Sensitivity and Specificity of In situ Proximity Ligation for Protein Interaction Analysis in a Model of Steatohepatitis with Mallory-Denk Bodies

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Abstract

The in situ proximity ligation assay (isPLA) is an increasingly used technology for in situ detection of protein interactions, post-translational modifications, and spatial relationships of antigens in cells and tissues, in general. In order to test its performance we compared isPLA with immunofluorescence microscopy by analyzing protein interactions in cytoplasmic protein aggregates, so-called Mallory Denk bodies (MDBs). These structures represent protein inclusions in hepatocytes typically found in human steatohepatitis and they can be generated in mice by feeding of 3,5-diethoxy-carbonyl-1,4-dihydrocollidine (DCC). We investigated the colocalization of all three key MDB components, namely keratin 8 (K8), keratin 18 (K18), and p62 (sequestosome 1) by isPLA and immunofluorescence microscopy. Sensitivity and specificity of isPLA was assessed by using Krt18\(^{-/-}\) and Krt18\(^{-/-}\) mice as biological controls, along with a series of technical controls. isPLA signal visualization is a robust technology with excellent sensitivity and specificity. The biological relevance of signals generated critically depends on the performance of antibodies used, which requires careful testing of antibodies like in immunofluorescence microscopy. There is a clear advantage of isPLA in visualizing protein co-localization, particularly when antigens are present at markedly different concentrations. Furthermore, isPLA is superior to confocal microscopy with respect to spatial resolution of colocalizing antigens. Disadvantages compared to immunofluorescence are increased costs and longer duration of the laboratory protocol.

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Introduction

In order to obtain insights in molecular mechanisms of diseases it is increasingly important to investigate not only levels of expression of proteins and their subcellular localization, but also their interactions with other proteins. This is particularly important because proteins may exert different functions depending on their presence in multi-protein complexes and their localization in cellular compartments. Techniques available for investigating the colocalization of proteins within cells and tissues include double-label indirect immune fluorescence (DIIF) microscopy and Förster resonance energy transfer (FRET) microscopy [1]. More recently, a new technique for visualizing proteins and their interactions based on the proximity ligation mechanism, was added to the spectrum of microscopy techniques [2].

The in situ proximity ligation assay (isPLA) is a technology that extends the capabilities of traditional immunoassays to also include protein interactions, post-translational modifications, and spatial relationships of antigens in cells and tissues. Protein targets can be readily detected and localized with single molecule resolution and objectively quantified in unmodified cells and tissues. Utilizing only a few cells, even transient or weak interactions are revealed in situ and sub-populations of cells can be distinguished. Within hours, results from conventional co-immunoprecipitation and co-localization techniques can be independently confirmed by isPLA.

isPLA can make use of pairs of primary antibodies raised in different species to recognize the target protein or protein complex of interest. Species-specific secondary antibodies, called PLA probes, each with a unique short DNA strand attached to it, bind the primary antibodies. When the PLA probes are in close proximity, the DNA strands can interact through a subsequent addition of two other circle-forming DNA oligonucleotides. After joining of the two added oligonucleotides by enzymatic ligation, the DNA circles are copied via rolling circle amplification using a polymerase. In the amplification reaction, long DNA strands, each containing several hundred complements of the DNA circle, are formed. These products are easily visualized by microscopy as brightly fluorescent spots using fluorescence-labeled complementary oligonucleotide probes (Fig. 1) [2].

isPLA has been used for investigation of a wide variety of disease conditions, for instance for quantification of HER2-protein complexes in breast cancer [3], and for detecting osteopontin-interactions in resectable gastric cancer.

The increasing use of isPLA as a tool in pathology prompted us to investigate its sensitivity in detail and specificity in comparison...
to that of DIIF confocal microscopy. For this purpose, we used experimentally induced multi-protein aggregates in mouse liver as a test system. This model is based on chronic feeding of mice with a diet containing 3,5-diethoxy-carbonyl-1,4-dihydrocollidine (DDC, also known as diethyl 1,4-dihydro-2,4,6-trimethyl-3,5-pyridinedicarboxylate), which leads to the formation of histopathological structures called Mallory-Denk bodies (MDBs) in hepatocytes and to ballooning of the hepatocytes, which are associated with a disruption of the keratin intermediate filament cytoskeleton. MDBs are protein aggregates in the cytoplasm of hepatocytes, which occurs in alcoholic and non-alcoholic steatohepatitis (ASH, NASH), copper storage diseases, chronic cholestasis, some types of which occur in alcoholic and non-alcoholic steatohepatitis (ASH, NASH), copper storage diseases, chronic cholestasis, some types of which occur in alcoholic and non-alcoholic steatohepatitis (ASH, NASH), rubber anti-K8 (TROMA1 tissue culture supernatant, neat) were used as primary antibodies. Primary antibodies were incubated for 30 min at room temperature, followed by 3×5 min washings in PBS slides were rinsed in 100% ethanol, air-dried and mounted in Mowiol R (Sigma-Aldrich). For the additional DIF staining, rabbit-anti-K8 (TROMA1 tissue culture supernatant, neat) were used as primary antibodies. Primary antibodies were incubated for 30 min at room temperature, followed by 3×5 min washings in PBS slides were rinsed in 100% ethanol, air-dried and mounted in Mowiol R (Sigma-Aldrich). Fluorescence signals from isPLA and DIF were detected by Zeiss LSM 510 META scanning laser confocal microscope. Excitation wavelength for Alexa 488 was 488 nm (Argon Laser), and for the isPLA reagent 594 nm (HeNe Laser) (analysis software: ZEN 2009) was used. A summary of all reaction steps for the combination of isPLA and DIF is shown in Figure 1.

As controls for specificity of isPLA a series of technical and biological negative controls were performed. Technical controls
were based on the omission of first antibodies (to exclude cross-reactivity of secondary antibodies or non-specific PLA-signal generation). Biological controls involved knock-out mice for two of the antigens present in MDBs. In this case all primary and secondary antibodies were applied, however because of the absence of one of the antigens detected in PLA, no PLA-related signals should be detected. Sensitivity of PLA was assessed by comparison with single and double-label immunofluorescence analyzed by confocal microscopy. A summary of all controls performed is shown in Table 1.

Results

In a first approach, the reactivity of the antibodies was tested. All antibodies (ABs) used in this study were applied for single and double-label indirect immunofluorescence (IF) microscopy in different sequential orders. Additionally, the primary ABs were tested by using different fluorochrome-labeled secondary ABs in various combinations to ensure equivalent reactivity (data not shown). Irrespective of the experimental setup identical results were observed for antibodies raised in different species but directed to the same antigen (i.e., K8 and K18, respectively) (Fig. 2).

This was essential because two different AB pairs, raised in different species (e.g., mouse and rat), were used in the combined iPLA and IF staining. Only if it was ensured that both ABs, namely the keratin AB used in IF and the keratin AB used in iPLA had identical staining properties, could differences between IF and iPLA be evaluated. Otherwise any observed differences between IF and iPLA could depend on different staining properties of the primary ABs.

Figure 2 shows a double–label IF of wild type Swiss Albino mice before and after DDC treatment using the optimal AB combinations as identified in the pre-testing phase. Normal mouse livers did not show any protein aggregations positive for p62, but exhibited a filamentous architecture of the keratin intermediate filament cytoskeleton in the hepatocytes. After DDC treatment ballooned hepatocytes were present with a disrupted keratin cytoskeleton and MDBs involving K8, K18 and p62 were observed (Fig. 3). In addition to the classical MDB, which is positive for keratin and p62, small p62 positive but K8/K18 negative structures were present [8]. However, the nature of these structures has not been characterized yet. Furthermore, small keratin positive MDB-like aggregates were seen in which p62 was only weakly present or even undetectable. It was not possible to draw clear conclusions about p62 positivity in these structures, since the observed results were influenced by the threshold and amplification settings for the different fluorescence channels during confocal microscopy (Fig. 3 B).

The same liver used in the experiment shown in Fig. 3, and the same combinations of ABs were applied for iPLA alone, and iPLA in combination with IF (Fig. 4). For the iPLA, two ABs (m-a-K8 and gp-a-p62) were used to detect protein aggregations in which p62 and K8 co-localized. A third AB (rat-a-K8) was applied on the same tissue sample as the primary AB for the IF. Since iPLA only gives a positive signal if both components (keratin, p62) were present, every MDB detected by the iPLA would also be expected to be positive for keratin 8 in the IF stain. The combined iPLA and IF stain confirmed the presence of K8 in every iPLA positive MDB. Moreover, it was notable that iPLA detected the smallest MDBs, visible by DIIF. However, some keratin aggregates resembling MDBs were negative for p62 both in iPLA and IF (Fig. 3 and Fig. 4). In summary, the experiments demonstrated that the sensitivity of iPLA is at least as high as the sensitivity of

Table 1. Technical controls performed on mice with and without DDC treatment.

| Sample | Standard diet | Standard diet | DDC diet | DDC diet | DDC diet | DDC diet | DDC diet |
|--------|---------------|---------------|----------|----------|----------|----------|----------|
| AB PLA m+a-K8 | x             | x             | x        | x        | x        | x        | x        |
| AB PLA gp-a-p62 | x             | x             | x        | x        | x        | x        | x        |
| PLA Probe PLUS PLA Probe MINUS | x             | x             | x        | x        | x        | x        | x        |
| PLA Detect. Kit | x             | x             | x        | x        | x        | x        | x        |
| Rhod. (a-rat) | x             | x             | x        | x        | x        | x        | x        |
| Alexa 488 (a-gp) | x             | x             | x        | x        | x        | x        | x        |

In order to complement the biological controls (knockout mice) we performed a number of technical controls to make sure that no cross reactions between the different reagents might lead to false results.

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As previously published [8], demonstration of equivalent binding properties of ABs used in IF and isPLA. IF staining of normal mouse liver tissues (Swiss Albino, SWA fed a standard diet) with antibodies to K8 (green), p62 (red) (A) and for K18 (green) and p62 (red) (B). Image (C) shows that the two different ABs directed to K8 (rat-anti-K8 [Troma I] and mouse-anti-K8 [Ks 8.7 Progen]) used for isPLA and IF or DIIF recognize identical structures in liver from a DDC fed SWA mouse. Scale bars: 20 μm.

Figure 2. Testing of ABs to keratin and p62 in mouse liver and demonstration of equivalent binding properties of ABs used in IF and isPLA. IF staining of normal mouse liver tissues (Swiss Albino, SWA fed a standard diet) with antibodies to K8 (green), p62 (red) (A) and for K18 (green) and p62 (red) (B). Image (C) shows that the two different ABs directed to K8 (rat-anti-K8 [Troma I] and mouse-anti-K8 [Ks 8.7 Progen]) used for isPLA and IF or DIIF recognize identical structures in liver from a DDC fed SWA mouse. Scale bars: 20 μm.

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K8 IF  
p62 IF  
Merge

K8 IF  
p62 IF  
Merge

K18 IF  
p62 IF  
Merge

DiIIF. As technical negative controls for the isPLA the first ABs to p62 and keratin, respectively, were omitted. All negative controls constantly showed negative isPLA signals (negative controls not shown).

Reactivity of ABs was further assessed in K18 and K18 knock-out mice. In both knock-out mice hepatocytes were negative for keratin intermediate filament cytoskeleton because, in the absence of one partner (e.g., K8) the other still expressed partner (e.g., K18 in K8−/− mice) is unstable and does not form intermediate filaments (Fig. 5). In the absence of a polymerization partner, K8 or K18 is quickly degraded. The only structures that were stained positive for the expressed keratin partner in mice fed a standard diet were bile ducts since in bile duct epithelia additional keratins are expressed that form intermediate filaments [8,9]. DIIF microscopy of DDC-fed krt8−/− mice showed positive cytoplasmic structures which were negative for keratin. In contrast, krt18−/− mice developed MDBs that were composed of K8 and p62 [6] (Fig. 5). These mice were then tested by applying combined isPLA and IF for p62 and K8 or K18 (Figs. 6 and 7). The krt8−/− mice were negative both in the K18+p62 and K8+p62 are PLAs, which underlines the absence of keratin in the small p62 positive structures observed in DIIF (Fig. 3). Furthermore immunofluorescence demonstrated keratin positive aggregates with typical MDB structure which were negative in isPLA. This is in line with previous results demonstrating by double-label immunofluorescence microscopy that some MDB-like aggregates were p62-negative [7]. Thus our isPLA results support these previous findings with an independent technology. In the krt18−/− mice isPLA revealed positive MDBs only in case of using K8 and p62 ABs as primary ABs for the isPLA. In contrast, the isPLA stain with ABs to K18 and p62 was negative. In krt18−/− mice all components of the isPLA (K18 and p62 ABs, amplification system, detection system) were applied but no reaction products were observed. This is consistent with the fact that only one of the two antigens was present, namely p62, while K18 was absent, demonstrating that isPLA is highly specific. Furthermore, these data demonstrated that no cross reactivity occurred between the ABs used for isPLA and those used in IF (in the DIIF a polyclonal rabbit-AB to K8+18 was used, whereas for isPLA a mouse monoclonal AB to K18 was used). This underlined the species

Figure 3. Testing of ABs to keratin and p62 in mouse liver cells after DDC treatment. Immunofluorescence stain on SWA mouse livers after 12 weeks DDC treatment using ABs to K8 (green), p62 (red) (A) and K18 (green), p62 (red) ABs (B). For image (A), note the presence of small (arrowhead) and large (empty arrow) MDBs. Additionally, small granules positive for p62 only can be observed (arrow). For image (B), note that small MDB-like keratin aggregates can be observed that are negative for p62 (empty arrow). Scale bars: 20 μm.

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Figure 4. isPLA for 12 week-DDC treated mice. Images (A, B) show isPLA signal (red) using ABs to p62 and K8 (A) and p62 and K18 (B). (C) shows a combined visualization of p62 and K8 in isPLA (red) and K8 in IF (green). (D) shows a combined visualization of p62 and K18 in isPLA (red) and K18 IF (green). Images (C) and (D) note that even very small isPLA positive structures are visible (arrowhead), demonstrating the high sensitivity of isPLA. However, there were also larger MDB-like keratin aggregates that are negative for isPLA stain (empty arrow), suggesting the absence of p62. Scale bars: 20 μm.

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specificity of the ABs used in the iPLA system and that there is no cross reactivity between iPLA and DIIF.

Discussion

In this study we have performed a comprehensive investigation of clinically relevant morphological alterations by using iPLA. We have chosen a mouse model for steatohepatitis and MDB formation for testing various methodologies because the main molecular components and interaction partners of MDBs, namely K8, K18, and p62, are well known and characterized, and a broad spectrum of antibodies and models were available. In order to demonstrate the sensitivity and specificity of iPLA we have performed technical as well as biological controls, by including gene knock-out mice for the MDB components K8 and K18. We applied PLA for K8 and p62. Arrows indicate a MDB-like aggregate that was constantly positive for K8 but negative for p62. Arrowheads indicate MDBs which were positive in iPLA for p62 and K8 but IF showed only presence of K8 (yellow merged signal) in

investigating the relationship between proteins and their association with each other in tissues. In general, findings of iPLA were similar to those of DIIF. However, iPLA led to much clearer results when one antigen was more abundant than the second one because iPLA only yields a signal in case of antigen colocalization (e.g., small MDB-related keratin aggregates in the presence of the keratin intermediate filament cytoskeleton). In contrast, results of DIIF analyzed by confocal microscopy depended on the settings of the different fluorescent channels. Therefore we recommend iPLA as a preferred technique for visualization of protein colocalization in small structures, particularly when larger,

Figure 5. Demonstration of keratin and p62 in DDC treated krt8<sup>−/−</sup> and krt18<sup>−/−</sup> mice. IF staining on krt8<sup>−/−</sup> (A, B) and krt18<sup>−/−</sup> (C, D) mouse livers after 12 weeks DDC treatment. Images (A) and (C) show the localization of K8 (green) and p62 (red). Images (B) and (D) show the localization of K18 (red) and p62 (red). A positive colocalization of p62 and K8 in krt18<sup>−/−</sup> mice was observed, which indicates presence of MDBs. In contrast krt8<sup>−/−</sup> mice did not develop MDBs after DDC feeding, therefore neither K18 nor p62 accumulated. ABs to K18 show positivity in bile ducts (BD) in krt8<sup>−/−</sup> mice (B). The green reaction in image (D) relates to plasma cells. Scale bars: 20 μm. doi:10.1371/journal.pone.0096690.g005

Figure 6. iPLA and IF for 12 week-DDC treated krt8<sup>−/−</sup> and krt18<sup>−/−</sup> mice. (A, B) krt8<sup>−/−</sup> mouse liver. (A) Combined iPLA (K8+ p62, red) and IF (K8, green). (B) Combined iPLA (K18+ p62, red) and IF (K8+ K18, green). (C, D) krt18<sup>−/−</sup> mouse liver. (C) Combined iPLA (K8+ p62, red) and IF (K8, green). (D) Combined iPLA (K18+ p62, red) and IF (K8+ K18, green). Note that krt8<sup>−/−</sup> do not form MDBs after DDC feeding and there is no iPLA positive signal. In DDC fed krt18<sup>−/−</sup> mice iPLA yielded only positive signals with K8 ABs demonstrating specificity of the whole iPLA system. Scale bars: 20 μm. doi:10.1371/journal.pone.0096690.g006

Figure 7. Influence of channel amplification on demonstration of antigen colocalization in iPLA and IF in 12 week-DDC treated krt18<sup>−/−</sup> mice. (A, B, C) Show different levels of digital amplification of the green channel (IF for K8) and constant amplification of the red channel showing iPLA for K8 and p62. Arrows indicate a MDB-like aggregate that was constantly positive for K8 but negative for p62. Arrowheads indicate MDBs which were positive in iPLA for p62 and K8 but IF showed only presence of K8 (yellow merged signal) in iPLA positive MDBs after maximal amplification of the green channel. Scale bars: 20 μm. doi:10.1371/journal.pone.0096690.g007
strongly positively stained structures are present as well (this situation causes problems in setting the appropriate background thresholds in DIIF). For instance, because of these properties of iPLA our analysis provided evidence that p62 was lacking in some MDB-like keratin aggregates. This new observation may shed new light on the role of p62 in MDB pathogenesis.

Furthermore, since iPLA signals depend on the molecularly defined maximal distance between the antigens, iPLA provides better defined resolution for protein colocalization than DIIF. iPLA is estimated to permit identification of pairs of epitopes located within less than approximately 40 nm. This is a 5-fold higher resolution compared to DIIF, which allows the detection of distinct signals with a distance of approximately 0.2 μm (along the x- and y-axes) due to limitations of standard light microscopy. Since the optical section thickness of confocal microscopy is in the range of 0.5 μm (z-axis), iPLA is of particular advantage for demonstrating colocalization of antigens along the z-axis. However, the resolution of iPLA is lower than that of FRET which detects molecular interactions at a distance of even below 0.5 nm [10].

In recent years other techniques like Array Tomography with High-Resolution Three-Dimensional Immunohistochemistry have also been developed. This is a volumetric microscopy method based on physical serial sectioning where ultrathin sections are cut, stained and imaged. The resulting two-dimensional images are then further reconstructed by using computational methods in order to generate three-dimensional volume images, suitable for quantitative analysis [11]. This method is more difficult to realize than iPLA.

In conclusion, we found that the iPLA provides more accurate results compared to DIIF because of the signal specificity, the better spatial resolution and the independence of the results and exposure time and digital amplification of microscope channels used (Fig. 7; pros and cons of iPLA are summarized in Table 2). As the function of proteins critically depends on their interactions with other molecules and the localization of multi component complexes in specific subcellular components, techniques for in situ detection of protein complexes become important. Furthermore, personalized medicine application does not only depend on the expression of the drug target but also on the activation of the target-related pathway, which is characterized by specific interactions between cellular components. Therefore application of iPLA might become increasingly relevant for molecular diagnosis and research requiring a carefully controlled and robust protocol, for which examples and practical guidance is provided in this study.

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Author Contributions

Conceived and designed the experiments: BZ KZ JH. Performed the experiments: BZ IK AT. Analyzed the data: BZ IK AT UL KZ JH. Contributed reagents/materials/analysis tools: KZ JH. Wrote the paper: BZ UL KZ JH.

Table 2. Comparison of DIIF microscopy and iPLA for analyzing the colocalization of antigens.

| Feature                  | Double-label IF | PLA |
|--------------------------|-----------------|-----|
| Sensitivity              | ++              | +++ |
| Specificity              | ++              | +++ |
| Dynamic range*           | +               | +++ |
| Spatial resolution       | ++              | +++ |
| Protocol time            | +++             | +   |
| Costs                    | +++             | +   |

Relative advantages are indicated in + to ++. *: dynamic range is particularly important for visualizing colocalization of high and low abundance antigens, which also impacts on overall sensitivity and specificity.

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