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Indirect Proximity Ligation Assay (PLA) - Fluoresence

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ABSTRACT

Indirect Proximity Ligation Assay (PLA) is a powerful molecular technique used to detect and visualize protein-protein interactions, protein modifications, and protein complex formations within cells or tissues. This method is based on the principles of proximity-dependent ligation and utilizes specific antibodies to detect the nitration of proteins on free-floating brain sections. Here we describe the PLA protocol that we routinely use in our laboratory to detect nitrated alpha-synuclein and nitration of mitochondrial enzymes such as SOD2 and the mitochondrial complex 1 subunit NDUFB8.

Protocol status: Working
We use this protocol and it's working

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MATERIALS

Blocking solution: Vector Lab SP-6000-100

Duolink In Situ Wash Buffers, Fluorescence: DUO82049-20L

Duolink InSitu PLA probe anti-rabbit PLUS kit: DUO92002-100RXN

Duolink InSitu PLA probe anti-mouse MINUS kit: DUO92004-100RXN

Duolink In Situ Detection Reagents Red: DUO92008-100RXN

Duolink *In Situ* Mounting Medium with DAPI: DUO82040-5ML

Antibodies:
mouse anti-3-NT: 1:250; ab61392, Abcam
rabbit anti-human alpha-synuclein (clone MJFR1): 1:4000, ab138501, Abcam
rabbit anti-SOD1: 1:1000; ADI-SOD-110, Enzo Life Sciences
rabbit anti-NDUFB8: **1**:300; 14794, Proteintech

Day 1

1 Pick 35um cut brain sections and transfer them to 1.5 mL Eppendorf tubes:
Note: all incubation and wash steps are performed by shaking Eppendorf tubes at 250rpm (e.g., thermomixer)

2 Wash 2x 00:05:00 with Tris-HCl 5m

3 Wash 3x 00:05:00 with wash buffer A (see materials) 5m

4 **Antigen-retrieval** with citrate buffer (+tween, pH = 6) at 95°C for 00:04:00 4m

Note: Heat the solution to 95°C before adding on the samples

5 Wash 3x 00:05:00 with wash buffer A 5m

6 **Blocking:** Incubate in Duolink Blocking Solution (300 µL)at 37°C for 01:00:00 1h

7 **Primary Antibody Incubation**: Dilute antibodies in Duolink Antibody Diluent (200 µL) and incubate Overnight room temperature. 5m

for nitrated alpha-synuclein: mouse anti-3-NT and rabbit anti-h-alpha-synuclein

for nitrated SOD2: mouse anti-3-NT and rabbit anti-SOD2

for nitrated NDUFB8: mouse anti-3-NT and rabbit anti-NDUFB8

see materials for dilutions and catalog numbers

Day 2 10m

8 Wash 3x 00:10:00 with wash buffer A 10m

9 **PLA probe incubation**: 1h 30m

Prepare anti-mouse MINUS and anti-rabbit PLUS probes following manufacturer's instructions, add solution on sections and incubate at 37°C for 01:30:00

10 Wash 3x 00:10:00 with wash buffer A 10m

11 **Ligation:** 1h 15m
Dilute the Duolink Ligation Buffer 1:5 in high-purity water
Add the ligase (diluted 1:40) just before incubation (keep cold on freezer block!)
add the ligation solution on sections and incubate at 37°C for 01:15:00

12 Wash 3x 00:05:00 with wash buffer A 5m

13 **Amplification**: This step is light-sensitive! incubation is performed using aluminum foil to protect 2h 30m
samples from light.
Dilute the Duolink Amplification Buffer (red) 1:5 in high-purity water
Add the polymerase (diluted 1:80) just before incubation (keep cold on freezer block!)
Add the amplification solution to the sections and incubate at 37°C for 02:30:00

14 Wash 2x 00:05:00 with wash buffer B 6m
Wash 1x 00:01:00 with wash 0.1x buffer B

15 **Additional immunohistochemical staining (optional):** if immunohistochemical counter-staining is going to be performed it is important to avoid the use of detergents in wash buffers and perform long incubations in cold.

16 Mount the samples on slides and let them air-dry for 5 mins

17 Coverslip samples with Duolink *In Situ* Mounting Medium with DAPI, store samples in the dark at 4°C