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Analyzing human epidermal growth factor receptor family dimerization and activation using Duolink®

Olink Bioscience has developed the Duolink® assay, which allows precise detection and quantification of proteins, protein interactions and modifications in fixed cells and tissue samples in their correct cellular context at physiologically relevant expression levels. This tool may ultimately be used in conjunction with new-generation anti-cancer therapies for inhibiting specific receptor dimerizations.

The importance of the human epidermal growth factor receptor (HER) family in the development and progression of various cancers is widely recognized. Standard immunohistochemistry yields acceptable semi-quantitative data for total protein levels; however, it cannot be used to analyze receptor dimerization patterns. To allow detailed studies of protein-protein interactions, we have developed Duolink, a product based on *in situ* PLA®. Duolink detects proximal proteins in their normal context at normal expression levels and also allows rare events to be detected by generating cell-to-cell statistics[1]. Here we give an example of how Duolink can be used to study epidermal growth factor (EGF) receptor (EGFR) and HER2 receptor dimerization and activation patterns in cells and tissue samples. Depending on the interaction partner for a specific HER receptor, different signaling cascades are activated. Measuring HER-family interaction patterns may have great potential as a companion diagnostic for the new-generation anti-cancer therapies aimed at inhibiting specific receptor dimerizations[2].

The Duolink reagents and *in situ* PLA technology

The Duolink kit series allows the user to combine any pair of immunofluorescence- or immunohistochemistry-validated antibodies. Duolink readout is performed with either a fluorescent label (for fluorescence microscopy) or horseradish peroxidase (HRP; for bright-field detection). The resulting distinct spots are derived from single-molecule protein-interaction events (**Fig. 1**).

Studying HER-family receptor dimerization and activation in cells

At least 11 different ligands are known that regulate HER-family receptor activation and dimerization[2]. Here we used EGF, which exclusively binds and phosphorylates EGFR, to validate EGFR-HER2 dimerization and activation. Two different cell lines, SKBR3 (known to express EGFR and high levels of HER2) and MDA-MB-468 (known to express EGFR and very low levels of HER2), were stimulated with the ligand EGF. Both cell lines responded to EGF stimulation by phosphorylation of EGFR (**Fig. 2**), which was measured using an anti-EGFR antibody (Y-1068). However, only the SKBR3 cell line gave a detectable signal after EGF stimulation when the interaction between phosphorylated EGFR (pEGFR) and HER2 was measured. This was expected, given that the MDA-MB-468 cell line has very low levels of HER2 expression. To further confirm that EGFR-HER2 interactions took place, phosphorylated HER2 (pHER2) was measured using an anti-HER2 (Y1221/1222) antibody. EGF does not bind to HER2; therefore, HER2 can be activated after EGF stimulation only via a complex with

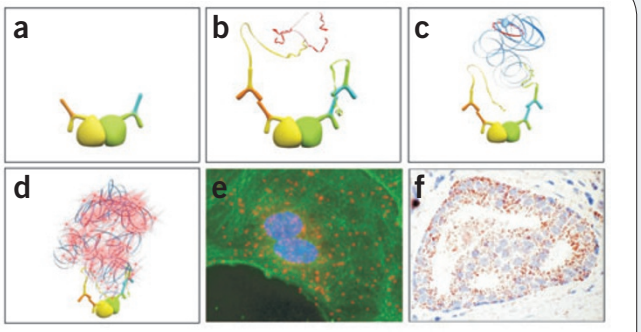

Figure 1 | The Duolink assay principle. (**a**) Two primary antibodies raised in different species and supplied by the user—for example, yellow/red (left) against a target protein (yellow) and green/blue (right) against a second target protein (green)—recognize the target antigen of interest. (**b**) Each species-specific secondary antibody, provided in the Duolink kit, has a unique short DNA strand attached to it (yellow and green, respectively). When the secondary antibodies are in close proximity, the DNA strands can interact through a subsequent addition of two other circle-forming DNA oligonucleotides (red). (**c**) After joining of the two added oligonucleotides by enzymatic ligation, they are amplified via rolling circle amplification using a polymerase. (**d**) After the amplification reaction, several-hundredfold replication of the DNA circle has occurred, and labeled complementary oligonucleotide probes highlight the product. (**e**,**f**) The resulting high concentration of fluorescence or HRP-converted chromogenic substrate in each single-molecule amplification product is easily visible as a distinct bright spot in a fluorescence (**e**) or bright-field microscope (**f**). Nuclei are stained with Hoechst (**e**) or hematoxylin (**f**) (blue).

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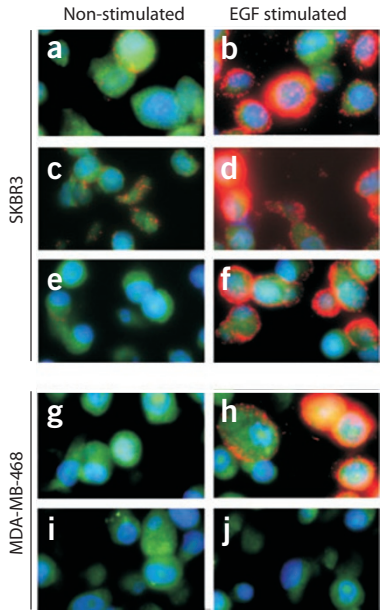

Figure 2 | Duolink assay of HER-family dimerization and activation on EGF-stimulated cells. (**a**–**j**) Two breast cancer cell lines, SKBR3 (**a**–**f**) and MDA-MB-468 (**g**–**j**), are assayed with Duolink before (non-stimulated) and after EGF treatment (EGF stimulated) for three different targets: pEGFR (**a**,**b**,**g**,**h**), pHER2 (**c**,**d**) and pEGFR-HER2 (**e**,**f**,**i**,**j**). The cells are formalin fixed and paraffin embedded. Duolink signals are visualized in red; autofluorescence in the sample (green) is used to visualize the cell confinement; and nuclei are stained with DAPI (blue). Images were taken pairwise with the same exposure time of EGF-stimulated and non-stimulated samples for each assayed analyte.

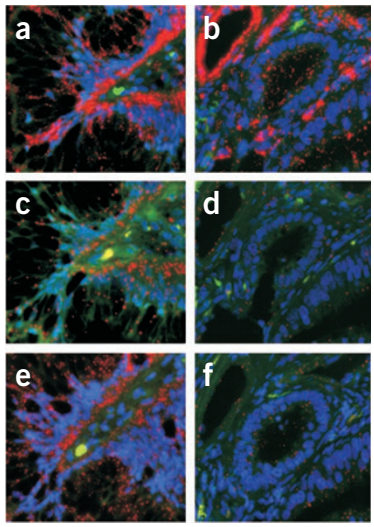

Figure 3 | Duolink assay of HER-family dimerization and activation in tissue samples. (**a**–**f**) Images of one serrated adenoma (**a**,**c**,**e**) and one primary tumor (**b**,**d**,**f**) of colon-tissue origin stained for activated HER-family receptors using Duolink. (**a**,**b**) Both samples contained high levels of pEGFR. (**c**,**d**) Only the sample from the serrated adenoma showed high levels of pEGFR-HER2 complexes. (**e**,**f**) High levels of pHER2 were detected in the same area of the tissue as in the serrated adenoma that stained strongly for pEGFR-HER2 complexes, validating the pEGFR-HER2 data. In this selected case, the primary tumor did not contain high levels of pHER2. Duolink signals are visualized in red; autofluorescence in the sample (green) is used to visualize the cell confinement; and nuclei are stained with DAPI (blue).

EGF-stimulated EGFR. A strong phosphorylation of HER2 was observed. The use of an antibody targeting pEGFR instead of total EGFR ensured that it was an activated EGFR-HER2 complex that was measured and not an inactive, preformed complex that does not elicit downstream signaling. The existence of inactive, preformed complexes has been suggested in the literature[3,4].

Studying tyrosine kinase receptor dimerization and activation in tissue

An advantage of Duolink compared to systems based on tagged proteins is that an assay validated on cells can be transferred and applied directly to tissue samples. In **Figure 3**, two different tissue samples shown to contain high levels of pEGFR were assayed for pEGFR-HER2 complexes using the same assay as shown on cells in **Figure 2**. Only one of the samples exhibited high levels in the pEGFR-HER2 assay. The presence of a complex between phosphorylated EGFR and HER2 would lead to phosphorylation of the HER2 receptor. A Duolink assay measuring pHER2 accordingly showed high levels of pHER2 in the same tissue areas showing high levels of pEGFR-HER2 complexes (**Fig. 3e**). Another example of how Duolink can be used to study HER-family interactions was recently published for the HER2-PTK6 complex detected in FFPE material[5]. The result indicates prognostic relevance of the HER2-PTK6 complex, showing the importance of investigating protein-interaction events for diagnostic use.

The concept described here is of course applicable to any receptor in the HER family. Care need only be taken that the two antibodies accept compatible antigen-retrieval conditions and are still able to bind their target when the target protein is interacting with its partner.

Conclusions

The Duolink assay offers a unique opportunity to decipher HER-family receptor interactions. It holds great promise for further fine-tuning companion diagnostics for HER family receptor–targeted therapies and as a means of measuring the effects of new drugs targeting specific interactions. *In situ* PLA using Duolink reagents is a straightforward process for reporting protein interactions and homodimerization events with very high specificity in their natural context at physiological expression levels.

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