	Rabbit	SP44	RTD #790-4430	60 mins	16 mins
GRB2	Mouse	81	BD Transduction Laboratories #610112 *(Diluted 1:100 in Antibody Diluent RTD #251-018)*	60 mins	32 mins

IHC experimental for Figure 4

To confirm that the caging group of the caged hapten-antibody conjugate is present and blocking an anti-hapten antibody from binding to the respective native (i.e. uncaged) hapten, the caged hapten conjugate was tested with single IHC detection. This confirms the ability of the caging group to block anti-hapten antibody recognition, and the ability to uncage the hapten with an enzymatic step. To look for very low levels of recognition of the caged hapten, a biomarker with a high expression level was selected, on FFPE samples with very high signal for the native detections.

Caged hapten – with enzymatic decaging step (Figure 4(H))

FFPE prostate tissue was deparaffinized and antigen retrieved (CC1, 24 minutes). Mouse-anti-PSA (clone ER-PR8) (RTD, #760-4271) antibody incubation (37°C, 16 minutes) and washing were followed by secondary antibody incubation with a goat polyclonal anti-mouse antibody conjugated to multiple caged nitropyrazoles (37°C; 12 minutes). The sample was then exposed to conditions to facilitate the enzymatic dephosphorylation and elimination of the caging group. To achieve this 100 µL of a 500 mM solution of Tris buffer (pH 10.0), 100 µL of a 1 mM solution of $MgCl_2$, and 100 µL of a 50 µg/mL solution of alkaline phosphatase in water were added to ~ 300 µL of residual Reaction Buffer. The slide was warmed up to 45°C for 32 minutes. After washing, the sample was incubated with a mouse-anti-NP HRP conjugate (37°C; 12 minutes). Tyramide amplification was performed with an Amp HQ kit (RTD, 760-052, 37°C, 8 minutes), followed by incubation with a mouse-anti-HQ HRP conjugate (RTD, #760-4602, 37°C, 8 minutes). The signal was visualized with DAB and the stained tissue sections were then counterstained. The slides were then dehydrated through a graded ethanol series, cleared with xylene, and coverslipped.

Caged hapten – no enzymatic decaging step (Figure 4(G))

The no enzyme control was carried out as described for experiment with enzymatic decaging, with the exception that the alkaline phosphatase solution was replaced with only water in the 'decaging step'. The conditions for this step were 100 µL of a 500 mM solution of Tris buffer (pH 10.0), 100 µL of a 1 mM solution of $MgCl_2$, and 100 µL of water, *with no alkaline phosphatase*, added to ~ 300 µL of residual Reaction Buffer. The slide was warmed up to 45°C for 32 minutes.

Proximity IHC general procedure

All formalin fixed, paraffin embedded (FFPE) tissue and cell line samples were mounted on Superfrost Plus glass slides (Fisher Scientific, #12-550-15). These were deparaffinized using 1x EZ Prep (RTD, #950-101) and heat induced epitope retrieval (HIER), or antigen retrieval (AR), was performed with Cell Conditioning 1 (CC1, RTD, #950-124). The general steps after deparaffinization and AR are: (1) inactivation of endogenous peroxidases with Inhibitor CM (RTD, #760-4307); (2) co-incubation with the primary antibodies (37 °C, time ranging from 8 – 32 minutes depending on the antibodies); (3) incubation with a goat-anti-mouse secondary antibody conjugated to alkaline phosphatase (AP); (4) incubation with a goat-anti-rabbit secondary antibody conjugated to caged haptens; (5) establish decaging conditions onslide: 100 µL of a 500 mM solution of Tris buffer (pH 10.0) and 100 µL of a 1 mM solution of magnesium chloride added to ~ 300 µL of residual Reaction Buffer. Warm up slide to 45°C for 32 minutes; (6) incubation with a mouse-anti-hapten HRP conjugate; (7) signal amplification with tyramide-HQ and H_2O_2 (RTD, #760-052); (8) incubation with a mouse-anti-HQ HRP conjugate (RTD, #760-4602); (9) detection with 3,3'-diaminobenzidine (DAB), hydrogen peroxide (H_2O_2), and toning with copper; (10) counterstaining with Hematoxylin II (RTD, #790-2208) and Bluing (RTD, #760-2037) to stain the nuclei; (11) dehydration with gradient alcohols and xylenes, followed by coverslipping. The slides were washed with 1x Reaction Buffer (RTD, #950-300) between each of the assay incubation steps.

Table S2: Primary antibody conditions for proximity assays

Antibody (Species)	AR time	Incubation time
E-Cadherin (Rabbit) **Beta-Catenin (Mouse)**	60 mins	32 mins
EGFR (Rabbit) **Beta-Catenin (Mouse)**	60 mins	32 mins
EGFR (Rabbit) **GRB2 (Mouse)**	60 mins	32 mins
c-MET (Rabbit) **GRB2 (Mouse)**	60 mins	32 mins

Proximity IHC experimental for Figure 5(B) – E-Cadherin:Beta-Catenin positive proximity

FFPE tonsil tissue was deparaffinized and antigen retrieved (CC1, 60 minutes). Rabbit-anti-E-Cadherin and mouse-anti-Beta-catenin were co-incubated (37°C, 32 minutes). After washing, a goat polyclonal anti-mouse antibody conjugated to alkaline phosphatase was applied (37°C; 12 minutes). Following washing, the sample was incubated with a goat polyclonal anti-rabbit antibody conjugated to multiple caged nitropyrazoles (37°C; 12 minutes). The sample was then exposed to conditions to facilitate the enzymatic

dephosphorylation and elimination of the caging group. To achieve this 100 µL of a 500 mM solution of Tris buffer (pH 10.0), and 100 µL of a 1 mM solution of $MgCl_2$, were added to ~ 300 µL of residual Reaction Buffer. The slide was warmed up to 45 °C for 32 minutes. After washing, the sample was incubated with a mouse-anti-NP HRP conjugate (37°C; 12 minutes). Tyramide amplification was performed with an Amp HQ kit (RTD, 760-052, 37°C, 8 minutes), followed by incubation with a mouse-anti-HQ HRP conjugate (RTD, #760-4602, 37°C, 8 minutes). The signal was visualized with DAB and the tissue sections were then counterstained. The slides were dehydrated through a graded ethanol series, cleared with xylene, and coverslipped.

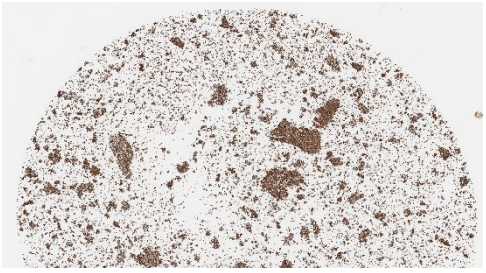
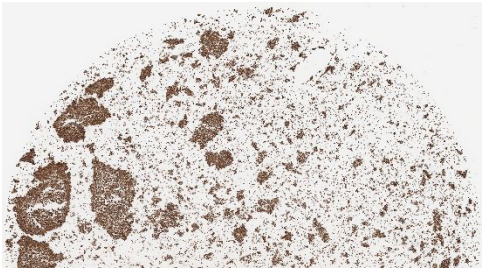
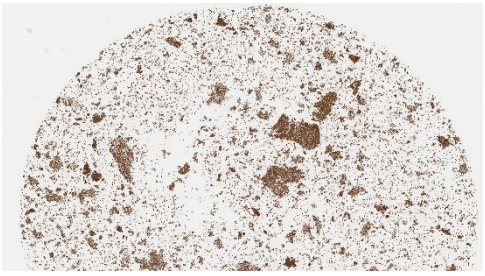
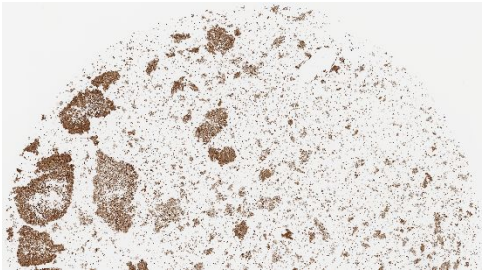
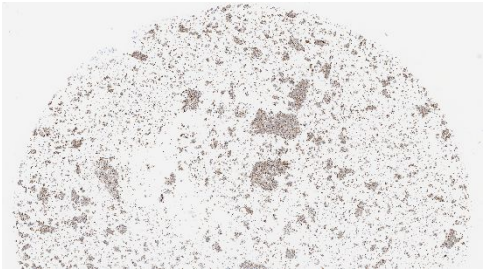
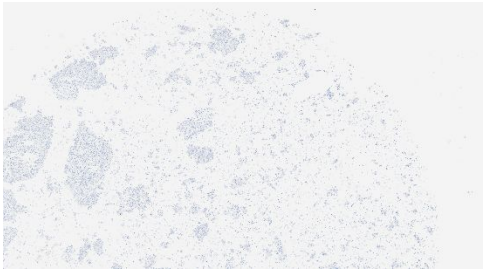
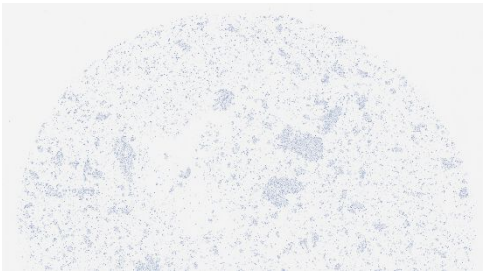
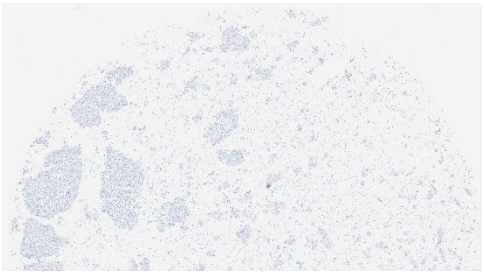
	No drug treatment	Drug treated
EGFR Single IHC		
GRB2 Single IHC		
EGFR GRB2 Proximity assay		
EGFR only Proximity Assay control		

Figure S1: Single IHC and proximity assay images of PC9 cells lines treated with DMSO (no drug) *or* erlotinib (drug). 2x magnification.

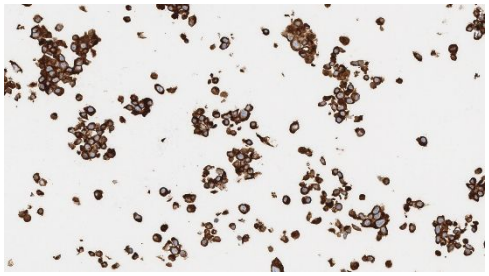
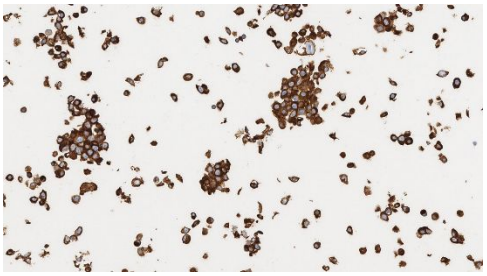
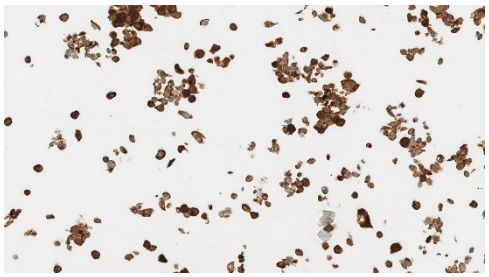
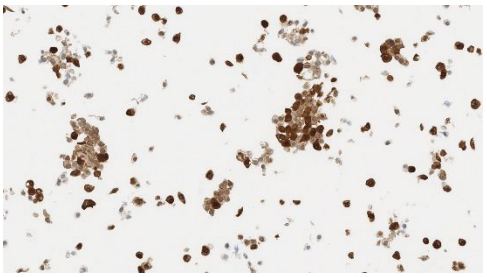
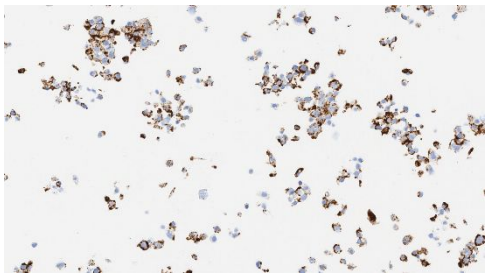
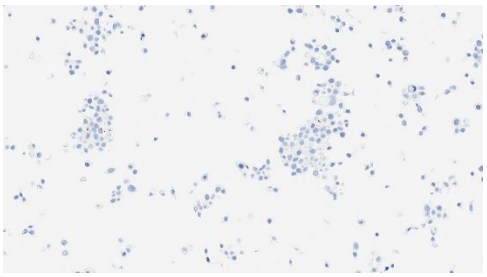
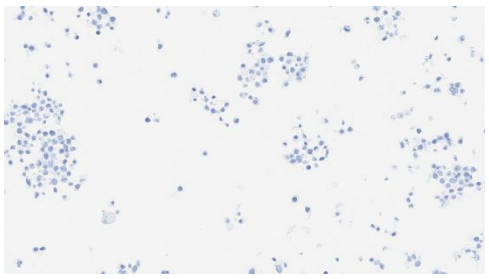
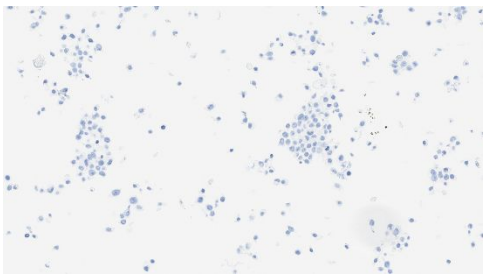
	No drug treatment	Drug treated
EGFR Single IHC		
GRB2 Single IHC		
EGFR GRB2 Proximity assay		
EGFR only Proximity assay control		

Figure S2: Single IHC and proximity assay images of PC9 cells lines treated with DMSO (no drug) *or* erlotinib (drug). 20x magnification.

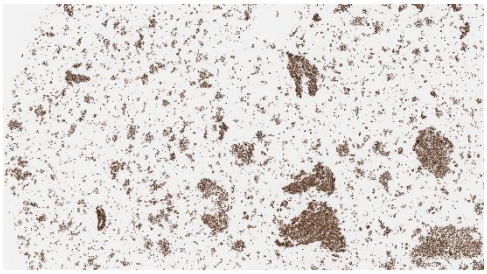
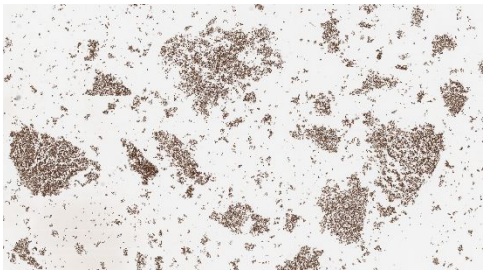
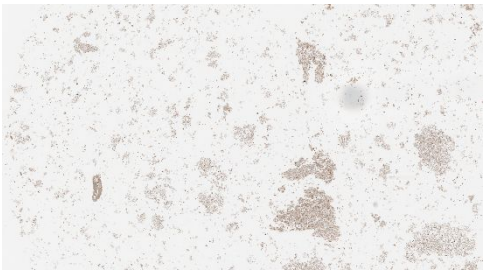
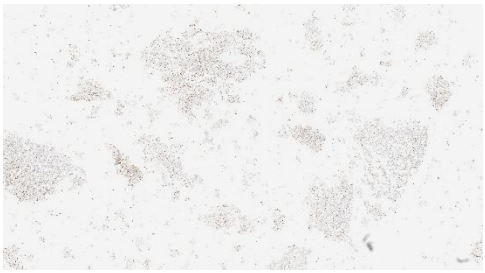
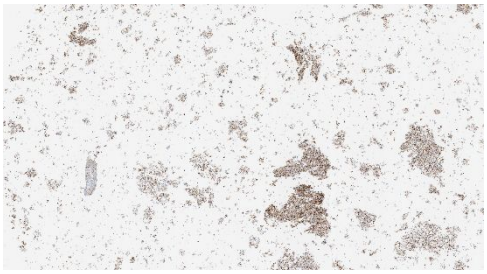
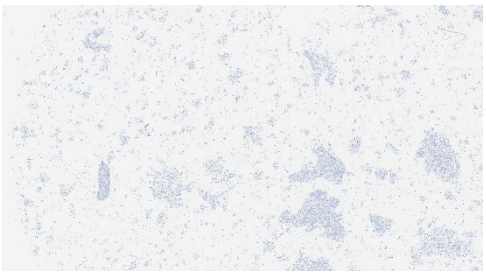
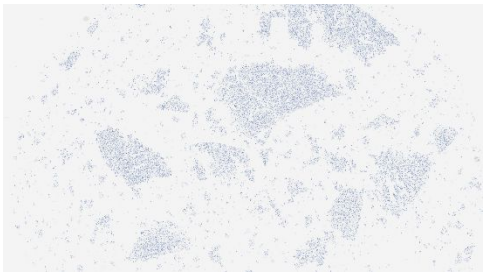
	No drug treatment	Drug treated
c-MET Single IHC		
GRB2 Single IHC		
c-MET GRB2 Proximity assay		
c-Met only Proximity assay control		

Figure S3: Single IHC and proximity assay images of EBC1 cells lines treated with DMSO (no drug) *or* crizotinib (drug). 2x magnification.

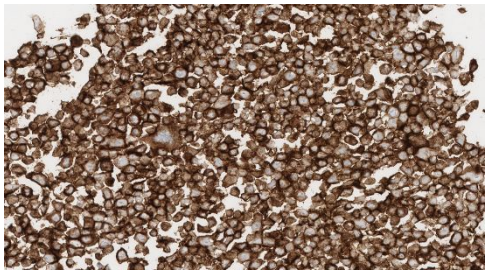
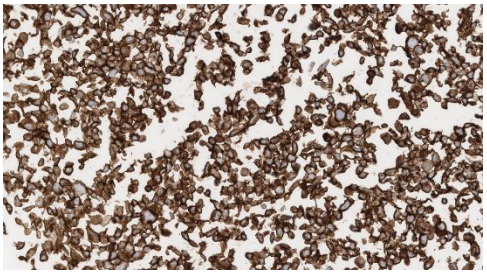
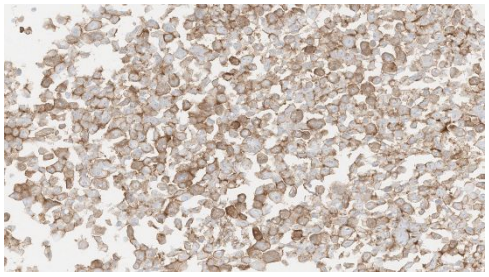
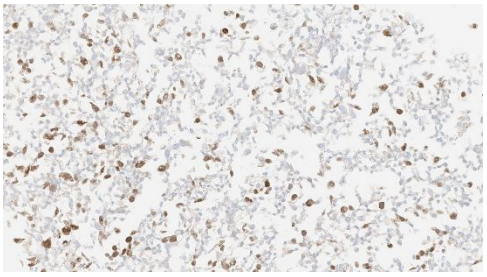
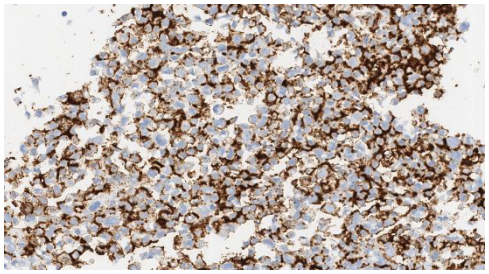
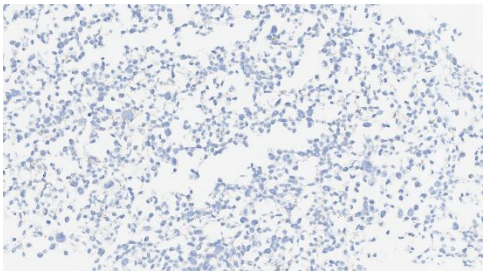
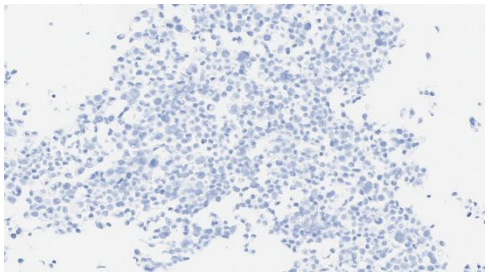
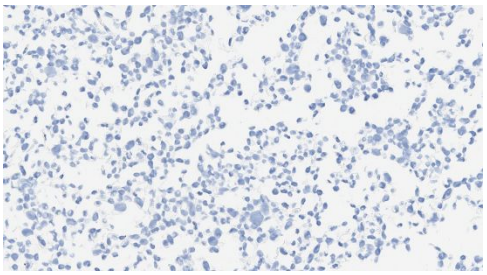
	No drug treatment	Drug treated
c-MET Single IHC		
GRB2 Single IHC		
c-MET GRB2 Proximity assay		
c-Met only Proximity assay control		

Figure S4: Single IHC and proximity assay images of EBC1 cells lines treated with DMSO (no drug) *or* crizotinib (drug). 20x magnification.

Cell line experimentals

PC9 cells harbor a deletion in exon 19 of the *EGFR* gene and are frequently utilized as an *in vitro* model of constitutive EGFR kinase activity. Treatment with the EGFR kinase inhibitor, erlotinib, leads to dose-dependent abrogation of survival-associated signaling and cell death. Similarly, EBC1 cells harbor an amplification of the *MET* gene and are an *in vitro* model of constitutive c-MET kinase activity. Treatment with the c-MET kinase inhibitor, crizotinib, leads to dose-dependent abrogation of survival-associated signaling and cell death. We previously demonstrated that EGFR:GRB2 and c-MET:GRB2 complexes were disrupted by treatment with erlotinib or crizotinib, respectively, and correlated data obtained with a fluorescent-based proximity ligation assay and co-immunoprecipitation immunoblots (Smith *Sci Sig 2015,* Smith *CCR 2017*). Here, we prepared FFPE pellets from PC9 and EBC1 cells treated with either DMSO or 1 μM of the respective inhibtors for 3h. Cells were cultured to approximately 80% confluency in RPMI-1640/5% fetal bovine serum using two plates per condition. Cells were pelleted, washed twice in PBS, then resuspended in 10% neutral formalin and processed into FFPE blocks following the "2+2" fixation protocol (Chafin, *et al*, *PLOS One* 2013). 5 μM sections were cut and floated onto Superfrost Plus glass slides (Fisher Scientific, #12-550-15) and analyzed by either single IHC or caged hapten assay as described above.

Proximity ligation assay (PLA) method comparison

The most widely used commercial technology for detecting PPC's in FFPE samples is the Duolink Proximity Ligation Assay kit (PLA) from Millipore Sigma (example kit P/N #DUO92101). As noted in Table S3, while both technologies utilize primary antibodies in the initial in situ proximity-dependent detection of the protein complex, the subsequent steps are quite divergent. The PLA technology uses DNA oligonucleotides to form a circularized DNA moiety, which is then amplified via rolling circle amplification and detected through hybridization. The caged hapten assay does not require DNA oligonucleotides or nucleic acid ligation, amplification or hybridization. The technology and associated reagents and conjugation chemistry are entirely novel. The principal advantage with the caged hapten approach is that it can be incorporated into existing clinical grade diagnostic hardware used in pathology labs for immunohistochemistry. The automated nature of the staining steps and detection via traditional light microscopy provide a platform suited for existing workflows.

Table S3: Comparison of steps in PLA & caged hapten assay technologies

Assay Step	**Proximity ligation assay**	**Caged hapten assay**
Initial target binding	Primary antibodies (2 species)	Primary antibodies (2 species)
Protein complex proximity mechanism	Oligonucleotide-labeled secondary antibodies, linker oligonucleotide, circularization oligonucleotide, T4 ligase-mediated ligation.	Caged hapten conjugated secondary antibody, alkaline phosphatase-conjugated secondary antibody; phosphatase-mediated liberation of caging group.
Signal generation/ Amplification	Phi29-mediated rolling circle amplification; fluorescently-labeled oligonucleotide probe	Antibody-mediated detection of "uncaged" hapten; tyramide amplification; HRP-mediated deposition of chromogen
Visualization	Fluorescence microscopy	Light microscopy
Automatable	No	Yes