Parallel Visualization of Multiple Protein Complexes in Individual Cells in Tumor Tissue*§

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Cellular functions are regulated and executed by complex protein interaction networks. Accordingly, it is essential to understand the interplay between proteins in determining the activity status of signaling cascades. New methods are therefore required to provide information on different protein interaction events at the single cell level in heterogeneous cell populations such as in tissue sections. Here, we describe a multiplex proximity ligation assay for simultaneous visualization of multiple protein complexes in situ. The assay is an enhancement of the original proximity ligation assay, and it is based on using proximity probes labeled with unique tag sequences that can be used to read out which probes, from a pool of probes, have bound a certain protein complex. Using this approach, it is possible to gain information on the constituents of different protein complexes, the subcellular location of the complexes, and how the balance between different complex constituents can change between normal and malignant cells, for example. As a proof of concept, we used the assay to simultaneously visualize multiple protein complexes involving EGFR, HER2, and HER3 homo- and heterodimers on a single-cell level in breast cancer tissue sections. The ability to study several protein complex formations concurrently at single cell resolution could be of great potential for a systems understanding, paving the way for improved disease diagnostics and possibilities for drug development. Molecular & Cellular Proteomics 12: 10.1074/mcp.O112.023374, 1563–1571, 2013.

A far greater understanding of proteins interacting in complexes in cells and tissues is needed to explain the functional states of cells. Accordingly, there is a pressing need for improved methods to study protein interaction complexes to explain disease mechanisms; however, suitable methods have been lacking, particularly for clinical material. As an example, proteins in the epidermal growth factor receptor (EGFR) family have traditionally been used as clinical markers. However, in many cases this has proven of limited prognostic value, and activity markers such as receptor interactions are attracting increasing interest (1, 2). Methods such as FRET-based detection (3) or the VeraTag assay (4) can be used to investigate protein complex formations in patient tissues. However, such techniques are not suitable to measure several concurrent protein complexes as required to characterize the balance between different segments of a signaling pathway or between different pathways. FRET-based methods are difficult to use with clinical material and have very limited multiplexing capabilities. The VeraTag assay can be multiplexed, but it fails to provide spatial information of the complexes and thus cannot distinguish between cancer cells and surrounding stroma. Similarly, bulk measures of protein complexes via e.g. co-immunoprecipitation and mass spectrometry (5) disregard cell-to-cell variations and the subcellular distributions of protein complexes. Moreover, such methods are poorly suited for analyzing precious clinical material as too much sample material is needed for the analysis.

To enable parallel analyses directly in tumor tissue of multiple protein complexes involved in signaling pathways, we have developed a multiplex version of the in situ proximity ligation assay (PLA) (6). In situ PLA has previously been used for localized detection of proteins, protein complexes, and post-translational modifications in cells and tissues (6). Because of its intrinsic requirement for dual target recognition by pairs of antibodies and the use of rolling circle amplification (RCA) to substantially amplify signals, the assay allows detection of endogenous protein complexes or post-translational modifications in fixed cells and tissue sections (7, 8) or Western blot membranes (9). The basis of in situ PLA is the detection of a target molecule through the use of a pair of PLA probes, i.e. target-specific affinity reagents such as antibodies to which DNA oligonucleotides have been attached (Fig. 1). We describe herein how tag sequences in the oligonucleotides of each PLA probe, uniquely identifying these probes,
Antibodies and oligonucleotides used in PLA, for creating PLA probes and visualizing RCA products

| Oligo | Description | Oligonucleotide vendor | Conjugated antibody | DNA sequence |
|-------|-------------|------------------------|---------------------|--------------|
| A     | Circularization oligo long | Integrated DNA Technologies |                       | 5'-phosphate-CTA TTA GCG TCC AGT GAA TGC GAG TCC GTC TAA GAG AGT AGT ACA GCA GCC GTC AAG AGT GTC TA |
| B     | Circularization oligo long (single version) | Integrated DNA Technologies |                       | 5'-phosphate-GTT CTG TCA TAC AGT GAA TGC GAG TCC GTC TAA GAG AGT AGT ACA GCA GCC GTC AAG AGT GTC TA |
| C     | Circularization oligo short | Integrated DNA Technologies |                       | 5'-phosphate-GTT CTG TCA TAT TTA AGC GTC TTA A |
| D     | Tag-specific oligo for EGFR | Integrated DNA Technologies |                       | 5'-phosphate-AGC GAT CTG CGA GAC CGT AT |
| E     | Tag-specific oligo for HER2 | Integrated DNA Technologies |                       | 5'-phosphate-EGFR Ab-15 (LabVision) |
| F     | Tag-specific oligo for HER3 | Integrated DNA Technologies |                       | 5'-phosphate-HER2 Ab-8 (LabVision) |
| G     | Tag-specific detection oligo | Integrated DNA Technologies |                       | 5'-Alexa 488-GTA TCT GCT TAT GTC GCC CG |
| H     | Tag-specific detection oligo | Integrated DNA Technologies |                       | 5'-Cy5-CTA GTG CTG GAT GAT CGT CC |
| I     | Tag-specific detection oligo | Integrated DNA Technologies |                       | 5'-Alexa 555-AGC GAT CGT CGA GAC CGT AT |
| J     | EGFR general PLA probe oligo | TriLink Biotechnologies | EGFR Ab-15 (LabVision) | 5'-aldehyde-GAC GCT AAT AGT TAA GAC GCT T |
| K     | EGFR-specific PLA probe oligo | TriLink Biotechnologies | EGFR Ab-15 (LabVision) | 5'-aldehyde-AAA AAA AAA ATA TGA CAG AAC CGG GCG ACA TAA GCA GAT ACT AGA CAC TCT T |
| L     | HER2 general PLA probe oligo | TriLink Biotechnologies | HER2 Ab-8 (LabVision) | 5'-aldehyde-GAC GCT AAT AGT TAA GAC GCT T |
| M     | HER2-specific PLA probe oligo | TriLink Biotechnologies | HER2 Ab-8 (LabVision) | 5'-aldehyde-AAA AAA AAA ATA TGA CAG AAC ATA CGG TCT CGG AGA TGG CTT AGA CAC TCT T |
| N     | HER3 general PLA probe oligo | TriLink Biotechnologies | HER3 Ab-2 (LabVision) | 5'-aldehyde-GAC GCT AAT AGT TAA GAC GCT T |
| O     | HER3-specific PLA probe oligo | TriLink Biotechnologies | HER3 Ab-2 (LabVision) | 5'-aldehyde-AAA AAA ATA TGA CAG AAC GGA CGA TCA TCC AGC ACT AGA CAC TCT T |
can be propagated into the single-stranded RCA products that result when two PLA probes have bound complex-forming proteins. The amplified tags in the RCA products can then be visualized using detection oligonucleotides, labeled with different fluorophores, to uniquely recognize the tag sequences. This multiplex readout makes it possible to compare levels of protein complexes between individual cells by identifying the PLA probes that gave rise to the signals.

To test our probe design, we targeted the well characterized EGFR family. This family consists of four transmembrane tyrosine kinase receptors (EGFR, HER2, HER3, and HER4), involved in the regulation of fundamental cellular functions such as cell growth, survival, death, differentiation, and proliferation (10). Increased expression, or aberrant regulation, of the receptors has been implicated in a broad range of human malignancies, including breast cancer, where overexpression of HER2 is associated with a poor prognosis (11). Members of the EGFR family can interact in different constellations, with HER2 as the preferred interaction partner (12), activating several signaling pathways. These interactions between different members of the EGFR family and with associated proteins have been studied extensively in many different types of cells and tissues with a range of methods (2–4, 13), including in situ PLA (14–17).

Using multiplex in situ PLA, we successfully visualized multiple protein complexes in cultured cells and in fresh frozen tissue sections, illustrating the potential to study the balance between alternative protein complexes in clinical specimens to identify cellular phenotypes.

**EXPERIMENTAL PROCEDURES**

**Preparation of PLA Probes**—For parallel detection of multiple protein complexes, PLA probes for each target protein were created by covalently attaching oligonucleotides, including antibody-specific DNA tags to the corresponding antibodies. The conjugated antibodies and oligonucleotides are described in Table I. The conjugation procedure was performed essentially as described previously (8);
Fig. 2. Selectivity of the PLA probes was verified with immuno-RCA on cell lines stably transfected to express various combinations of EGFR, HER2, and HER3. The following cell lines were used: a, PAE cells expressing EGFR and HER2; b, PAE cells expressing EGFR and HER3, and c, PAE cells expressing HER2 and HER3. Red indicates RCA products; gray indicates cell nuclei, and green indicates cytoplasm. Scale bars, 20 μm.

however, to increase conjugation efficiency we replaced the MES conjugation buffer with a phosphate buffer (100 mM phosphate, 150 mM NaCl, pH 6.0). In addition, 10 mM aniline (Sigma-Aldrich) was included as a catalyst in the conjugation reaction. All conjugates were purified by HPLC on a Superdex-75 column (GE Healthcare) to remove unreacted oligonucleotides and aniline. After purification, the concentrations of the PLA probes were 1 mg/ml.

Cell Cultures and Fresh Frozen Tissue—A selection of porcine aortic endothelial (PAE) cell lines stably transfected with different combinations of EGFR, HER2, and HER3 (PAE-EGFR.HER2, PAE-EGFR.HER3, PAE-HER2.HER3, PAE-HER2.EGFR and PAE-HER2) were cultivated according to published protocols (18). SK-BR-3 cells (origin confirmed by Uppsala Genome Center with AmpliFISTR® from Applied Biosystems; data not shown) were cultured in RPMI 1640 medium with 10% FBS. Cell lines were tested mycoplasma-negative using the mycoplasma detection kit from Roche Applied Science (catalog no. 11296744001; data not shown), and expressions of EGFR, HER2, and HER3 were verified by Western blot (supplemental Fig. 7). Before in situ PLA, cells were seeded on Lab-Tek II chamber slides (Thermo Fisher Scientific Nunc) overnight, then washed with PBS, and fixed with ice-cold 70% ethanol for 60 min.

Fully anonymized fresh frozen human tissue sections were obtained from the Fresh Tissue Biobank at the Department of Pathology, Uppsala University Hospital, in accordance with the Swedish biobank legislation. The breast cancer tissue sections had been previously characterized by HercepTest (Dako) and scored according to the legislation. The breast cancer tissue sections had been previously characterized by HercepTest (Dako) and scored according to the legislation. The breast cancer tissue sections had been previously characterized by HercepTest (Dako) and scored according to the legislation. The breast cancer tissue sections had been previously characterized by HercepTest (Dako) and scored according to the legislation.

Multiplex Quantification of Protein Complexes Involving EGFR, HER2, and HER3 in Cultured Cells and Fresh Frozen Breast Cancer Tissues—To reduce the likelihood of unspecific binding of PLA probes, the fixed cells and tissues on glass slides were first incubated with a blocking solution (1× TBS with 10% sterile filtered goat serum and 2.5 ng/μl sonicated salmon sperm DNA) for 60 min at 37°C. The PLA probes were then diluted 1:50 (EGFR probes), 1:150 (HER2 probes), and 1:250 (HER3 probes) in blocking solution with 0.05% Tween 20 added and applied to the slides for overnight incubation at 4°C. For the SK-BR-3 cells, the probe concentrations were varied as indicated in Fig. 3. After the incubation, the slides were washed three times for 5 min with TBST (1× TBS with 0.05% Tween 20) to remove unbound probes. Hybridization and ligation of linear oligonucleotides to form DNA circles were performed by incubating the slides with 125 nM circularization oligonucleotides A and C (Table I) and 125 nM tag-specific oligonucleotides D, E, and F in ligation buffer (10 mM Tris acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 250 mM NaCl, 0.25 μg/μl BSA, 0.05% Tween 20, 1 mM ATP (Fermentas), pH 7.5) with 0.05 unit/μl T4 DNA ligase (Fermentas) for 30 min at 37°C. To test the binding of the PLA probes, PAE cells were analyzed for protein expression. In these experiments, a single circularization oligonucleotide B was added to the bound PLA probes at a concentration of 125 nM. The slides were washed twice for 5 min with TBST. RCA was performed by incubating the slides with 0.125 unit/μl ψ-29 DNA polymerase (Fermentas) in RCA buffer (33 mM Tris acetate, 10 mM magnesium acetate, 66 mM potassium acetate, 0.1% Tween 20, 1 mM DTT, 0.25 μg/μl BSA, 250 μM dNTP (Fermentas), pH 7.9) for 100 min at 37°C. The slides were washed twice for 5 min with TBST. To detect the RCA products, the slides were incubated with 25 nM fluorescence-labeled tag-specific detection oligonucleotides G, H, and I in hybridization buffer (1× SSC, 0.25 μg/μl BSA, and 0.05% Tween 20) with 1 μM Hoechst 33342 (Sigma) for 60 min at 37°C. Finally, the slides were washed twice for 5 min with TBST and 5 min with PBS before they were dried by centrifugation, and mounted with Vectashield (Vector Laboratories). Single representative pictures were captured as indicated below before the slides were stored at −20°C.

To scan the complete tissue sections, the slides needed rehybridization with fresh detection oligonucleotides. The slides were first heated to 65°C for 2 min and then washed in 70% EtOH for 1 min or until the coverslip fell off, after which the slides were transferred to TBST. Subsequently, the RCA products were re-detected by repeating the protocol mentioned above from the detection step where the tag-specific detection oligonucleotides were used, and until mounting with cover slips.

Image Management—Single tissue images and images of cultured PAE cells were acquired using an Axioplan 2 epifluorescence microscope (Zeiss) equipped with a 100-watt mercury lamp, a cooled CCD camera (AxioCam HRM, Zeiss), and a computer-controlled filter wheel with excitation and emission filters for visualization of DAPI, FITC, Cy3, and Cy5. A ×20/NA 0.8 (Plan-Apochromat 40x/0.90, Zeiss) or a ×40/NA 1.3 oil (Plan-Neofluar 102–818, Zeiss) objective was used for capturing the images. Images were collected as Z-
stacks using the AxioVision software (version 4.8, Zeiss). The Image-Xpress Micro XL (Molecular Devices) was used to acquire images of SK-BR-3 cells (see below). Image analysis and RCA product quantification were performed with the open-source cell image analysis software CellProfiler (19) for representative tissue images and cultured SK-BR-3 cells. The image analysis was performed on the projected images by first using the EnhanceOrSuppressFeatures module to enhance the spot-like RCA products and then counting the number of signal spots.
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(a) (b) (c) (d) (e) (f)

(g) (h)
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of spots with the IdentifyPrimaryAutomatic module. For visualization, the different image channels were pseudo-colored and merged in ImageJ (Fig. 4 and supplemental Figs. 1–6).

The ImageXpress Micro XL (Molecular Devices) was used for tile-scanning of the tissues and acquiring images of SK-BR-3 cells. It was equipped with an S Fluor ×40/0.9 (Nikon) objective and filter cubes compatible with DAPI, FITC, Cy3, and Cy5 excitation and emission wavelengths. The image tiles were saved in a grayscale 16-bit TIFF format. Sets of images representing subregions with both cancerous and stromal areas, selected and annotated by a pathologist based solely on the nuclei presented in the DAPI channel, were manually exported for further processing. All images except for the DAPI images were run through CellProfiler 2.0.0 (revised 5c781e) to distinguish the signals from tissue autofluorescence. The pipeline was primarily composed of the modules ImageMath (brightening the images), EnhanceOrSuppressFeatures (enhancing speckle-like features), IdentifyPrimaryObjects (identifying the signals), MeasureSizeAndShape, and FilterObjects (filtering out all detected objects smaller than or equal to 16 pixels). Afterward, all three signal channels were overlaid and pseudo-colored in Fiji (20) together with the DAPI channel, which was also subjected to background subtraction. Finally, each set of image tiles representing a subregion of the tissue was stitched together with Microsoft® image composite editor.

RESULTS

To enable multiplex combinatorial in situ PLA, we included a tag-specific sequence within the PLA probe. This design utilizes the same oligonucleotides as the version published previously of in situ PLA, with the added requirement of a tag oligonucleotide to create a circular ligation product. By introducing a specific tag for each PLA probe, the ligation product will contain information of the identity of the PLA probe (Fig. 1). To test the new oligonucleotide design, we chose to detect proximity between HER family receptors, visualizing complexes of EGFR, HER2, and HER3. Antibodies against EGFR, HER2, and HER3 were conjugated with oligonucleotides to convert them into PLA probes. To verify the selectivity of the PLA probes, stably transfected PAE cells expressing different combinations of EGFR, HER2, and HER3 were investigated. The three PLA probes were used one at a time to detect the expression levels of all three proteins in the different cells. The binding of the PLA probes was in accordance with results obtained by Western blotting (18; supplemental Fig. 7), demonstrating selective binding of the probes (Fig. 2). To determine how the probe concentrations influence the ratio of observed protein complexes, we then analyzed SK-BR-3 cells while varying the PLA probe concentrations (Fig. 3). As expected, the amount of RCA products for each protein complex varied accordingly. We continued to investigate the reproducibility of the assay by analyzing the mean number of signals per cell obtained when staining three different wells of SK-BR-3 cells using the same master mix (intra-experimental set), compared with three consecutive experiments with individual reagent mixes (inter-experimental set). No significant difference between the intra-experimental and the inter-experimental means of signals per cell was detected (supplemental Fig. 8).

After the validation steps of the PLA probes on cells, we continued to demonstrate the multiplex combinatorial in situ PLA on fresh frozen tissue. We chose to detect proximity between the EGFR family members EGFR, HER2, and HER3 in fresh-frozen human breast cancer tissue. Breast cancer tissues are routinely characterized by immunohistochemistry to assess HER2 expression and are scored from 0−, indicating no visible staining, to 3+, indicating strong staining. Breast cancer tissue sections are heterogeneous, and the staining patterns will vary depending on the fraction of cancer cells present in the image. We performed the assay multiple times in different tissue sections to confirm reproducibility. We present representative images of the different tissues from patients of the two extremes (0− and 3+) and analyzed for all possible EGFR-HER2, HER2-HER3, and HER3-HER3 combinations, together with quantification of these selected images. First, we used a general HER2-specific PLA probe in combination with HER2-, HER3-, and EGFR-specific probes to evaluate the level of complex formation of HER2 with all three receptors. We observed high levels of HER2 complexes, especially HER2 homodimers, in the 3+ tissue but not in the 0− tissues (Fig. 4, a and b). A specific staining pattern was observed, with tumor areas displaying very high levels of complexes, whereas much fewer complexes were observed in the surrounding stroma (supplemental Figs. 9–14). We then replaced the HER2-specific general PLA probe with probes recognizing EGFR or HER3 to detect all pairwise associations between EGFR and HER3 with EGFR, HER2, and HER3 (Fig. 4, c and d and e and f). The numbers of observed complexes involving EGFR and HER3 were generally moderate to low in both tissues. Distinct staining patterns across different cells were discerned, even though differences were not as clearly defined as for complexes involving HER2.

DISCUSSION

The ability of in situ PLA to visualize endogenous protein complexes in clinical tissue specimens provides unique opportunities to characterize molecular processes at cellular resolution to explain cellular reactions and for diagnosis of disease states. As is true for other proximity-based methods used to monitor protein complexes in situ, e.g. FRET, the...
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VeraTag assay, or protein fragment complementation assays such as BIFC, the detected proximity between two proteins is not proof of direct physical interactions between the molecules. Instead, the assays may provide information that the molecules participate in the same protein complexes and thus contribute to the actions by these protein complexes. So-called scaffold proteins can coordinate binding in single complexes by many proteins whose co-location could be measured by methods like in situ PLA (21). However, highly expressed proteins should have an increased risk of false positive PLA signal production as more copies will reside close to the interaction partner by chance. This is a fate shared with the other mentioned proximity-based detection methods. The distance that can separate two molecules recognized in a proximity ligation reaction depends on a number of factors such as the location of the recognized epitopes on the protein surfaces, the size of the affinity reagents used, the site of the attached oligonucleotides, and the distance over which the oligonucleotides can give rise to a DNA circle and hence detect a signal via RCA. Under the conditions reported herein, we estimate that epitope distances of a few tens of nanometers permit production of detection signals.

Just as with immunohistochemistry, the detection efficiency of in situ PLA depends on factors such as choice of fixation and pretreatment in combination with the antibodies used, as well as the concentration of each probe. It is therefore important to optimize all of these parameters for each probe set to ensure optimal conditions. To be able to use in situ PLA for formalin-fixed paraffin embedded (FFPE), the antibodies used need to recognize the fixed epitopes, and antigen retrieval may be required (22). Another concern with PLA is that at higher concentrations of recorded PLA signals, these RCA products will start to coalesce, preventing counting of individual signals. Recent developments have made possible the detection of RCA products with adjustable sensitivity to expand the dynamic range of the assay (23).

Here, we describe a novel oligonucleotide design that facilitates multiplexed analyses of combinatorial protein interactions in fixed cells and tissue, using in situ PLA. The level of multiplexing in this advancement of in situ PLA could be increased substantially from the three simultaneous detection reactions that we demonstrate herein, simply by designing larger sets of orthogonal oligonucleotides. By using a serial hybridization approach, as has been described previously for RCA products deposited on slides (24), the number of recorded protein complex identities can be increased beyond the limit set by the number of fluorophores that can be spectrally distinguished by microscopy. We have used an oligonucleotide design suitable to detect complexes of one protein and each of three other proteins (Fig. 1), but other designs could be used that would allow detection of all pairwise combinations by including a detection tag in both PLA probes (supplemental Fig. 15) and assay for double-labeled RCA products (25). Multiplex assays generally save time and effort and, where applicable, precious clinical material, while providing opportunities for internally controlled reactions. More importantly, however, the method demonstrated herein allows simultaneous assessment of several concurrent molecular events at cellular and subcellular resolution in cells and tissues. This provides entirely new opportunities for snapshot analyses of molecular events that will help elucidate intricate protein networks, revealing protein complexes that jointly exert cellular responses. Multiplex in situ PLA can measure the balance between protein complex formations along a pathway or between different pathways, and it can provide a foundation for a systems understanding of cellular functions. Multiplex in situ PLA also has the potential to reveal entirely new biomarkers that reflect activity states rather than the mere presence or absence of individual proteins. This promises to result in improved disease diagnostics and efficient prediction of responses to molecularly targeted treatment. The method could also prove of great importance in drug discovery, for example in network pharmacology (26) where drugs are designed to act on several targets simultaneously. The ability to simultaneously assess several protein complexes may prove important in approaches to perturb robust underlying networks (27) and to avoid escape mutations in malignancy (26, 28).

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