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Title: Detection of Protein Interactions by Subcellular Localization Assay

Maria-Magdalena Mocanu[1*], Péter Nagy[2], János Szöllősi[2,3]

[1]*Department of Biophysics, "Carol Davila" University of Medicine and Pharmacy, 050474 Bucharest, Romania*
[2]*Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen, 4032 Debrecen, Hungary*
[3]*MTA-DE Cell Biology and Signaling Research Group, Faculty of Medicine, University of Debrecen, 4032 Debrecen, Hungary*

**Corresponding author: magda.mocanu@umfcd.ro*

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Several techniques have been developed to study protein association or interactions, such as co-immunoprecipitation, co-localization, fluorescence resonance transfer energy (FRET) or yeast-two-hybrid system (1). Nevertheless, each of the aforementioned techniques has its advantages and disadvantages. Patient-derived tissue samples are unique, in a sense they cannot be modified by exogenous expression or overexpression of genetically modified proteins and they typically have high background fluorescence intensity (1,2). To circumvent all these impediments, a Swedish research group developed Proximity Ligation Assay (PLA), a proteomic tool which allows the visualization of protein interactions and their post-translational modifications with high sensitivity with the help of amplified DNA (3,4). The technique was initially established as a tool to detect cytokines (5,6) and was further adapted to study protein post-translational modification *in situ* (2,3,7).

The PLA method uses two antibodies conjugated with oligonucleotides (proximity probes), each antibody being specific for a protein of interest or a cellular structure (3). Two connector oligonucleotides are added to the reaction mixture leading to the formation of circular DNA followed by joining the free ends of DNA by the addition of DNA ligase. In the next step, Rolling Circle Amplification (RCA) will generate a single-stranded DNA product which is connected to one of the proximity probes and it contains several concatenated copies of the connector oligonucleotides. Detection of the amplified DNA is carried out by hybridization with a short, fluorescently conjugated oligonucleotide (detection oligonucleotides) complementary to a short segment of the connector oligonucleotide appearing as a bright spot having a high signal-to-noise ratio in fluorescent microscopy (2,3,6). Interpretation of the results of PLA are based on the assumption that a PLA signal is detectable if the proximity probes are located at a distance of

tens of nanometers (7,8). However, PLA was also considered a “molecular ruler” suitable for measuring distances between two epitopes in the range of 10-30 nm (3). Nevertheless, for the quantitative measurements in the range of 2-10 nm, Förster resonance energy transfer (FRET) should be considered as a more reliable tool (9-13) and at the same time, the saturation features of PLA should be taken into account (14). It is known that photobleaching or autofluorescence of tissue samples can represent drawbacks for FRET, so researchers should take these facts into account when selecting the optimal method to answer their scientific questions (1). Nevertheless, a couple of measurement techniques can minimize the autofluorescence effects on FRET. The accuracy of FRET measurements can be improved by cell-by-cell correction for autofluorescence (15) and by the use of FRET in combination with another technique: total internal reflection fluorescence microscopy (16).

The development of new instruments, faster computers and different software tools led to the adaptation of PLA to a statistically more robust technique, flow cytometry (2,8). When the post-translational modifications of PDGF receptor were analyzed by fluorescence microscopy, the number of quantified cells varied in the range of 100 – 130 cells/sample (4). Statistical improvements became visible when dimerization of EGFR or HER2 was studied by PLA applied to flow cytometry allowing the measurement of thousands of cells per second (8). Furthermore, the technique was developed to allow simultaneous detection of mRNA molecules and protein complexes or post-translational modifications at the single cell level (17).

The paper by Burns *et al*. recently published in Cytometry Part A introduced a new version of PLA, Subcellular Localization Assay (SLA), which allows the identification of the subcellular localization of the proteins of interest using microscopy and a high throughput method, flow cytometry (18). The technique requires two proximity probes: (i) an antibody recognizing a tag, which must be an abundant molecule characteristic of an organelle or part of an organelle (e.g. damaged DNA in the nucleus); (ii) another antibody against the protein of interest. The flow cytometric version of SLA not only offers high-throughput detection by measuring more than 10000 cells/ second, but it is also within reach in many research laboratories or hospitals as opposed to the restricted availability and low-throughput capability of imaging cytometers. Additionally, simultaneous measurement of subcellular localization by SLA and another parameter detectable by fluorescence labeling is also possible. However, the widespread application of SLA may be hindered by the complexity of the protocol for preparing the proximity probes and for performing SLA (18).

SLA was used to study protein localization in two main subcellular compartments, nuclear and mitochondrial. The most developed part of the paper was the application of SLA in the nuclear

compartment for studying the nuclear import of transcription factors in cell populations or in different cell subsets as a function of time and for the localization of the tumor suppressor protein breast cancer 1 (BRCA1) to damaged DNA. Nuclear import of the transcription factors: nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and nuclear factor of activated T-cells (NF-AT) was studied in different cell lines and in peripheral blood mononuclear cells by SLA. In these experiments an antibody against the transcription factors and another antibody against double-stranded DNA served as proximity probes. SLA not only revealed the nuclear translocation of NF-κB and NF-AT as a function of time, but it also allowed the authors to analyze these events in different cell subpopulations (18).

Besides the research applications of SLA, the technique can have clinical use as shown by its ability to detect the interaction between the tumor suppressor protein, BRCA1 and the marker for DNA double strand breaks, gamma-H2A histone family, member X (γH2AX) after DNA damage. Double strand breaks represent a marker for the evaluation of the DNA repair mechanisms after cancer therapy (19). Lymphocytes, skin or tumor biopsies can be collected from patients before and after therapy and investigated for the number of γH2AX foci (20). In their experiments the authors showed that the BRCA1-γH2AX interaction was increased in S and G2/M phases as compared to G1 phase in both control and irradiated samples. Measurement of the nuclear co-localization of BRCA1 and histone γH2AX by confocal microscopy confirmed the SLA results. Since SLA is a high throughput method the authors suggested that this technique can complement the classical microscopy counting method (18).

Taken together, the authors presented a new technique based on the principles of proximity ligation assay which is able to quantify the localization of a protein of interest to different subcellular compartments. Besides the multiple research applications ranging from the detection of nuclear localization of transcription factors to the investigation of the kinetics in multiple cell populations simultaneously in a high-throughput and multiplexed manner, the technique has a high potential to be useful in clinical practice.

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