Detection of SMAD complex formation by Duolink™ provides qualitative information on the activation status of TGF-β signaling pathway on single molecular interactions in unmodified cells.

Detection of SMAD complex formation with Duolink identifies activated TGF-β signaling pathways. Duolink goes further by visualizing the subcellular localization of complexes. Duolink provides a detailed and qualitative means of detecting interactions of SMAD proteins with other signaling components.

Studies of SMAD protein interactions are limited by the fact that traditional technologies neither offer the means to work on unmodified cells nor the sensitivity to visualize individual interactions in modified cells. Duolink™ resolves these limitations and thereby provides an excellent method for detailed and qualitative studies of SMAD protein interactions. As SMAD complex formation predefines an active TGF-β signaling pathway, we can assess the activation status of the pathway by observing SMAD complexes.

TGF-β is a growth factor that controls a number of cellular processes, and is implicated in a number of pathological situations, among others cancer and fibrosis. After the ligand binds to its receptors, it activates downstream effectors of the SMAD protein family. SMAD proteins are transcription factors that once phosphorylated by the TGF-β receptors become activated, translocate to the nucleus where they hetero-oligomerize and regulate transcription of target genes (for an extensive review see Feng, et al. 2005).

Although the TGF-β signaling pathway has been extensively studied, several questions about the regulation of the pathway remain unanswered, due to the lack of methods sensitive and specific enough to detect endogenous protein interactions. Furthermore, detecting an activated TGF-β signaling pathway in unmodified tissue samples is essential in a number of pathological conditions.

Olink Bioscience is a privately held company based in Uppsala, Sweden, founded in 2004 by Prof. Ulf Landegren and partners. Olink focuses on innovative methods for detecting endogenous proteins and protein complexes for basic research and high-content screening to deliver accurate information on disease mechanisms and drug response. Our mission is to contribute to a better understanding of the interactome. For more information, visit www.olink.com.
The **in situ** Proximity Ligation Assay and the Duolink detection kit

Traditional cell imaging methods like immunohistochemistry (IHC) can detect the presence of specific proteins, but observations are semi-quantitative at best and are limited to detecting presence of proteins but not protein interactions. There has long been a need for better methods to quantify the interaction of proteins objectively as well as to visualize interactions of proteins **in situ** at naturally occurring levels.

The **in situ** Proximity Ligation Assay (**in situ** PLA) recently described by Söderberg *et al.* (Nature Methods, 2006) resolves these limitations by providing a sensitive means to detect and visualize proteins as well as individual protein-protein interactions **in situ**. Importantly, **in situ** PLA allows point detection of individual endogenous proteins which reveals both their subcellular locations and the frequency of occurrences. This opens completely new opportunities to accurately quantify proteins and protein interactions in unmodified single cells and tissues.

Duolink™ from Olink Bioscience provides a generic approach to using the **in situ** Proximity Ligation Assay with an existing pair of validated antibodies, based on secondary antibody labelling. Duolink is based on the recognition of proteins by the binding of two secondary antibodies to two primary antibodies, which in turn are bound to selected epitopes on the target protein(s). This is followed by DNA amplification that occurs when the secondary antibodies are in close proximity and fluorescent labeling of the DNA product (for a detailed technical description, see the Principle of Duolink note). No modification of cells or tissues is required beyond fixation using standard methods.

**Evaluation of the primary antibodies used for in situ PLA**

A pre-study was carried out to confirm that the primary antibodies used for **in situ** PLA showed sufficient affinities for their respective proteins, SMAD1/2/3 (Figure 1) and SMAD4 (Figure 2) in the cell system studied. Figure 1 shows a translocation of SMAD2 and SMAD3 proteins to the cell nucleus after 45 minutes stimulation with TGF-β. Similarly, Figure 2 shows a translocation of SMAD4 proteins to the nucleus after stimulation. Neither of these experiments can, however, demonstrate the formation of complexes. This pre-study was useful in several respects. It confirmed that the fixation/blocking steps in sample preparation made the protein epitopes available, that the antibodies showed affinity for the epitopes, and that the cells responded to TGF-β stimulation, thereby verifying the activation of the pathway by TGF-β.
Fig. 1. Immunofluorescence staining of endogenous SMAD1/2/3 in unstimulated mouse embryonic fibroblasts (MEF) (A-C) compared to cells stimulated with TGF-β for 45 minutes (D-F). Increased signal is observed in the nuclei of the stimulated cells (D) when compared to the signal in unstimulated cells (A). Cells were incubated with mouse SMAD1/2/3-antibody and secondary FITC-anti-mouse antibody (green). Nuclei were stained with DAPI (blue).

Fig. 2. Immunofluorescence staining of endogenous SMAD4 in unstimulated mouse embryonic fibroblasts (MEF) (A-C) compared to cells stimulated with TGF-β for 45 minutes (D-F). Increased signal is observed in the nuclei of the stimulated cells (D) when compared to the signal in unstimulated cells (A). Cells were incubated with rabbit SMAD4-antibody and secondary TRITC-anti-rabbit antibody (red). Nuclei were stained with DAPI (blue).
Analysis of Heterodimerization of SMAD1/2/3 and SMAD4 by Duolink

In situ PLA enables point detection, i.e. it generates a discrete signal for each detected event. The discrete nature of the assay facilitates an objective approach to image analysis and comparisons of SMAD complex numbers in unstimulated cells to TGF-β stimulated cells.

After stimulation, a higher frequency of events is observed in the cytoplasm, and events localized in the nucleus are clearly discernable. This confirmed that SMAD1/2/3-SMAD4 complexes are formed after TGF-β stimulation as well as that complexes are translocated to the nucleus (Figures 3 and 5).

Fig. 3. Images of mouse embryonic fibroblasts in the absence (A) and presence (B) of TGF-β stimulation for 45 minutes. In situ PLA signals with primary antibodies against SMAD1/2/3 and SMAD4 proteins show interactions as visible red dots. Nuclei are stained with Hoechst 33342 (blue) and actin stained with FITC-anti-actin (green). Images were enhanced identically.

Fig. 4. Image analysis of SMAD1/2/3-SMAD4 interactions. A and B show the segmentation of the images into zones of nuclei and cytoplasm and detection of the interaction events in the cells shown in Figures 3A and 3B respectively, using the BlobFinder software. Signals that fell within a zone around a detected nucleus (between the green and yellow rings) were attributed to a cell, whereas those outside of the boundary or not in conjunction to a detected nucleus were attributed to background and not included in the analysis.
Fig 5. Scatter plot of data output from single cell image analysis by the BlobFinder program. The SMAD1/2/3-SMAD4 interaction events were counted and allocated to either nucleus or cytoplasm. The graph plots the number of nuclear signals/cells (x-axis) vs. the number of cytoplasmic signals/cell (y-axis) for each analyzed cell. Sixty-five unstimulated cells (red squares) and sixty TGF-beta-stimulated cells (grey triangles) were analyzed.

Methodological considerations for the design of in situ PLA assays
As for any method used to detect specific molecules in cells and tissues, special considerations must be taken that may be unique to your particular material and analyte to make them amenable to treatment and detection. You may need to optimize analyte-specific aspects such as the permeability of cellular compartments and the availability of epitopes through fixation, and the elimination of non-specific background by the use of special blocking agents. We have found that assay design is greatly improved by testing different combinations of antibodies, and by having access to appropriate control samples. Depending on the protein or interaction being assayed, it may be important to evaluate the choice of epitopes that are available for labeling.

Methods
Sample preparation
Mouse embryonic fibroblasts (MEF) were seeded on glass slides in regular culture medium (DMEM supplemented with 10% FBS in the presence of antibiotics) and allowed to adhere overnight. The TGF-β signaling pathway was inhibited using 5 µM of the GW6604 low molecular weight inhibitor for 2 h prior to stimulation. Then the cells were rinsed twice with DMEM and either were stimulated with 20 ng/ml TGF-β or remained unstimulated for 45 min. Then the cells were rinsed briefly with PBS and fixed with freshly prepared 3% PFA for 30 min, rinsed with PBS, and the cells were permeabilized with 0.5% TritonX-100 in PBS for 10 min, again rinsed with PBS and 70% ethanol, air dried and stored at -20°C until analyzed with the Duolink assay according to the manufacturer’s instructions (available at www.olink.com).
**Imaging**

Image acquisition was made at 20X magnification (Zeiss Plan-Apochromat, 20X/0.8NA dry) with the Zeiss Axio Imager M1 fluorescence microscope and the AxioVision 4.5 software (Carl Zeiss). Images were captured with an Axio-Cam MRm-camera with an extension tube of 1X (Zeiss 60N-C 1” 1x). Filters used were 575/605 nm (in situ PLA signal) and 350/461 nm (nuclear stain). A Z-stack of 20 images was taken at 20X magnification of the in situ PLA image channel. We acquired one image of nuclei in the Hoechst/DAPI channel by auto-exposure and auto-focus. Typically, an image field contained 25-30 cells.

**Image Analysis**

Cell images acquired using the AxioVision software were exported in TIFF format for subsequent analysis in the BlobFinder image analysis software. The analysis program first performs a signal enhancement of the stack, upon which the rest of the image analysis is based. To perform batch analysis of image stacks, one representative stack of images was selected and a configuration file was created and applied on the batch. Output from the analysis contained data showing both the dots per single cell as well as a maximum intensity projection of the raw image data side-by-side with the segmented image showing identified nuclei and the Duolink assay signals.

**References and footnotes**

In collaboration with Katerina Pardali, Uppsala University

Allalou A. and Wählby C. BlobFinder image analysis software: www.cb.uu.se/~amin/BlobFinder

