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Visualizing signal transduction pathways by quantifying protein-protein interactions in native cells and tissue

Olink Bioscience has developed a new protein detection assay that revolutionizes the possibilities of investigating protein-protein interactions in fixed cells and tissue samples. The Duolink® immunoassay reagents are based on the *in situ* proximity ligation assay technology capable of reporting protein-protein interactions in natively expressing cells as countable bright fluorescent spots visualized in a standard fluorescence microscope.

A wealth of tools is available today to study cell-signaling mechanisms. When it comes to quantifying transient protein-protein interactions within a single native cell, however, adequate tools are missing. Duolink immunoassay reagent kits now provide a means of visualizing and quantifying such interactions.

The preexisting techniques for quantifying transient protein-protein interactions require lysing a large amount of the cells to be studied and then performing assays such as the classical co-immunoprecipitation followed by western blotting. This workhorse of cellular biology has been extensively used for many years but is limited in some essential respects. For example, weak and transient interactions may not survive the required washing procedures, and the technique consumes large amounts of cultured cells—which are difficult to obtain for stem cells and tissue material. Also, cell lysis does not maintain the subcellular localization of a particular signaling event, and the commonly used approach of overexpressing target proteins with tagged constructs may itself perturb the natural interactome balance.

Standard *in situ* protein detection protocols such as immunofluorescence and immunohistochemistry involve the use of only a single primary antibody recognizing its target protein. To study two different interacting proteins, one would need to devise an assay using two different primary antibodies. To provide this capability, Olink Bioscience released Duolink, a kit series enabling the use of two primary antibodies for *in situ* immunoassays, thereby bringing the element of dual recognition to localized analyses. Historically, dual recognition by two antibodies, exemplified by sandwich immunoassays such as ELISA, has drastically enhanced the sensitivity, specificity and ability to study protein interactions in solution.

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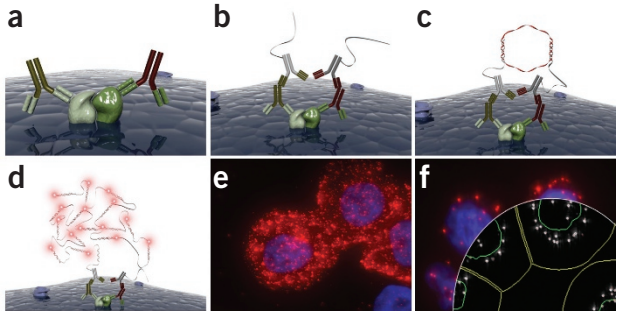

Figure 1 | The Duolink assay principle. (**a**) Two primary antibodies supplied by the user—for example, green (left) from mouse and red (right) from rabbit—recognize the two target antigens of interest. (**b**) Each species-specific secondary antibody provided in the Duolink kit has a different short DNA strand attached to it (light and dark gray, anti-mouse and anti-rabbit, respectively). (**c**) When the secondary antibodies are in close proximity, the DNA strands can interact through the addition of two other circle-forming DNA oligonucleotides, which are then joined by enzymatic ligation. (**d**) Upon addition of Amplification solution (containing nucleotides and polymerase), several hundred–fold replication of the DNA circle can subsequently occur, and fluorescent probes (added as Detection solution) highlight the product. (**e**,**f**) The resulting high concentration of fluorescence in each single-molecule amplification product is easily visible as a discrete bright spot in a fluorescence microscope (**e**), and freeware software can be used to count the number of spots per cell, providing quantitative information and subcellular localization (**f**). Nuclei are stained with Hoechst (blue).

The Duolink reagents and *in situ* PLA technology

The Duolink user first applies two primary target-specific antibodies to cells on microscopy slides. These primary antibodies are selected from two different host species—such as one mouse and one rabbit antibody—so that they are compatible with the generic secondary species-specific antibodies provided in the Duolink kit. These secondary antibodies contain unique DNA strands that hybridize to oligonucleotides, which upon addition of ligase and when in close proximity (<40 nm) form a circular template—hence the name of the assay, *in situ* proximity ligation assay (PLA™)[1–3]. This template is subsequently amplified and then detected using fluorescent

APPLICATION NOTES

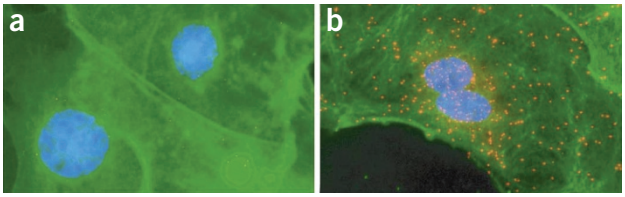

Figure 2 | Visualization of SMAD protein interactions. (**a**,**b**) Images of mouse embryonic fibroblasts without (**a**) or with (**b**) TGF-β stimulation. Each red dot represents an interaction, detected by *in situ* PLA using the Duolink kit, between the SMAD1/2/3 and the SMAD4 protein, respectively targeted by two primary antibodies. Nuclei are stained with Hoechst 33342 (blue), and actin is stained with FITC–anti-actin (green). Data were collected in collaboration with Katerina Pardali, Uppsala University.

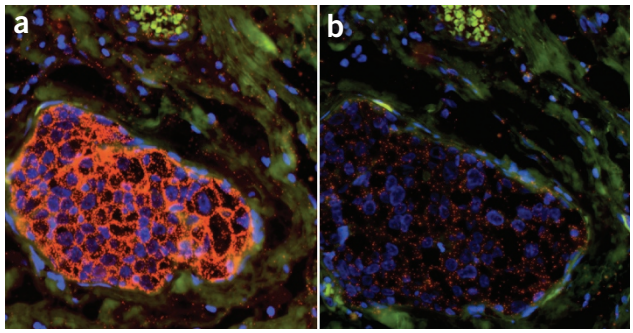

Figure 3 | Analysis of receptor dimerization in formalin-fixed paraffin-embedded tissue. (**a**,**b**) Two serial sections of a breast cancer tissue analyzed for total Her2 protein (**a**) and the Her2-Her3 interaction (**b**) using *in situ* PLA and the Duolink reagents.

oligonucleotide probes. The resulting bright fluorescent spots are derived from single-molecule events of protein-protein interactions, which are visualized using a standard fluorescence microscope (**Fig. 1**).

Applying *in situ* PLA for studying protein interactions

The Duolink reagent kits can be used in numerous applications and are particularly suitable for protein-protein interaction analyses[4,5]. When investigating signaling pathways and protein interactions, one often stimulates the cells to promote the interaction, as illustrated by TGF-β stimulation of mouse embryonic fibroblasts, resulting in SMAD dimerization (**Fig. 2**).

An excellent example of how *in situ* PLA can complement data derived from coimmunoprecipitation and western blotting was recently described in a report[4] of the interaction between PDGF-Rβ and VEGF-R2. The *in situ* PLA results confirmed this newly discovered signaling mechanism as well as provided quantitative localization data. A natural next step for investigating such new interactions would be to study these in tissue samples, but for such experiments western blot detection rarely is sufficiently sensitive.

The quantitative power of Duolink—derived from single-molecule counting—can be used in time-course experiments to study interaction formation and receptor activation upon ligand binding, providing insights into signaling events[5]. Also, in combination with stimulation, one may add substances capable of disrupting protein-protein interactions and study their respective efficiencies. These substances may be monoclonal antibodies or small-molecule drug candidates.

Highly specific phosphorylation detection

The dual recognition element of *in situ* PLA can also be used to gain higher specificity when detecting receptor phosphorylation events. As phosphospecific antibodies are notorious for nonspecific binding to closely related receptors, the use of two antibodies per assay has provided higher specificity when studying such modification events[3,6].

Interaction-based biomarker assays

Individual proteins have long been used as diagnostic markers in cancer tissue samples, such as Her-2 detection for trastuzumab (Herceptin) treatment guidance. To complement these single-protein assays, protein-protein interactions within signal transduction pathways show great promise as a new class of biomarker. Such interaction-based markers could be used to predict and monitor drug response as companion diagnostics, particularly in cancer, where signaling pathway disruption can lead to uncontrolled growth. The Duolink reagents offer novel and efficient means to study interaction-based putative biomarkers at high throughput in archival tissue microarrays. In these applications, the single-cell resolution aids the objective analysis of heterogeneous tissue samples. **Figure 3** illustrates the analysis of the interaction between Her2 and Her3 in breast cancer tissue, as visualized by Duolink.

Conclusions

The Duolink reagent series provides a powerful yet simple means to quantitatively visualize even weak or transient protein interactions in fixed cells and tissue with high specificity and sensitivity. There is no need to overexpress target proteins, and data interpretation is facilitated by countable fluorescent spots representing single molecular events. These generic secondary reagents can be obtained toward multiple species of primary antibody to fit the unique application of the user.

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