Alpha-synuclein proximity ligation assay (AS-PLA) in brain sections to probe for alpha-synuclein oligomers

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Running title: AS-PLA technique for alpha-synuclein oligomers

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Abstract

Alpha-synuclein oligomers are thought to be toxic mediators of Parkinson's disease and other alpha-synucleinopathies, but their histological detection *in situ* in diseased brain has been a challenge in the field for some time. Here we describe a method, the alpha-synuclein proximity ligation assay (AS-PLA), to detect alpha-synuclein oligomers in paraffin-embedded brain sections. Using AS-PLA previously unobserved alpha-synuclein oligomeric pathology is revealed.

Keywords

Alpha-synuclein, oligomers, aggregates, proximity ligation assay, pathology, Parkinson's disease, synucleinopathy

1. Introduction

Lewy bodies, proteinaceous inclusions that contain fibrillized alpha-synuclein (Spillantini, Schmidt et al. 1997), are the pathological hallmark of Parkinson's disease (PD),. Studies over the past two decades have provided evidence that Lewy bodies are markers of a late stage of the disease and may form as a protective mechanism, placing smaller aggregates of alpha-synuclein, or oligomers, as the toxic species mediating disease (Caughey and Lansbury 2003). However, while Lewy bodies can be easily detected using alpha-synuclein immunohistochemistry, alpha-synuclein oligomers have proved more challenging to detect in patient brain.

To address this, we developed the alpha-synuclein proximity ligation assay (AS-PLA) (Roberts, Wade-Martins et al. 2015). Proximity ligation assays (PLAs) were originally developed for the sensitive detection of proteins, but soon adapted to detect protein-protein interactions (Gullberg, Gustafsdottir

et al. 2004, Soderberg, Gullberg et al. 2006). PLA probes are generated from antibodies raised against the protein(s) of interest, one for each of the proteins involved in the putative interaction, which are conjugated to short oligonucleotides. If the probes bind interacting proteins, the oligonucleotides are sufficiently close to prime an amplification reaction, which can be detected by tagged oligonucleotides and observed as punctate signal, with each punctum representing an interaction.

We adapted PLA in order to detect alpha-synuclein oligomers by using the same epitope-blocking anti alpha-synuclein antibody to generate both probes (Figure 1) and performed extensive *in vitro* characterization to demonstrate the specific detection of alpha-synuclein oligomers. Using AS-PLA we were able to describe for the first time the presence of abundant diffusely deposited oligomeric pathology in the medulla and cingulate cortex of Parkinson's disease post-mortem brain tissue (Roberts, Wade-Martins et al. 2015) (Figure 2). AS-PLA also labelled very early perikaryal aggregates in morphologically intact neurons that may precede the development of classical Parkinson's disease, whereas brain stem LBs, considered heavily compacted late lesions were only stained on their periphery, if at all, indicating that oligomers exist in the cytoplasm surrounding the more compact structures of LBs (Figure 2). AS-PLA is a robust technique for the detection of alpha-synuclein oligomers and can be performed in a similar time-frame to and using equipment for standard immunohistochemistry, making it widely applicable.

2. Materials

2.1. Tissue

Sections of formalin-fixed paraffin-embedded tissue should be cut using a microtome at 4-6 µm (see **Note 1**) and deposited on positively charged slides (e.g. Fisher SuperFrost slides).

2.2. Preparation of tissue for staining

- Solutions for dehydration and rehydration of tissue: Xylene, histoclear, ethanol. Make up solutions for rehydration and dehydration of tissue in coplin jars. Make ethanol and water solutions fresh each time and reuse xylene and histoclear up to a maximum of five times.
- For mounting: DPX and rectangular coverslips
- 30% hydrogen peroxide – store at 4 °C in dark
- Antigen retrieval buffer – citrate buffer pH 6.0 (e.g. Abcam catalogue #: ab93678)
- Hydrophobic barrier pen (e.g. Vector Laboratories catalogue # : H-4000)

2.3. Reagents for alpha-synuclein proximity ligation assay

- Anti alpha-synuclein antibody 211 (1mg/ml). Abcam catalogue #: ab80627 (see **Note 2**)
- Duolink Probemaker kit (Sigma catalogue #: DUO92009 and DUO92010) (see **Note 3**). Components: lyophilized probe oligonucleotides, conjugation buffer, stop reagent, storage solution, blocking solution, probe diluent
- Duolink brightfield detection reagents (Sigma catalogue #: DUO92012)(see **Note 3**). Note that some reagents are stored at -20 °C and some at 4 °C. Components: 5x ligation stock, ligase, 5x amplification stock, polymerase, 5x detection stock, substrates A, B C and D
- Tris-buffered saline (0.01 M Tris, 0.137 M NaCl) + 0.05% tween (TBS-T)

3. Methods

3.1. Preparation of AS-PLA probes

AS-PLA probes should start to be prepared at least the day before experiment, as an overnight incubation is required. Testing each new batch of probes using, for example, Parkinson's brain tissue, before running experiments is advised. The protocol described here utilizes the Duolink Probemaker kit.

- Add 20 µl of the antibody syn211 to 2 µl of conjugation buffer and mix by gently pipetting, then add the solution to the freshly opened PLUS or MINUS lyophilized probe oligonucleotides and mix by gently pipetting
- Incubate at room temperature overnight (16 hours minimum)
- Add 2 µl of stop reagent to the reaction and incubate at room temperature for 30 minutes
- Add 24 µl of storage solution and store 4°C

3.2. Preparation of tissue

- Warm slides for 30 minutes on a hot plate at 55 °C
- Dewax by dipping slides in xylene 5 times, leaving for 2 minutes, then dipping 5 times again
- Repeat with histoclear
- Rehydrate by dipping slides in 100% 5 times, leaving for 2 minutes, then dipping 5 times again. Repeat with 95% ethanol, then 70% ethanol, then RO water for 3 x 1 minute.
- Quench endogenous peroxidases with 10% H_2O_2 diluted in PBS for 15 minutes at room temperature in the dark.
- Wash in running RO water for 5 minutes.
- ***CRITICAL STEP*** (see **Note 4**). Antigen retrieval: Put slides in microwaveable box filled with citrate buffer pH 6 and microwave on a standard cycle for 4 minutes followed by 5 minutes rest. We use a Sharp 900W microwave. Microwave again for 1.5 minutes, then rest for 5 minutes and top up the buffer. Repeat microwaving for 1.5 minutes followed by a 5 minute rest three times.
- Cool the box on ice until cool (approximately 45 minutes), then wash the slides under running RO water
- Put slides onto humidified staining plate and circle the sample with a hydrophobic barrier pen

- Wash a couple of times with TBS + 0.05 % Tween (TBS-T)

3.3. AS-PLA staining

General points:

Do not allow the tissue to dry at any point during the staining protocol as this will increase background.

Defrost stock solutions fully at room temperature and mix before use. Always prepare immediately before use. Do not use diluted solutions unless fresh.

For steps with enzymes, add them to the mix immediately before addition to the sample. Once out of the freezer, ensure they are placed on an ice block.

- Cover the tissue with an appropriate amount of blocking solution (see **Note 5**) and incubate for 1 hr at 37 °C. Blocking at lower temperatures or for shorter times will increase background. If 1 h at 37 °C results in diffuse background that is not punctate (especially noticeable in the nucleus) block for longer.

- Dilute AS-PLA probes appropriately in PLA probe diluent (1:100 for brightfield). Add mix to slides and incubate overnight at 4 °C. (see **Note 6**)

- Wash 4 x 5 minutes with TBS-T

- Prepare the ligation solution consisting of 5x ligation stock diluted to 1x in ddH_2O and ligase (1U per 40 ul reaction, stock is 1U/ul). Add the solution to the sections and incubate for 1 hr at 37 °C

- Wash 4 x 5 minutes with TBS-T

- Prepare the amplification solution consisting of 5x amplification stock diluted to 1x in ddH_2O and polymerase (5U per 40ul reaction, stock is 10U/ul) and incubate for 2.5 hours at 37 °C

- Wash 4 x 5 minutes with TBS-T
- Prepare the detection solution consisting of 5x detection stock diluted to 1x in ddH_2O and incubate for 1 hour at room temperature
- Wash 4 x 5 minutes with TBS-T
- Prepare the substrate solution consisting of 66.6x substrate A stock, 100x substrate B stock, 100x substrate C stock and 50x substrate D stock all diluted to 1x in ddH_2O and incubate for 20 minutes at room temperature
- Wash 3 x 5 minutes in ddH_2O
- Add hematoxylin for 5 minutes (see **Note 7**)
- Wash under running tap water for 5 minutes
- Dehydrate (RO water, 95% EtOH, 100% EtOH, xylenes) and mount using DPX

4. Notes

Note 1

We have tested using 20 µm thick frozen tissue and found there was an order of magnitude less signal. Therefore, for optimal results we recommend using thinly cut paraffin embedded sections.

Note 2

The antibody must be in an amine-free buffer, ideally PBS. The manufacturer suggests that the buffer should be carrier- and preservative-free, but may contain up to 0.1% BSA, 5% trehalose, and 0.02% sodium azide.

It is best if the antibody is bought at a high concentration (ideally 1 mg/ml) as the yield of antibody following concentration by filter column is poor.

For murine applications alpha-synuclein 4D6 (Abcam catalogue #: ab1903) enables the detection of alpha-synuclein oligomers detecting mouse, rat and human alpha-synuclein(McMillan, Murray et al. 2017).

Please note that detection of different alpha-synuclein species requires careful validation of the conjugated antibodies, as the epitope of each antibody will determine which alpha-synuclein species is/are detected by the assay. It is also essential to perform *in vitro* validation of novel antibodies to ensure that they block the epitope following binding, and spurious signal does not arise from multiple antibody molecules binding a monomer. This can be achieved using the antibody for both capture and detection in an ELISA with monomeric protein.

Note 3

It is possible to make the PLA reagents in house, as previously described (Soderberg, Gullberg et al. 2006), but for most laboratories using kits is more convenient. As the sequence of the oligonucleotides for the PLA probes provided in the Probemaker kit are proprietary, it is not known if this is the same as in previous publications.

Note 4

Antigen retrieval is a critical step in the success of AS-PLA. We have found that antigen retrieval needs to be more extensive than what is required for immunohistochemistry, and is essential for the observation of AS-PLA signal. The antigen retrieval method described here has given us consistent results, but may require optimization in each laboratory according to the microwave. Other tools, such as an autoclave, may be used as an alternative, but the specific conditions must be adjusted according to the tools available.

Note 5

Ensure that the tissue is fully covered by the reagents. We have found that 120 ul is enough volume to cover tissue sections that cover most of a standard slide, and 60 ul is used for half a slide. If a tissue section covers the full area of the slide, 150ul may be required. Volumes should be adjusted according to the size of tissue for each assay. Other trays or staining systems may be used. For example, we have achieved similar results using the Shandon Sequenza Coverplates. The technique is also amenable to automation.

Note 6

Note that while we have described brightfield AS-PLA here, it is also possible to utilize fluorescent AS-PLA and complement with immunofluorescence to undertake co-localization studies. For this, a standard immunofluorescence protocol is undertaken first, followed by washing with TBS + 0.05% Tween. Then the steps for AS-PLA described in this protocol are followed, until the amplification step and the amplification and detection are done together (use Duolink fluorescent reagents appropriate to your imaging solutions). Following amplification, wash with TBS (0.2 M Tris and 0.1M NaCl) including DAPI, then a further 3 times with TBS and mount using an aqueous solution.

Note 7

It is important not to over develop the hematoxylin so its signal does not obscure the PLA signal.

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