

Enzyme-activated Proximity of Oligonucleotides Sensing (EPOS), for precise monitoring of protein interactions

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Enzyme-activated Proximity of Oligonucleotides Sensing (EPOS), for precise monitoring of protein interactions

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Abstract

Protein–protein interactions (PPIs) govern diverse cellular processes, which raise demands for better methods to investigate them. We have herein developed a method for high-resolution mapping of endogenous PPIs – Enzyme-Activated Proximity of Oligonucleotides Sensing (EPOS) – an antibody-based proximity assay that integrates enzymatic activation with the hybridization chain reaction (HCR). EPOS utilizes deoxyuridine-containing DNA hairpins conjugated to antibodies; following target binding, uracil-DNA glycosylase and endonuclease IV degrade blocking regions, thereby unmasking initiator sequences that drive HCR amplification. This design generates compact, fluorophore-dense products, by that improving subcellular localization compared with rolling circle amplification-based *in situ* proximity ligation assays (*in situ* PLA). Benchmarking against *in situ* PLA across multiple cellular models demonstrated EPOS’s superior spatial resolution and broader dynamic range. EPOS offers a robust and versatile platform for quantitative, high-resolution visualization of protein complexes *in situ*.

Introduction

Methods for determining protein-protein interactions (PPIs) are essential for analyzing cellular activity states. Over the years, a multitude of techniques have been developed to monitor protein-protein interactions in individual cells. Several of those methods are based on genetic constructs in which candidate proteins are fused to reporter molecules that, upon interaction, reconstitute a functional reporter, i.e., protein fragment complementation assays. Examples of such methods include yeast two-hybrid (Y2H) ¹, split-ubiquitin assays, e.g., mammalian membrane two-hybrid (MaMTH) ² and ClpS-Linked Luminescent Ubiquitin eXchange (CLIP-LUX) ³, and bimolecular fluorescence complementation (BiFC) ⁴. Another common approach is to use Förster resonance energy transfer (FRET) to determine interactions between fluorescent fusion proteins ⁵. To infer interactions between native proteins, several antibody-based methods are available, in which antibodies are conjugated with functional groups to enable the detection of protein complexes, such as antibody-based FRET ⁶. Although these methods have been widely used and have enhanced our understanding of biology, they are limited in their ability to detect endogenous protein-protein interactions.

Our aim over the last two decades has been to develop methods that enable the analysis of endogenous protein-protein interactions using antibody-oligonucleotide conjugates, facilitating targeting, proximity sensing, and providing a means to visualize these interactions. *In situ* proximity ligation assays (*in situ* PLA) ⁷ combine dual recognition of affinity reagents with potent signal amplification, utilizing DNA-modifying enzymes to generate a signal that serves as a surrogate marker of the targeted protein or protein complex. The method is based on pairs of proximity probes (prox probes) (i.e., antibodies conjugated to DNA oligonucleotides) to target the proteins of interest. Only through proximal binding of these probes can they create an amplifiable DNA strand by templating the ligation of subsequently added circularization oligonucleotides. The ligation creates a circular DNA molecule, which is then amplified by rolling circle amplification (RCA). This process generates long single-stranded DNA concatemers that collapse into bundles of DNA, still connected to one of the prox probes. These can be visualized by hybridizing fluorophore-labeled detection oligonucleotides, enabling localized single-molecule detection *in situ*. We have developed several variants of *in situ* PLA aimed at achieving increased dynamic range ⁸, enhanced efficiency ⁹, multiplexing capability ¹⁰, identification of protein-DNA interactions ¹¹ and combined analysis of protein interactions and mRNA ¹². Recently, we have published yet

another method, MolBoolean¹³, which provides information on both levels of the individual proteins and their level of interactions.

In situ PLA provides several advantages: it provides the ability to monitor endogenous protein-protein interactions and it does not require advanced instrumentation as the signal amplification with RCA allows detection of single protein complexes. However, the disadvantage of RCA is the size of its product, with a diameter of approximately 1 μm ¹⁴. Hence, each individual protein complex will be visualized as an 1 μm sized dot. This negatively affects the resolution of the images and makes it impossible to locate interaction events to/in fine subcellular structures. Furthermore, the size of the RCA product also limits the dynamic range of *in situ* PLA. As the number of *in situ* PLA signals increases, it becomes difficult to separate individual objects. We have hence explored alternative strategies of providing an amplifiable DNA signal. The hybridization chain reaction (HCR) method is an enzyme-free amplification method for nucleic acid analysis¹⁵, which is based on at least two different kinetically trapped hairpin structures. In the absence of an initiator oligonucleotide, both hairpin structures remain as monomers. Once the initiator is introduced, however, it will hybridize with one of the hairpin structures by invading it via a random-walk process. This process is driven by the release of potential energy stored in the oligonucleotide's structure. The hybridization between the initiator and the first hairpin structure exposes a part of the first hairpin sequence (a foothold) that can bind and invade the second hairpin structure, which then, in turn, can bind and invade another molecule of the first species. The hybridization chain reaction will generate a long, nicked double-stranded DNA molecule, which will continue to grow linearly until the hairpin species are exhausted.

We and others have developed methods where proximity sensing facilitates signal generation by HCR¹⁶⁻¹⁹, retaining the dual binding requirement of *in situ* PLA while providing a signal generation that does not require any enzymatic steps. In proximity-dependent initiation of HCR (proxHCR)¹⁵, we conjugate two new hairpin species (prox probe oligonucleotides) to antibodies. Once a pair of proximity-probe oligonucleotide-conjugated antibodies (prox probes) has bound to their targets, an activator oligonucleotide is added. The activator can invade the first prox probe oligonucleotide and thus expose its 3' end. Only if both prox probes are in close proximity will the liberated 3' end of the first prox probe oligonucleotide invade the second prox probe oligonucleotide, thereby exposing its 3' end, which will act as an initiator

for the subsequent HCR reaction. In unreacted prox probe oligonucleotides (*i.e.*, those that did not bind to proximal targets), the initiator sequence will remain hidden within the stem of the hairpin loop. By using fluorophore-labeled HCR hairpins, the fluorescence of the HCR signal will linearly increase over time. The chain reaction building up the nicked double-stranded reporter sequence will continue until the supply of one or both HCR hairpins is exhausted. The advantages of proxHCR are that it provides a better representation of where an interaction occurs and provides a better dynamic range, as HCR does not generate large objects as RCA does. The disadvantage is that signal amplification is less efficient, partly due to impurities in oligonucleotide synthesis ²⁰. In addition, the oligonucleotide design for proxHCR is complicated ²¹, to ensure that there will be a strict step-wise process of DNA strand invasions while retaining the metastability of the prox probe oligonucleotides (*i.e.*, they will only open up by invasion of their cognate DNA molecule), which forces a compromise between speed and stability.

We herein present a new method, Enzyme-Activated Proximity of Oligonucleotides Sensing (EPOS), that overcomes the limitations of proxHCR. EPOS employs an enzymatic step to partially degrade the prox probe oligonucleotides, facilitating efficient proximity sensing and HCR initiation. Hence, the prox probe oligonucleotides can be designed for high stability. Once bound, excess prox probes are removed by washes, and enzymatic digestion generates shorter oligonucleotides with a greater propensity for hybridization and invasion. We have herein benchmarked the EPOS method against *in situ* PLA and provided compelling evidence of the advantages of the EPOS method.

Results

Design of the EPOS method

We designed the EPOS method to contain several deoxyuridine residues in the prox probe oligonucleotides, that we could enzymatically degrade with a combination of uracil-DNA glycosylase (UDG) and endonuclease IV (EndoIV). Enzymatic digestion will partially degrade the hairpins, exposing a long foothold in one prox probe oligonucleotide (prox probe oligonucleotide PLUS) and the reverse-complementary sequence towards the foothold and the stem region in the other prox probe oligonucleotide (prox probe oligonucleotide MINUS). This approach enables us to design the oligonucleotides of the prox probes more flexibly, *i.e.*, we can utilize the stem region of the HCR hairpins in the prox probe oligonucleotides, combined

with unique footholds for activation. This enables a design where the prox probe oligonucleotides are almost entirely double-stranded until activated by digestion, with only a short loop region and a short linker, in which the oligonucleotides are conjugated to an antibody (Fig. 1a and suppl. Table 1). This makes the oligonucleotide design for EPOS straightforward. HCR hairpin 1 is composed of the regions: 5'- B' - A' - C' - A and HCR hairpin 2 of the regions: 5'- A - B - A' - C. Using the HCR hairpins as guides for the design, the prox probe oligonucleotide PLUS would hence have to have the following design: 5'- D - A' - C' - A - D'U (where the deoxythymidines in D'U are replaced with deoxyuridines) and the prox probe oligonucleotide (MINUS) will be composed of: 5'- D_U - A'_U - U - A - D' (where U is a loop containing deoxyuridines and for region D_U and A'_U the deoxythymidines are replaced with deoxyuridines). The double-stranded region in the prox probe oligonucleotides serves to block unintended interactions between the prox probe oligonucleotides before activation, *i.e.*, enzymatic digestion by UDG and EndoIV, making them as inert as possible. These blocking regions of the prox probe oligonucleotides are the regions where the deoxythymidines are replaced with deoxyuridines. In order to have a stringent requirement for proximity sensing, prox probe oligonucleotide PLUS is conjugated at its 5' end and prox probe oligonucleotide MINUS at its 3' end. Once the prox probes have bound their targets, and excess is removed by washes (Fig. 1b), the deoxyuridines are degraded by UDG and EndoIV (Fig. 1c). This will fragmentize the blocking region of the prox probe oligonucleotides, activating proximity sensing by exposing a long foothold on prox probe oligonucleotide PLUS and an activator sequence on prox probe oligonucleotide MINUS (Fig. 1d), facilitating a very efficient strand displacement if the prox probe oligonucleotides have bound in close proximity (Fig. 1e). As a consequence, the initiator sequence, which was previously hidden in the loop and hairpin structure of the prox probe oligonucleotide PLUS, will be exposed, allowing for the binding of HCR hairpins (Fig. 1f). The long, nicked double-stranded HCR product formed contains several fluorophores and is still connected to the prox probe oligonucleotides (Fig. 1g).

High-resolution imaging of protein-interactions

To validate EPOS, we conjugated the prox probe oligonucleotides to secondary species-specific antibodies (anti-mouse, anti-rabbit and anti-goat) and used these secondary species-specific prox probes to stain adherens junctions by investigating the interaction between E-cadherin and β -catenin in HaCaT human keratinocytes. To determine the efficiency and necessity of the enzymes in the de-blocking/activation step, the experiment was conducted by

treating the prox probe oligonucleotides by either UDG or EndoIV alone, or a combination of UDG and EndoIV. In the absence of the activating enzymes, the prox probes remained inactivated, as indicated by the lack of signal. Using UDG alone, which removes deoxyuridine bases, resulted in a substantial staining that was further increased by the addition of EndoIV, which cuts the abasic sites generated by UDG (Fig. 2a and 2b). The abasic sites created by UDG are prone to hydrolysis, which facilitates activation of some probes, resulting in a weak signal. However, for complete activation and removal of all abasic sites, EndoIV is required. The difference in mean integrated intensity is significantly higher in the samples with both UDG and EndoIV present, compared to the samples where UDG or both enzymes have been omitted (Fig. 2b) demonstrating the robustness of the method. Furthermore, we wanted to benchmark EPOS against *in situ* PLA and immunofluorescence (IF), which are commonly used methods for visualizing protein interactions. We herein chose to use the commercially available kit (NaveniFlex). What was apparent from this comparison was that EPOS, to a much greater extent than *in situ* PLA, provided a sharper image of the localization of the protein interactions (Fig. 2a and suppl Fig. 1). This is the result of the more compact size of the generated EPOS signals, which are much smaller than the *in situ* PLA signals. The higher resolution of EPOS compared with *in situ* PLA becomes even more apparent when using structural illumination microscopy (SIM) (Fig. 2c and suppl Fig. 1). The signal obtained with EPOS results in a fine line that represents the interaction of E-cadherin and β -catenin as a result of the formation of adherens junctions after cell-to-cell contact with minimal signal in the cytosol. Omitting any of the prox probes completely abolished activation (suppl. Fig. 1), demonstrating the specificity and the requirement for both prox probes to initiate HCR. Next, we compared IF and EPOS, and concluded that the E-cadherin and β -catenin expression determined by IF (Fig. 2d) exhibits a similar staining pattern to EPOS. Further, to determine the binding pattern of the prox probes (for EPOS staining), we stained cells with primary antibodies targeting either E-cadherin or β -catenin, and the single corresponding prox probe. After activation of the bound single prox probe, an activated soluble oligonucleotide MINUS, or an activated soluble oligonucleotide PLUS (i.e., oligonucleotides with an identical sequence as activated prox probe oligonucleotides) was added (suppl. Fig. 2 and suppl. Table 1). As the activated soluble oligonucleotide can diffuse freely, it will initiate an HCR reaction for all prox probes bound to the respective primary antibodies, producing an immuno-HCR staining pattern that mirrors the IF staining (Fig. 2e).

The superior resolution of EPOS, compared with *in situ* PLA, prompted us to investigate how well these methods can detect protein interactions in sub-cellular compartments. Cellular organelles such as mitochondria are often crowded and compartmentalized, making it difficult to study protein interactions in their natural state within these structures. To test whether EPOS could detect PPIs in mitochondria, we probed the mitochondrial transcription factors TFAM and TFB2M in A549 cells, which are involved in initiating the RNA polymerase POLRMT. The mitochondria were visualised using mtCO1, which targets the mitochondrial-encoded cytochrome c oxidase I. The signal for the TFAM/TFB2M interaction obtained with EPOS overlaps with the MitoTracker to a much higher degree than *in situ* PLA (Fig. 3a and 3b, and suppl. Fig. 3a), as visualized by the differences in the Pearson coefficient. Where a value of 1 indicates a perfect positive correlation (100 % co-localization), and -1 indicates a perfect negative correlation (no co-localization). Because the space inside the mitochondria is limited, the RCA product will be generated mainly outside the organelle. EPOS, on the other hand, generates a signal that co-localizes with mtCO1 to a much higher degree, as the smaller size of the HCR product allows it to be generated inside the mitochondria, where the TFAM/TFB2M interaction occurs.

As an additional example, we investigated the interaction between the RNA-binding protein Ras GTPase-activating protein-binding protein 1 (G3BP1) and N6-methyladenosine (m6A)-modified mRNAs (m6A-mRNAs), which are recruited during stress granule (SG) formation²². SGs are dynamic, cytoplasmic aggregates composed of, among others, RNA-binding proteins (RBPs) and RNAs that form upon cellular stress, for example, in response to arsenite treatment, and facilitate a global suppression of translation until cellular homeostasis is reestablished^{23,24}. Under normal culture conditions, EPOS produced faint, even cytoplasmic staining, but upon arsenite treatment, the staining increased significantly and condensed into stress granules (Fig. 3c and 3d, and suppl. Fig. 3b). In contrast, the *in situ* PLA staining exhibited a punctate pattern that was slightly increased and not visibly changed in the localization of RCA signals after arsenite treatment.

The distance requirement for proximity sensing with EPOS is reduced compared with *in situ* PLA as one of the proximity oligonucleotides is conjugated at its 5'-end and the other at its 3'-end. We therefore generated prox probes using single-domain antibodies (sdAbs, also known as nanobodies), which are only 15 kDa in molecular weight (10-fold smaller than conventional IgG antibodies) and 2.5 nm in diameter^{25,26}. Prox probes based on the previously described

HER2-binding sdAb 11A4²⁷ enabled us to visualize HER2 homodimers in the HER2+ cell line SKBR3, while no staining was observed in the HER2- cell line MDA-MB-231 (Suppl Fig. 4a and b).

Analysis of the dynamic range

As *in situ* PLA previously has been reported to have a limited dynamic range²⁸, we set out to determine how the dynamic range of EPOS compares to that of *in situ* PLA. To test this, HaCaT cells were probed with two primary antibodies recognizing the heavy chain of clathrin. While the mouse antibody was kept constant, the rabbit antibody concentration was decreased by 1/3 six times, resulting in dilutions ranging from 1:10 to 1:7290 (Fig. 4a). To account for background staining, a condition with no rabbit antibody (No ab) was included. The EPOS staining showed a stable decrease in signal strength and amount, corresponding to a decreased amount of rabbit primary antibody, following a linear regression curve across all dilutions ($R^2=0.9705$) (Fig. 4a and 4b). The *in situ* PLA signal also decreased linearly; however, to obtain the best coefficient of determination ($R^2=0.9963$), the first two dilutions had to be omitted. We conclude that EPOS provides a more extended dynamic range than *in situ* PLA.

Sensitive detection of protein-protein interactions

The nucleus is the most crowded organelle in the cell. As it contains high amounts of negatively charged DNA, it poses a challenge for DNA-dependent proximity methods such as *in situ* PLA. We thus wanted to examine how EPOS compares to *in situ* PLA for visualization of nuclear protein-protein interactions using SMAD and MYCN/MAX interactions. Upon activation of TGF- β receptors, SMAD3 and SMAD4 heterodimerize and translocate to the nucleus, providing an excellent model system where nuclear staining should be induced by stimulation with TGF- β . MDA-MB-231 breast cancer cells were stimulated with 5 ng/mL TGF- β resulting in the interaction between SMAD3 and SMAD4 and their translocation to the nucleus (Fig. 5a and suppl. Fig. 5). Both methods show a significant increase in signal after TGF- β stimulation, however EPOS was able to detect a substantially higher number of interactions inside the nucleus after TGF- β stimulation (Fig. 5b). Similarly, the interaction between MYCN/MAX could be demonstrated in Tet21N neuroblastoma cells, that carry a Tet-regulatable *MYCN* gene, under MYCN ON conditions both with EPOS and *in situ* PLA. EPOS, however, detected considerably more interactions (Fig. 5c and 5d, and suppl. Fig. 5). Upon downregulation of MYCN expression by the addition of doxycycline, there was a drastic significant reduction in

MYCN/MAX signal both with *in situ* PLA and EPOS, thus demonstrating the specificity of the signal. The high resolution obtained with EPOS, makes it even possible to observe details such as nucleoli with the EPOS staining in the images. This information is lost when *in situ* PLA is being used.

To test the performance of EPOS in staining PPIs in formalin-fixed paraffin-embedded tissue sections, we assayed the interaction between E-cadherin and β -catenin in breast cancer tissue sections²⁹. In analogy with experiments on cultured HaCaT cells, EPOS staining of tissue sections yielded sharp outlines of adherens junctions, whereas *in situ* PLA yielded a blurred, dotted depiction (Fig. 5e).

Detection and quantification of post-translational modifications

Antibody-based proximity detection methods can, in addition to detection of PPIs, be used to monitor post-translational modifications (PTMs) of proteins. We have frequently been using *in situ* PLA with pairs of antibodies where one targets the core protein and the other a phosphorylated or glycosylated epitope^{30,31}. We therefore wanted to ensure that the antibody-based detection method EPOS also performs well in detecting the presence of post-translationally modified proteins, and compare the achieved staining with *in situ* PLA. As a model system, we used AKT phosphorylation by probing with phospho-specific antibodies against phosphorylation on serine 473 (pS473-AKT) and antibodies against the total protein (AKT). Human lung fibroblasts (HFL1) were stimulated with 20 ng/ml PDGF-BB for 15 minutes and downstream activation of AKT was monitored by EPOS and *in situ* PLA with both methods showing a significant increase in signal after stimulation (Fig. 6a and 6b, and suppl. Fig. 6). We found that EPOS was able to detect more phosphorylated AKT molecules in the cells than *in situ* PLA, both at steady-state levels before stimulation with PDGF-BB and after PDGFR-mediated activation of AKT, thus better portraying the physiological activation of AKT. The images also clearly illustrate the difference in signal size with EPOS generating a more even signal.

Discussion

Herein, we present EPOS, an HCR-based method that overcomes key limitations of proxHCR. By using a cocktail of DNA-degrading enzymes, activation of proximity-sensing is maximized while maintaining an inert design for the prox probe oligonucleotides to minimize nonspecific

activation. This approach also simplifies oligonucleotide design, facilitating the generation of orthogonal probe sets for multiplexed applications.

Benchmarking against *in situ* PLA showed that EPOS provides superior spatial resolution, owing to its more compact amplification products and higher fluorophore density. *In situ* PLA generates an RCA product consisting of a single-stranded DNA molecule of approximately 100,000 nucleotides, with roughly one fluorophore per 100 nucleotides. By contrast, EPOS generates shorter, double-stranded HCR products that are more compact and fluorophore-dense; for a 36-mer hairpin, this corresponds to approximately one fluorophore per 18 base pairs. Although *in situ* PLA produces bright signals because of the strong amplification achieved by RCA, this also limits the number of events that can be resolved as discrete intracellular objects. Only a few hundred RCA products can typically be accommodated within a cell while remaining distinguishable as individual signals. Moreover, RCA products are not tightly compacted and may extend or bind to the glass slide at some distance from their site of origin, reducing spatial precision¹⁴. This limits the accurate localization of PPIs in subcellular structures such as mitochondria. In contrast, EPOS yields PPI patterns that resemble immunofluorescence images of overlapping proteins, providing a more precise means of determining PPI localization.

EPOS and *in situ* PLA also differ in dynamic range and in their ability to detect PPIs in regions of high local interaction density. For *in situ* PLA this can be caused coalescing RCA products⁸ which affects enumeration and reduced efficiency in generating circular ligation products. Most likely, it is a combination of both. In *in situ* PLA, only a subset of target molecules is detected because some ligation events produce non-amplifiable linear ligation products rather than circular ligation products. At low PPI density, prox probe pairs are less likely to have neighboring prox probes nearby, favoring formation of circular ligation products because the circularization oligonucleotides hybridize primarily to the cognate prox probe pairs. At high PPI density, however, multiple prox probes may be located in close proximity, increasing the likelihood that circularization oligonucleotides hybridize to adjacent non-cognate probes and generate non-amplifiable linear products. In EPOS, by contrast, all productive prox probe interactions expose the HCR initiator, enabling faithful detection even in regions with high local concentrations of protein interactions, such as SG and adherens junctions.

Since our development of *in situ* PLA twenty years ago⁷, the method has been widely used by both our group and the broader research community and has proven highly valuable. However,

as with all methods, improved alternatives eventually emerge. EPOS represents such an advance. The present work demonstrates that EPOS offers important advantages for future studies of endogenous PPIs and PTMs in fixed cells. We conclude that EPOS provides improved spatial resolution, broader dynamic range, and greater capacity to detect high local concentrations of PPIs, although individual molecules cannot be enumerated by standard microscopy.

Material and Methods

Design of EPOS oligonucleotides

The modular design, using defined domains, and design criteria for the design of prox probe oligonucleotides are detailed in the Results section. To provide specific sequences (Figure 1) we initially designed HCR hairpins, as previously described ³², and used these for the design of the prox probe oligonucleotides. For the analysis of the oligonucleotide system we used the NUPACK ³³ software.

Ligation of proximity probe hairpins

The oligonucleotides for the proximity probes were acquired from Integrated DNA Technologies (IDT) or Qiagen DNA Synthesis AB (QDS). QDS provided oligonucleotides of sufficient purity, so they were used unpurified and as one piece for the subsequent production of proximity probes. The proximity probe oligonucleotide purchased from IDT had to be purchased in two pieces and ligated before PAGE purification to achieve sufficient purity. To obtain the complete proximity probe oligonucleotides, the two parts were mixed (5000 pmol + 4000 pmol) with a surplus of the non-azide-modified part and ligated with T4 DNA ligase (0.025 U/ml T4 DNA ligase, 0.25 U/ml PNK and 1x T4 ligation buffer) by incubation for 16 hours in 30-minute intervals, cycling between 37 °C and 4°C. All from Thermofisher scientific. The HCR-hairpin 1 and HCR-hairpin 2 were purchased from IDT and PAGE-purified before use. The oligonucleotides used are listed in Supplemental Table 1.

PAGE purification of hairpins

To achieve high enough purity, all DNA oligonucleotides purchased from IDT were purified by TBE-Urea PAGE purification before use. The PAGE purification was conducted at an elevated temperature to ensure that all secondary structures in the DNA oligonucleotide had

denatured. The 10% TBE-Urea gels (EC68755BOX) and the XCell *SureLock*TM Mini-Cell (EI0001) were placed in a water bath set to 60°C and preincubated until the TBE buffer reached 50°C in the inner chamber. Before loading the DNA onto the gels (1500 pmol/gel), the DNA oligonucleotides were mixed with 2x Urea loading dye and boiled at 95°C for 10 minutes. The electrophoresis was performed for 45 minutes at 100V, after which the gels were rinsed in deionized water and DNA stained with SYBR Gold for 10 minutes (for TexasRed labeled oligonucleotides this step was omitted since they are already visible as a red band in the gels). The band at the correct size was excised and the gel was pushed through an 18G needle to shred the gel. DNA oligonucleotides were eluted at 37°C with 4x400µl H₂O. Residual gel pieces were removed by centrifugation using MicroSpin columns at 300xg until the eluate passed through the filter (Ultrafree-MC Centrifugal Filter 0.22, UFC30GV00, Sigma). The flow-through was collected in a 2 ml Eppendorf tube and concentrated by speed-vac without heating until 200µl remained. To precipitate the DNA, Sodium Acetate was added to a final concentration of 300mM and thereafter 2.5 volumes of ice-cold 99.5% ethanol was added and incubated overnight at -20°C. The precipitated DNA was pelleted by centrifugation for 10 minutes at -10°C and max speed. The pellet was thereafter washed three additional times with 70% ice-cold ethanol. DNA was resuspended in H₂O, and its concentration was measured using a Nanodrop. If not stated otherwise, all reagents are from Thermo Fisher Scientific.

Production of HER2-binding single-domain antibody with unpaired cysteine

As previously described ²⁷, HER2-binding sdAb 11A4 was equipped with a C-terminal C-Direct-tag containing a free thiol (cysteine) for directional labeling for EPOS. C-Direct-tagged 11A4 (named Q17c) was produced in *S. cerevisiae* strain VWK18 as described previously ³⁴. sdAbs were purified from the yeast supernatant using an ÄKTA Start (Cytiva) and a Protein A affinity chromatography column (10000176-C05, JSR Life Sciences), with PBS as the running buffer and citrate buffer (20 mM, with 150 mM NaCl, pH=3) for elution. Eluted proteins were neutralized with Tris buffer (1 M, pH 7.5, 10% of elution volume added) and desalted using size-exclusion chromatography using a HiPrep 26/10 desalting column (17508701, Cytiva) according to the manufacturer's protocols. The purified Q17c was filter-sterilized using a 0.22 µm filter and stored in PBS until labeling.

Making proximity probes

Secondary antibodies, Donkey anti-Mouse (715-005-150), Donkey anti-Rabbit (711-005-152) and Donkey anti-Goat (705-005-147) (all from Jackson ImmunoResearch) and anti-HER2 sdAbs were labeled with DBCO using EZ-Link™ DBCO Protein Labeling Kit (C20046, ThermoFisher Scientific) according to the manufacturer's recommendations. Briefly, after resuspension in 400 µL of anhydrous dimethyl sulfoxide (DMSO), the EZ-Link™ TFP Ester-PEG4-DBCO was incubated for 1 hour at room temperature at a 20x molar excess with the species-specific secondary antibodies or HER2 sdAbs. The DBCO-modified antibodies were thereafter purified and the excess linker was removed with a Zeba column. The DBCO-modified antibody and the purified azide-modified proximity probe oligonucleotides (see "PAGE purification of hairpins") were mixed (2:1 ratio) and incubated at room temperature for 4 hours and thereafter at 4°C overnight. The conjugated secondary antibodies (proximity probes) and sdAbs were purified using an ÄKTA Pure chromatography (GE Healthcare) using a Superdex 200 10/300 GL column (GE Healthcare), as previously described ¹³.

Cell culture

HaCaT, human keratinocytes (300493, Cyton), SK-BR-3, breast cancer cells (300333, Cyton) and MDA-MB-231, breast cancer cells (HTB-26, ATCC) were cultured in Dulbecco's Modified Eagle medium + GlutaMAX (DMEM+GlutaMAX) supplemented with 1xPEST (15140122) and 10 % FBS (A5256801). HFL1, human lung fibroblasts (89071902-1VL, Sigma) were cultured in Dulbecco's Modified Eagle medium + GlutaMAX (DMEM+GlutaMAX) supplemented with Non-Essential Amino Acids (11140050), PEST and 10 % FBS. A549, human lung adenocarcinoma cells (kind gift from Prof. Ingo Schmitz, Ruhr University Bochum, authenticated by Eurofins Genomics), were cultured in Dulbecco's Modified Eagle Medium (DMEM; PAN Biotech) supplemented with 10% FBS (PAN Biotech) and 1xPEST. HT22 mouse hippocampal neuronal cells (SCC129, Merck) were cultured in DMEM High Glucose (4.5g/l) w/o L-Glutamine with Na-Pyruvate (Nordic Biolabs), supplemented with 10% FBS (A5209402), 1× L-glutamine (25030081), and 1× PEST. Tet21N neuroblastoma cells containing a Tet-regulatable MYCN gene were kindly provided by Dr. Werner Lutz, DKFZ, Heidelberg ³⁵. The cells were cultured in RPMI medium + GlutaMAX supplemented with 10% FBS, and 1xPEST. To turn off MYCN expression, doxycycline (#D9891, Millipore) was added to the culture medium at a concentration of 1 µg/ml for 24 hours before the experiment started. If not stated otherwise, all from Thermo Fisher Scientific.

To perform experiments all cells were seeded in 8 well chamber slides and left to adhere overnight. For E-Cadherin+ β -catenin and Clathrin experiments, HaCaTs were seeded to confluence and fixed when adherent junctions had been formed. For HER2 homodimerization, SK-BR-3 and MDA-MB-231 cells were seeded to confluence and allowed to adhere overnight. To provoke stress granule formation, cells were exposed to 250 μ M sodium arsenite (NaAsO₂; S7400, Merck) for 60 min, washed twice with PBS and fixed in methanol for 10 min at room temperature. For pAKT+AKT experiments, HFL1 cells were starved overnight with culture medium with reduced serum (0.2% FBS) and thereafter stimulated with 20ng/ml PDGF-BB for 0 or 15 minutes. For SMAD3+SMAD4 experiments, MDA-MB-231 cells were starved overnight with culture medium with reduced serum (0.2% FBS) and thereafter stimulated with 5ng/ml TGF- β for 0 or 45 minutes. For TFAM–TFB2M, cells were allowed to adhere overnight and subsequently fixed the following day using pre-warmed (37 °C) PFA to improve mitochondrial staining.

If not stated otherwise, the cells were washed once with ice-cold PBS and thereafter fixed with ice-cold 4% PFA for 15 minutes, and washed three times with PBS. Permeabilization was performed with 0.2% Triton-x100 for 10 minutes. For the TFAM+TFB2M interaction, an additional treatment with 1% SDS for 5 minutes was performed. The antibodies used are listed in Supplemental Table 2.

Tissue sections

The formalin-fixed paraffin-embedded human breast cancer tissue sections were retrieved from the Pathology Department of the Portuguese Institute of Oncology in Porto (IPOP, Porto, Portugal). Their use for research purposes was approved by the Ethics Committee of IPOP (CES. 64/023), in accordance with national regulatory law on the handling of biological specimens from tumor banks, with the samples exclusively available for research purposes in retrospective studies, as well as with the international Helsinki declaration. Prior to permeabilization, the tissues were deparaffinized and subjected to antigen retrieval. This was done by two 10-minute incubations in Xylene; thereafter, the slides were rehydrated with three 5-minute washes in ethanol (100%, 100%, and 70%). Finally, the slides were immersed in water. Antigen retrieval was performed with a Decloaking chamber NxGen (Biocare Medical) using DAKO Target Retrieval Solution (S1699) at 95 °C for 40 min.

EPOS experimental procedure

Following cell treatments, EPOS was performed. The slides were blocked with 0.75x Intercept blocking buffer (LI-COR) and 2.5 mg/ml Salmon sperm DNA (EPOS blocking buffer) for 1 hour at room temperature, followed by overnight incubation at 4°C with primary antibodies (Suppl. Table 2) diluted in 0.25x EPOS blocking buffer in TBS. The excess of primary antibodies was washed away with two 5-minute washes with TBS+0.05% Tween-20 and one 5-minute wash with TBS. Next, the EPOS proximity probes (5µg/ml for secondary antibodies or 2µg/ml for HER2 sdAbs), diluted in 0.25x EPOS blocking buffer in TBS, were incubated for 1 hour at room temperature and washed away for 5 minutes with TBS+0.05% Tween-20, 10 minutes with TBS+0.05% Tween-20 with 1M NaCl (37°C) and 5 minutes with TBS. To activate the EPOS system, the cells were incubated with EndoIV (EN0591, Thermo Fisher scientific) and UDG (EN0361, Thermo Fisher scientific) for 45 minutes at 37°C (0.01 U/µl EndoIV, 0.05 U/µl UDG, 40mM Tris-HCl, 20mM Acetate, pH 7.6, 1mM EDTA, 300mM NaCl and 0.05% Triton-x100), followed by a brief wash with TBS and a second incubation with HCR hairpins to initiate the elongation step (0.1-0.5µM DH1, 0.1-0.5µM DH2, 40mM Tris-HCl, 20mM Acetate, pH 7.6, 1mM EDTA, 300mM NaCl, 0.05% Triton-x100, 5% dextrane sulfate and 0.02mg/ml Hoechst). The slides were finally washed three times with TBS for 5 minutes before being mounted with ProLong Glass antifade reagent (Thermo Fisher Scientific). For the TFAM+TFB2M interaction, the mitochondria-specific staining (Alexa Fluor® 488 conjugated anti MTCO1 antibody (clone 1D6E1A8, ab154477, abcam) was diluted 1:100 and incubated overnight in parallel with the primary antibodies. For the HER2+HER2 experiments, only TBS at RT was used for washing. For the activated prox probe/ activated soluble oligonucleotide experiments (Fig 2e), an additional incubation with the activated soluble oligonucleotide (see suppl. Table 1, 0.1µM) and a washing step were inserted between the proximity probe activation and the HCR step.

***In situ* PLA**

Following cell treatments, *in situ* PLA was performed using NaveniFlex Cell MR and NaveniFlex Cell GM kits (Navinci) according to the manufacturer's recommendations. Primary antibodies are listed in suppl. Table 2.

Immunofluorescent staining (IF)

The cells were fixed and permeabilized as described for EPOS, thereafter blocked with 1x Intercept blocking buffer (LI-COR), followed by overnight incubation at 4°C with primary antibodies diluted in 0.25x 1x Intercept blocking buffer in TBS. The excess primary antibodies were washed away with two 5-minute washes in TBS + 0.05% Tween-20 and one 5-minute wash in TBS. Next, secondary antibodies (Goat anti-Mouse IgG AF647+, A32728; Goat anti-Rabbit IgG AF555+, A32732) and 0.02mg/ml Hoechst diluted in 0.25x EPOS blocking buffer in TBS were incubated for 1 hour at room temperature. The slides were finally washed with two 5-minute washes in TBS + 0.05% Tween-20, followed by 5 minutes in TBS, before being mounted with ProLong Glass antifade reagent (Thermo Fisher Scientific).

Image acquisition

All images except the mitochondria staining were acquired with a Zeiss imager M2 microscope equipped with a Plan-Apochromat 63X/1.4 Oil objective, HXP 120 V light source, Hamamatsu C11440 camera and Zen 2 software (blue edition). Cube filter sets 31, 43HE, 49 and 50 were used (all from Zeiss). Each image was acquired as a z-stack spanning 4 μm with an interval of 0.25 μm . The mitochondria staining was acquired using a Zeiss Axio Observer 7 microscope equipped with Apotome 3 and ZEN lite software (version 3.9.101.0200; Zeiss). Signal strength was enhanced for visualization purposes; however, the analyses were performed on raw images.

3D structured illumination microscopy (3D-SIM)

Super-resolution 3D-SIM imaging was performed on a Carl Zeiss Elyra PS.1 microscope equipped with high-performance filter cubes and a 561 nm excitation laser. Images were captured using an Andor iXon DU 897 camera with a Plan-Apochromat 100x/1.46 NA, oil-immersion objective. Emission was collected through appropriate dichroic mirrors and bandpass filters set at 570–650 nm for 561 nm excitation. 3D-SIM processing was performed using the integrated ELYRA S system software (Zen 2011 SP2 Black), with automatic settings selected for evaluating the raw data (i.e., theoretical PSF, noise filter setting, frequency weighting, baseline settings, etc.). The optimal grid size was automatically assigned to each wavelength by the Zeiss Zen software, and the grid was rotated five times at five phases for each image. Calibration on 40 nm sub-resolution beads generated a lateral precision of about 85 ± 5 nm with 488 nm excitation, as shown before³⁶.

Image analysis

All image analysis was performed using CellProfiler 4.2.8. Prior to quantification, flatfield illumination correction was applied to all channels. For the EPOS datasets, an illumination correction function was calculated using a polynomial smoothing method across the complete image set per channel and applied by pixel-wise division of the raw images. For the *in situ* PLA datasets, a background-based illumination correction function was calculated across the full image set and applied by subtraction, to remove uneven background without distorting spot-level signals.

Cell segmentation was performed in two steps. Nuclei were identified using the *IdentifyPrimaryObjects* module on the illumination-corrected Hoechst 33342 channel. Cell bodies were subsequently delineated using the *IdentifySecondaryObjects* module, with the EPOS or *in situ* PLA signal serving as the reference channel. For datasets in which the signal of interest was confined to the nucleus (MYC/MAX and SMAD3/SMAD4), quantification was restricted to the primary nuclear objects. Cells touching image borders were excluded from all analyses.

For EPOS quantification, a per-replicate background threshold was established using one omitting control per biological replicate. Integrated intensity of the illumination-corrected EPOS signal was measured within segmented cell bodies using *MeasureObjectIntensity*, and the 99th percentile of the per-cell median pixel intensity distribution across all segmented secondary objects in the control was calculated. This threshold was subtracted from each pixel of the illumination-corrected EPOS signal image using the *ImageMath* module, setting sub-threshold pixels to zero. Integrated intensity was subsequently extracted from threshold-subtracted images, and per-image means were used for downstream analyses. One dataset (HER2 nanobodies) lacked an omitting control and was therefore quantified directly from illumination-corrected images without thresholding.

For *in situ* PLA quantification, individual protein–protein interaction events were detected as discrete fluorescent spots. The *in situ* PLA signal was enhanced using the *EnhanceOrSuppressFeatures* module (speckle enhancement, feature size 8 pixels), and signal outside segmented cell boundaries was excluded via the *MaskImage* module. Spots were then segmented using *IdentifyPrimaryObjects* (diameter range: 2–25 pixels; global Otsu thresholding, two-class method). Each detected spot was assigned to its parent cell using the *RelateObjects* module, and the mean number of spots per image was used for downstream analyses.

For colocalization analyses (TFAM/TFB2M/mtCO1 dataset), pixel-based colocalization between the mitotracker signal and the thresholded EPOS or *in situ* PLA signal was quantified using the *MeasureColocalization* module. The Pearson correlation coefficient was used as the primary colocalization metric, calculated at the image level as per-image means.

Statistical analysis

The statistical analysis involved first assessing whether the mean integrated intensity values or the RCP/cell values obtained from EPOS or *in situ* PLA, respectively, were normally distributed. This was tested using the Shapiro-Wilks test. Subsequently, statistical significance was evaluated with the Mann–Whitney test or the Kruskal-Wallis test, followed by Dunn’s multiple comparisons test.

Raw data availability

Image analysis pipelines and the output files for this study have been deposited at Zenodo (<https://doi.org/10.5281/zenodo.19553261>).

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Conflict of interest

OS and BK are the inventors of the proxHCR patent (US Patent 11,034,995), covering also the EPOS design. SMA and RH are affiliated with QVQ, a CRO offering sdAb services and reagents.

Author contributions

LWe, JH, BD, CJ, LWa, EW and AS performed experiments, the vast majority by JH & LWe. SMA, RH, JP, & MMC provided material and expertise in affinity reagents and breast cancer tissues, assisting in setting up experiments. ML assisted in oligonucleotide purification. AW &

JCP performed the image analysis. SB, LGL, BK & JL provided expertise in setting up experiments. JH, LWe & OS drafted the first version of the manuscript; all coauthors contributed to the final version of the manuscript. OS conceived the project, designed the oligonucleotides and supervised the work.

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Figure legends

Figure 1: Design and schematic representation of the EPOS method. **a)** A schematic overview of the oligonucleotide design of the activator (prox probe MINUS) and sensor (prox probe PLUS) proximity probes and HCR hairpins 1 and 2, with complementary domains in each stem of the prox probe oligonucleotides containing deoxyuridines (dUs) and loop domains complementary to the foothold regions of the HCR hairpins. All sequences are shown in 5'-3' direction. **b)** The prox probes bound to interacting proteins with intact dUs in the stem regions. **c)** Degraded prox probes after digestion with uracil-DNA glycosylase (UDG) and endonuclease IV (EndoIV), creating gaps in the stem regions where the dUs were located. **d)** Prox probe PLUS with a long foothold and prox probe MINUS with a single-stranded oligonucleotide containing the activator sequence. **e)** The exposed activator sequence of the prox probe MINUS hybridizes to and invades the prox probe PLUS. **f)** The now-exposed initiator sequence of the prox probe PLUS hybridizes to and invades HCR hairpin 2. **g)** Representation of the HCR reaction, in which several HCR hairpins 1 and 2 form a long, nicked, fluorophore-labelled DNA molecule.

Figure 2: EPOS produces a high-resolution staining of PPIs, which is dependent on enzyme-mediated activation. **a)** Method validation by visualisation of E-cadherin and β -catenin interactions in HaCaT human keratinocytes with EPOS proximity hairpins at different stages of the enzyme activation: Full enzymatic digestion (All), omitting EndoIV (only UDG), omitting UDG (only EndoIV) or no enzymatic digestion (no Enzymes) compared to the same interaction visualized with *in situ* PLA. **b)** Quantification of EPOS signal in a. **c)** E-cadherin and β -catenin interactions in HaCaT cells visualised with EPOS or *in situ* PLA imaged with structured illumination microscopy (SIM) and super resolution widefield microscopy **d)** IF staining of E-cadherin (magenta) and β -catenin (green). **e)** E-cadherin or β -catenin staining in HaCaT cells using a soluble oligonucleotide with the same sequence as an activated prox probe oligonucleotide to initiate HCR reaction of all bound proximity probes. The EPOS and *in situ* PLA signals are shown in yellow, and Hoechst 33342 nuclear staining is shown in blue in the merged images. The scale bar is 5 μ m. Statistical analysis was performed with the Kruskal-Wallis test. n= 3-4 experiments. Each dot represents an image frame, mean values per cell, *** = $p < 0.005$.

Figure 3: EPOS retains spatial signal pattern of PPIs. a) Visualization of the TFAM+TFB2M interaction (magenta) in mitochondria stained with Alexa Fluor® 488-conjugated anti-MTCO1 antibody (mitochondria, green) using EPOS, compared with *in situ* PLA. The signal obtained with EPOS overlaps to a higher degree with the mitochondrial staining than *in situ* PLA. b) Quantification of the degree of EPOS and *in situ* PLA signal overlap with mitochondria staining in a. c) G3BP1 and m6A-mRNA interactions (yellow) in cells after arsenite treatment. d) Quantification of the EPOS and *in situ* PLA signal in c. Hoechst 33342 nuclear staining is shown in blue in the merged images. The scale bar is 5 μ m. Statistical analysis was performed with the Mann–Whitney test. n = 3-4 experiments. Each dot represents an image frame, mean values per cell, * = $p < 0.05$, *** = $p < 0.005$.

Figure 4: EPOS provides an extended dynamic range. a) A dilution series of the rabbit antibody in HaCaT cells in combination with a constant level of mouse antibody demonstrates an increased dynamic range in EPOS when compared to *in situ* PLA. Both antibodies target the heavy chains in clathrin. The rabbit antibody concentration was diluted ranging from 1:10, 1:30, 1:90, 1:270, 1:810, 1:2430 to 1:7290 dilutions and no antibody. b) Quantification of the EPOS and *in situ* PLA signal in a. The EPOS and *in situ* PLA signals are shown in yellow, and Hoechst 33342 nuclear staining is shown in blue in the merged images. The scale bar is 5 μ m. Statistical analysis was performed using linear regression. n= 3 experiments. Each dot represents an image frame, mean values per cell.

Figure 5: EPOS staining of nuclear PPIs and in FFPE tissue sections. EPOS shows higher sensitivity in detecting nuclear interactions between a) SMAD3 and SMAD4 upon stimulation with 5 ng/ml TGF- β in MDA-MB-231 breast cancer cells, b) Quantification of the EPOS and *in situ* PLA signal in a. c) MYCN and MAX interactions in Tet21N neuroblastoma cells containing a Tet-regulatable *MYCN* gene downregulated with doxycycline. d) Quantification of the EPOS and *in situ* PLA signal in c. e) E-cadherin and β -catenin interactions in breast cancer tissue, either illustrated with EPOS or *in situ* PLA. The EPOS and *in situ* PLA signals are shown in yellow, and Hoechst 33342 nuclear staining is shown in blue in the merged images. The scale bar is 5 μ m. Statistical analysis was performed with the Mann–Whitney test. n= 3-4 experiments. Each dot represents an image frame, mean values per cell, ***= $p < 0.005$.

Figure 6: EPOS staining of PTMs. a) Visualisation of post-translational modifications in Human lung fibroblasts (HFL1) with antibodies against phosphorylation on serine 473 (pS473-AKT) and total AKT after the cells were stimulated with 20 ng/ml PDGF-BB for 15 minutes. EPOS shows higher detection levels compared with *in situ* PLA. b) Quantification of the EPOS and *in situ* PLA signal in a. The EPOS and *in situ* PLA signals are shown in yellow, and Hoechst 33342 nuclear staining is shown in blue in the merged images. The scale bar is 5 μ m. Statistical analysis was performed with the Mann–Whitney test. n= 4 experiments. Each dot represents an image frame, mean values per cell, ***=p<0.005.

Fig. 1

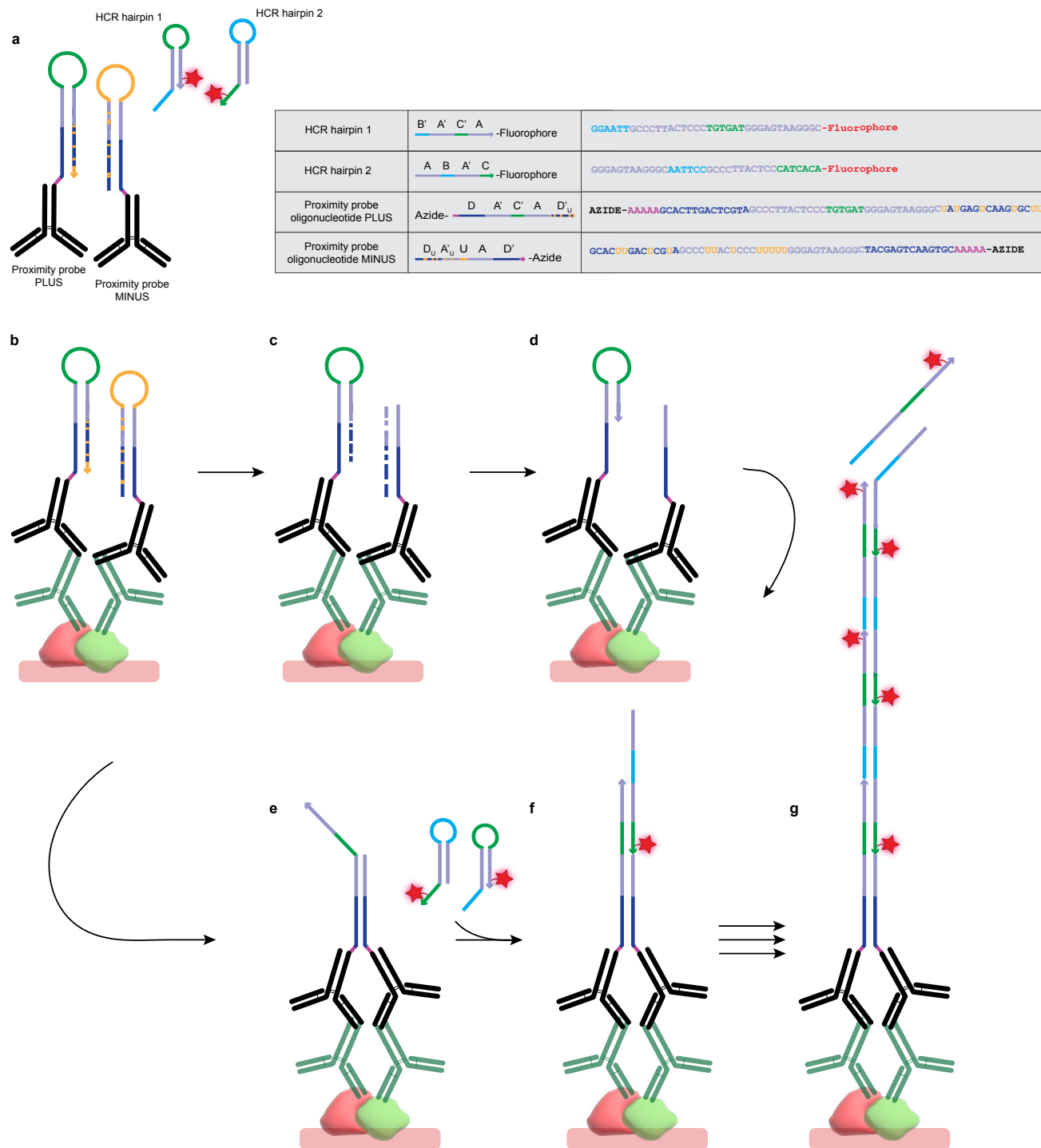


Figure 2

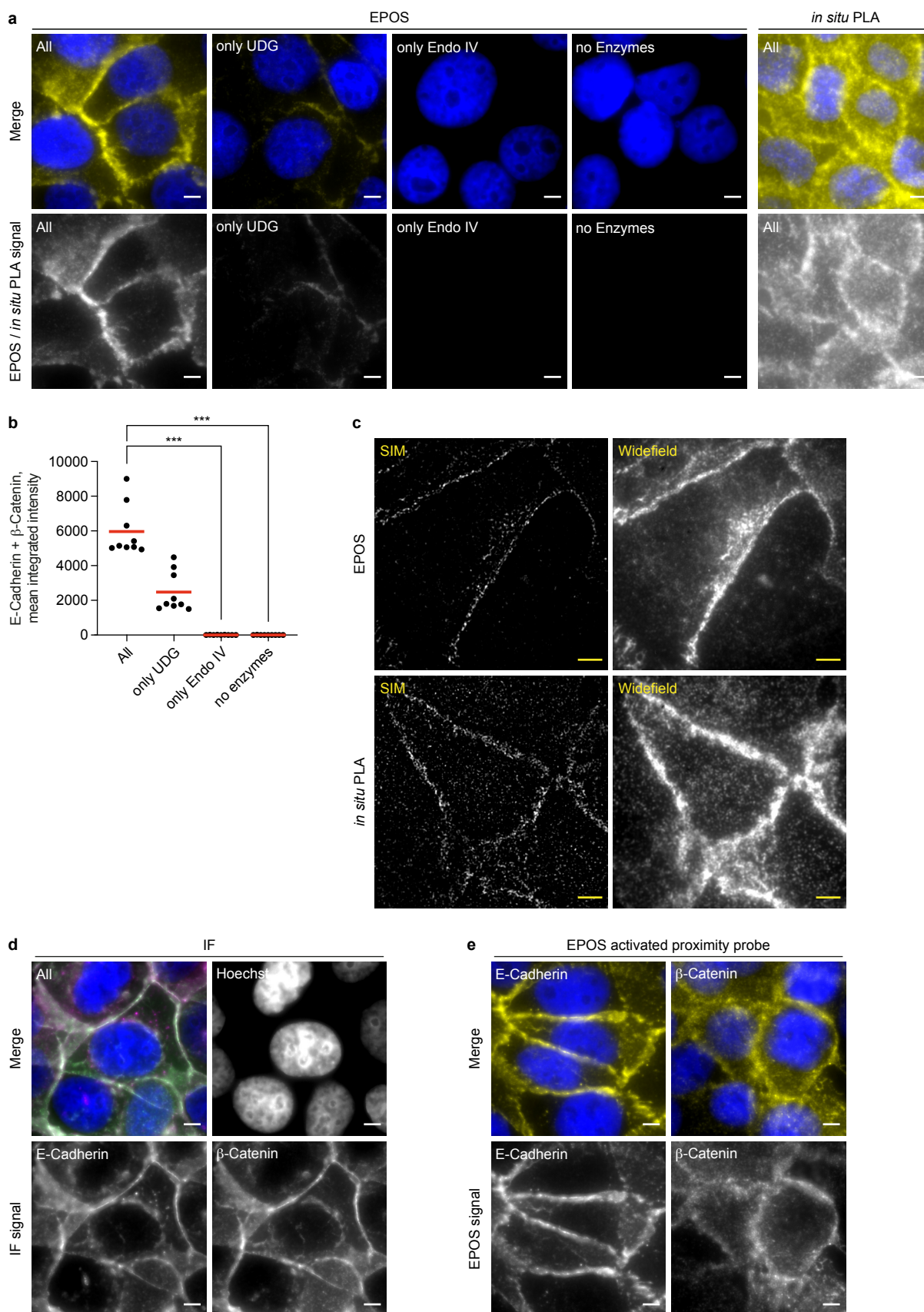


Figure 3

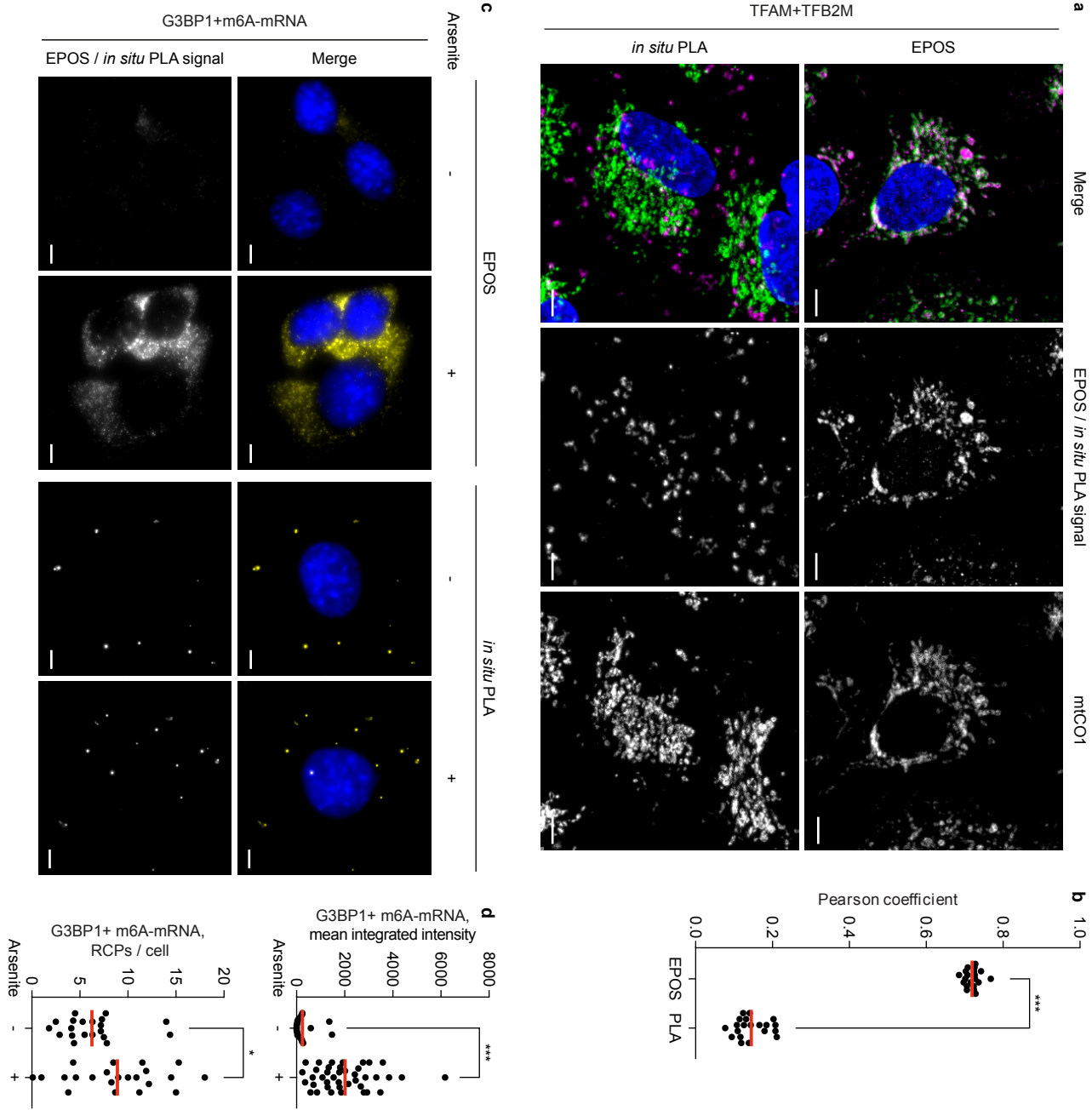


Figure 4

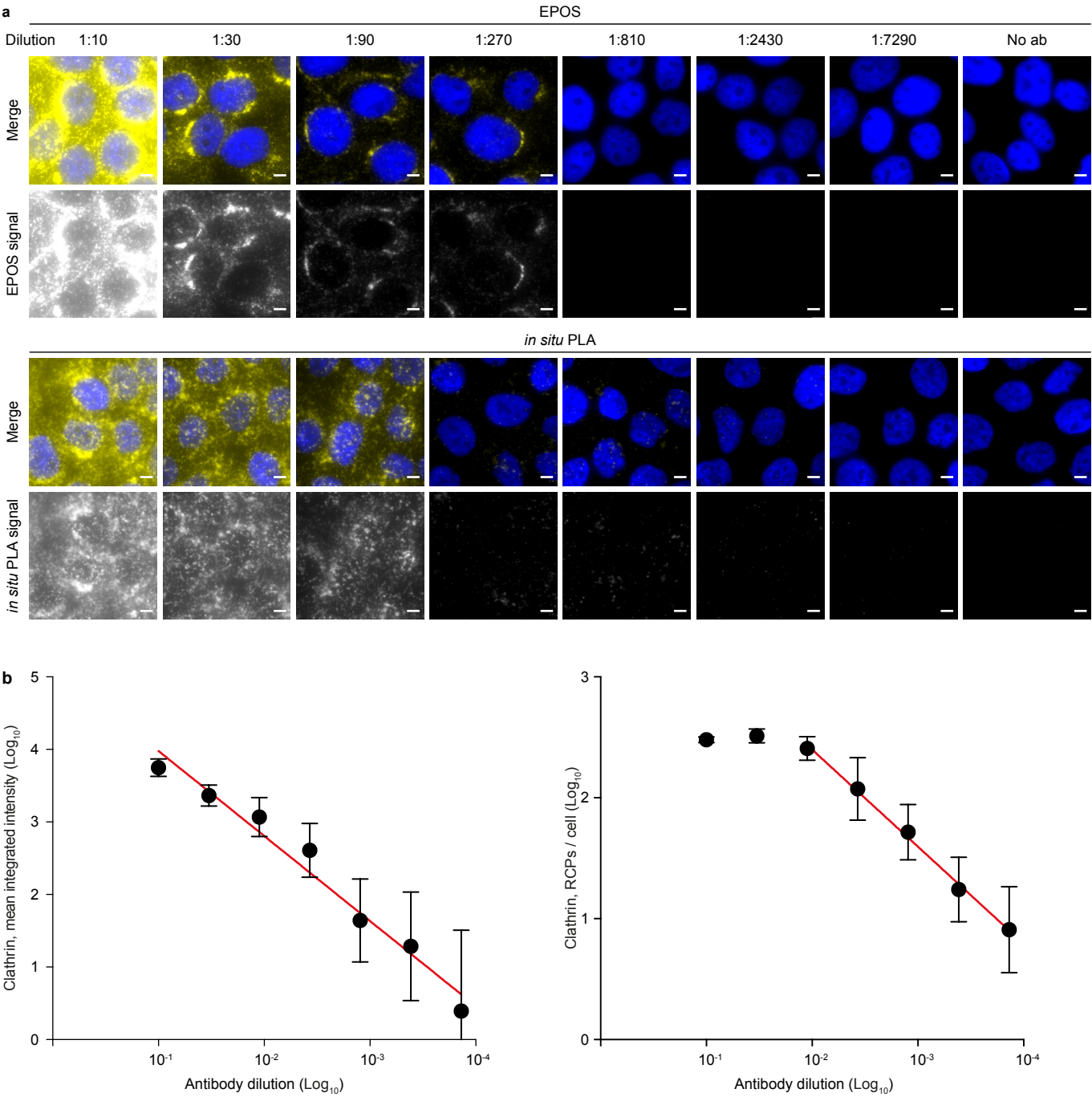


Figure 5

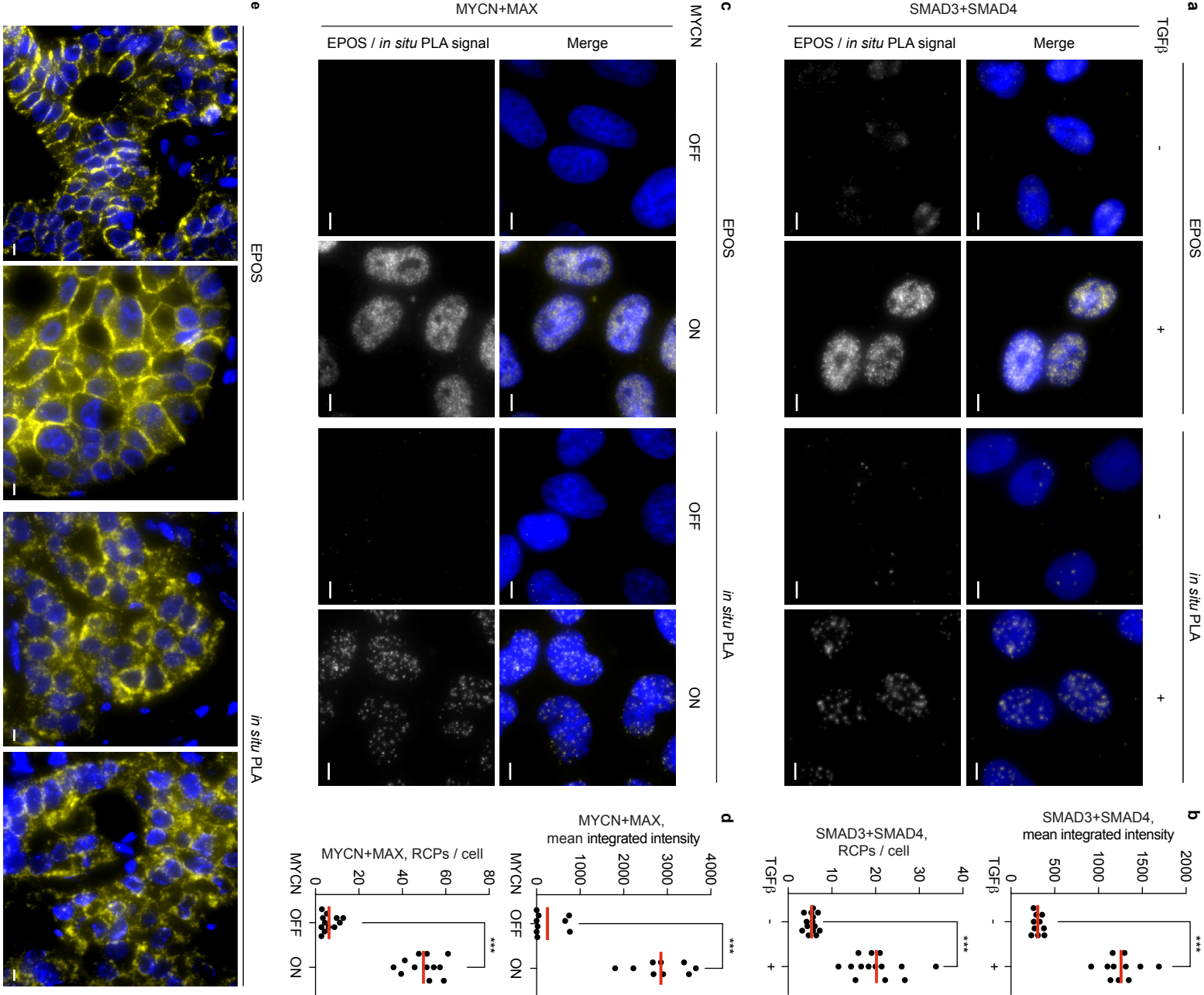
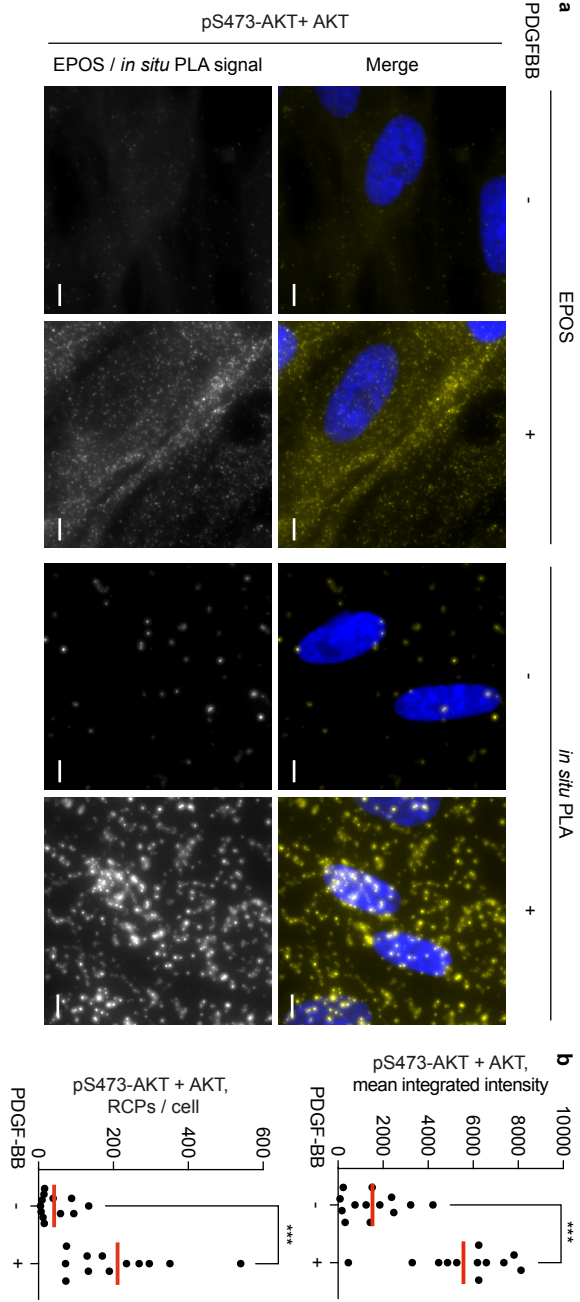


Figure 6



Supplementary Files

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