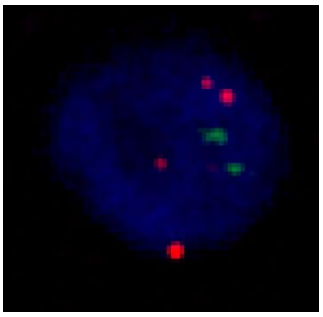
Targeted detection of *SNCA* CNVs in SOX10+ nuclei from oligodendrocytes containing alpha-synuclein inclusions isolated from human post-mortem brain

Cover photo:



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Intended purposes:

This protocol has been optimised for use on single-nuclei isolated from flash-frozen, human post-mortem brain tissue. It can be adapted to different SureFISH Agilent probes and antibodies for detecting nuclear markers.

Abstract

There has been a growing recognition of the complexity of the human genome, and the role somatic variation plays in disease. The brain is particularly vulnerable to genomic mosaicism, likely arising during complex neurodevelopmental and ageing processes. However, current genomic technologies often lack the sensitivity to detect low-level genomic mosaics that could contribute to disease. An alternative cytogenetic method is DNA fluorescence *in situ* hybridisation (FISH), which allows for a targeted analysis of rare, disease-relevant copy number variants (CNVs). FISH can be subsequently combined with immunofluorescence to characterize somatic CNVs in specific cell populations based on specific protein marker expression. This protocol describes a method combining FISH with immunofluorescence, which we name immuno-FISH, for the detection of CNVs in the *SNCA* gene of patients with synucleinopathies, such as Parkinson's disease (PD) and Multiple System Atrophy (MSA). This method is performed on nuclei isolated from frozen, human post-mortem brain tissue, which addresses potential sectioning artefacts and reduces protease digestion for epitope preservation. Our protocol is optimised to detect SOX10, a nuclear oligodendrocyte marker, and alpha-synuclein inclusions, which are frequently retained at the perinucleus in MSA (the so-called Papp-Lantos inclusions). This protocol also describes its use in affected PD and MSA brain regions such as the putamen, substantia nigra (SN) and cerebellum.

Keywords

Alpha-synuclein, a-Syn, *SNCA,* SOX10, FISH, Fluorescence in Situ Hybridisation, IF, Immunofluorescence, nuclei, nuclear suspension, CNV, Copy Number Variation, human, post-mortem, brain, tissue, DNA

Funders

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Disclaimers

This protocol was adapted from the following:

- Garcia-Segura, M.E., Perez-Rodriguez, D. and Proukakis, C. (2022) 'Combined fluorescence in situ hybridization (FISH) and immunofluorescence for the targeted detection of somatic copy number variants in Synucleinopathies', *Neuromethods*, pp. 229–243.
- Ester Kalef-Ezra, Diego Perez-Rodriguez, Christos Proukakis. Manual isolation of nuclei from human brain using CellRaft device and single nucleus Whole Genome Amplification. Protocols.io (https://protocols.io/view/manual-isolation-of-nuclei-from-human-brain-using-cx4mxqu6).

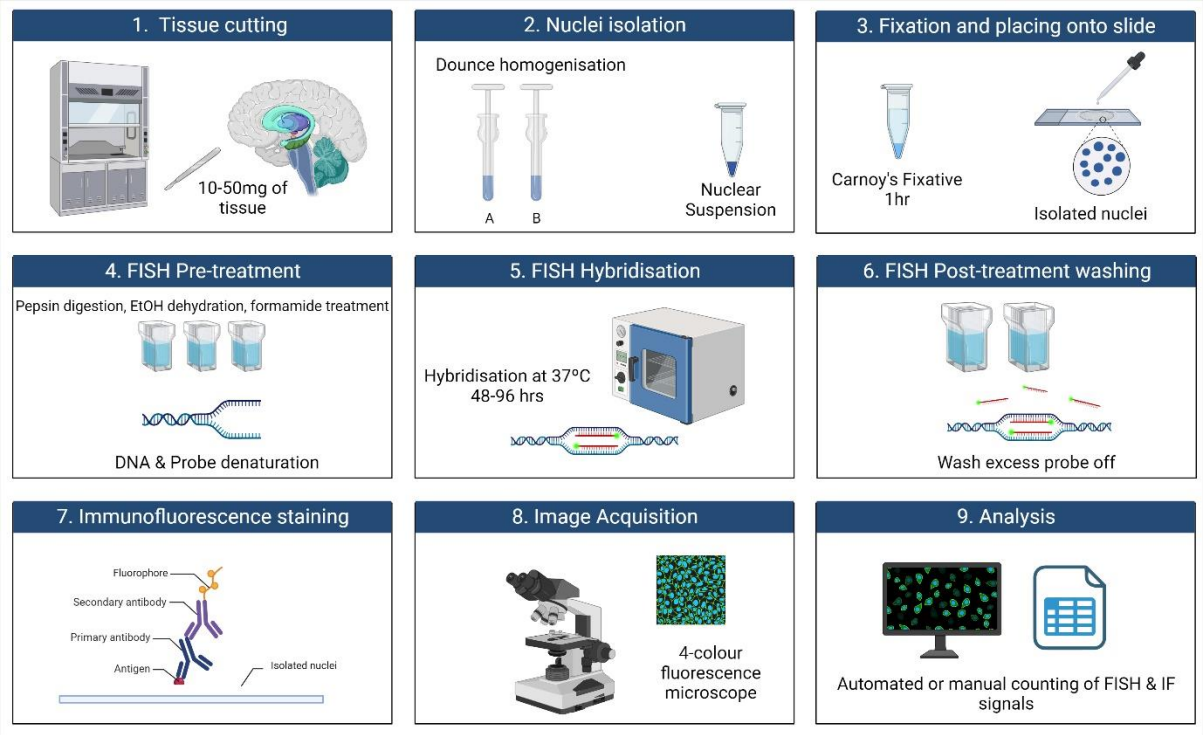

Figure 1. Overview of nuclei isolation and immuno-FISH protocol (created using BioRender)

Safety warnings

All tissue cutting and nuclei isolation steps must be performed in a Class II biosafety cabinet. Toxic chemicals such as formamide must be used in a fume hood. Refer to the SDS of each reagent for details on handling guidelines.

Steps

Section 1. Nuclei isolation from human post-mortem brain tissue using iodixanol gradient

1. Set the centrifuge to 4°C.
2. Prepare ice-cold Carnoy's fixative (3:1 Methanol: Glacial acetic acid) and 1X PBS.
3. Isolate nuclei manually:

   3a. See Table 1 for reagents and steps used for nuclei isolation. Refer to Kalef-Ezra, Perez-Rodriguez and Proukakis (dx.doi.org/10.17504/protocols.io.kxygxzjjov8j/v1) for details of the methods and solutions required for nuclei isolation implemented here.

   3b. Tissue guidelines: Use approximately 10–50 mg of brain tissue per nuclear suspension. Nuclei yield will vary between samples due to tissue collection, disease progression, and sub-regional differences between grey and white matter (cellular density and lipid composition among others).

Note 1 – For the putamen, 20-50 mg of tissue is recommended. For the cerebellum and substantia nigra, 10-30 mg is recommended due to overall higher cellular density and proportion of lipid content within these regions.

Note 2 – The granular layer of the cerebellar cortex cannot be fully disassociated by Dounce homogenisation and may cause clumps within the nuclear suspension.

Section 2 (optional). Nuclear yield check and visualization with DAPI:

1. Resuspend the pellet containing the isolated nuclei in 500µL of DAPI (1µg/mL in 1x PBS working concentration).
2. Leave the tube on a rotator disk for 20 minutes at 4°C.
3. Centrifuge at 800xg at 4°C for 5 minutes and remove the supernatant.
4. Resuspend in 100µL-200µL of 1X PBS.
5. Use a haemocytometer and an epifluorescence microscope to estimate yield and visualise the spread of nuclei. The nuclear suspension should be evenly distributed, appear as single nuclei and free of large debris (see Figure 2 for examples).
6. Centrifuge at 800xg for 5 minutes to pellet nuclei and remove the supernatant.

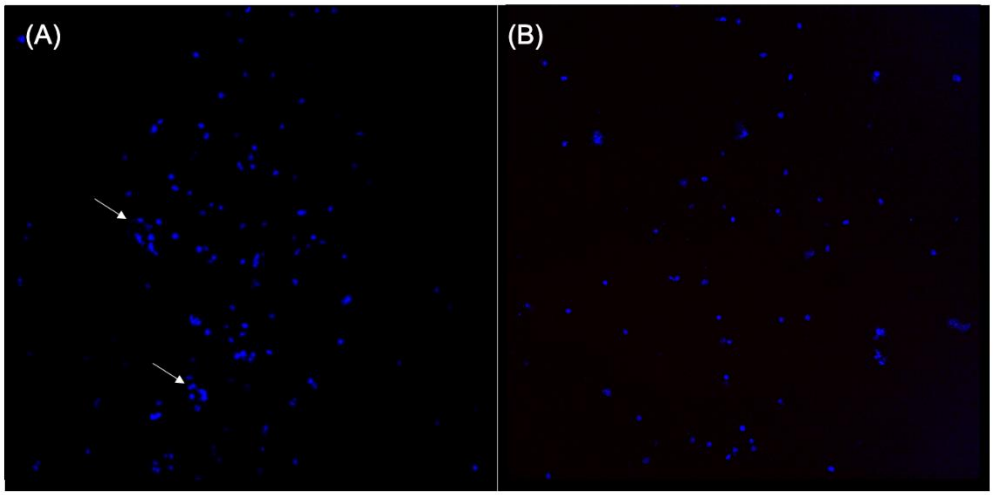

Figure 2. Examples of isolated nuclei stained with DAPI illustrating (A) areas of nuclei clumping and (B) evenly distributed, single nuclei

Section 3. Nuclei fixation and preparation onto slide

1. Resuspend pellet containing the isolated nuclei in 1mL of pre-chilled Carnoy's fixative and leave to fix on a rotator disk for 1hr at 4°C.
2. Centrifuge 800xg for 5 minutes and remove the supernatant.
3. Resuspend pellet in 100µL-200µL of Carnoy's fixative by pipetting up and down.
   Optional: A 70uM Flowmi cell strainer can be used to filter large clumps and debris.
4. Using a dropper or a pipette, place nuclear suspension onto an Epredia™ SuperFrost Plus Gold Adhesion slide and leave to evaporate for 20-60 min RT.

Note 1 The charge of the SuperFrost Plus Gold Adhesion Slides repels PBS, therefore we do not recommend dropping a nuclear suspension containing PBS as it will require hours to evaporate and forms crystallised salts on the slide.

Note 2 We recommend using a Super PAP pen to create a hydrophobic barrier prior to dropping the nuclei to contain the nuclear suspension within a small area on the slide.

5. Wash slides twice, 5 minutes each, at RT in an EasyDip slide staining jar containing 1X PBS.
6. Check under microscope to assess the spread of nuclei before proceeding with immuno-FISH.

Section 4. FISH Pre-treatment

1. Prepare slide staining jars for FISH Pre-treatment according to Table 5 of Materials.
2. Place the water bath in a fume hood and set it to 72°C.
3. Submerge the staining jar containing formamide solution into the water bath.
4. Set the oven to 37°C and place the jar with dH_2O inside (to which pepsin will be added afterwards), allow at least 30 minutes for solutions to reach the desired temperature.
5. Add HCl and pepsin to dH_2O jar (according to Table 5 of Materials), then immediately place the slides in the pepsin solution for 5 minutes in the oven.
6. Transfer the slides to the PBS/$MgCl_2$ solution and leave for 5 minutes at RT.
1. Wash with 1X PBS once for 5 minutes at RT.
2. Dehydrate the isolated nuclei in increasing concentrations of EtOH (70%, 90% and 100%) stored at RT for 2 minutes each.
3. Allow the slides to air-dry for 10 minutes on the bench at RT.

Note In the meantime, take out the FISH probes and hybridisation buffer from –20°C to equilibrate to RT, taking care to avoid exposure to direct light.

4. Incubate the slides in the formamide solution for 3 minutes at 72°C.
5. Dehydrate the nuclei in 70%, 90% and 100% EtOH (pre-chilled at -20°C) for 2 minutes each at RT.
6. Allow the slides to air-dry for 10 minutes on the bench at RT.

Note 1 In the meantime, prepare the FISH probe mixture as outlined in Table 6 of Materials.

Note 2 This protocol can be performed as a 1-colour or 2-colour FISH probe reaction depending on the number of protein markers being investigated. If two protein markers will be used for immunofluorescence, the reference probe can be excluded, and the volume of the reaction mix adjusted with Nuclease-free H_2O.

7. Denature the FISH probe mixture for 5 minutes at 72°C in the water bath.
8. Add 10µL of the probe mixture to the slide, evenly distributing small droplets onto the nuclear suspension.
9. Place a 22mm x 22mm coverslip and seal the edges with rubber cement.
10. The FISH probes can be left to hybridise to DNA in a humidified box kept in the dark at 37°C for 48-96hrs.

Section 5. FISH Post-hybridisation treatment & immunofluorescence staining

Prepare immunofluorescence solutions according to Table 7 of Materials

1. Place the water bath in a fume hood, then set temperature to 72°C
2. Add Wash Buffer 1 at least 30 minutes in the water bath.
3. Take out an aliquot of goat serum from –20°C and leave to thaw at RT.
4. Peel off the rubber cement manually, soak the slides in 2X SSC for 10 minutes and then remove the coverslips from the slides.
5. Wash the slides in FISH Wash Buffer 1 for 2 min at 72°C in the water bath.
6. Wash the slides in FISH Wash Buffer 2 for 1 min at RT.
7. Wash the slides three times, 10 min each, in 1X PBS at RT.
8. Hand-dry sections with tissue to remove PBS excess and create a hydrophobic barrier around the section using a Super PAP pen.

   Note 1 Be careful not to damage the nuclei on the slides.

   Note 2 If the barrier pen was previously used for containing the nuclear suspension, apply more in the same area.

9. Add 300µL of the blocking solution and leave the slides in a humidified chamber for 1hr at RT or overnight at 4°C.
10. Remove the blocking solution excess and apply 150µL of the primary antibody solution.
11. Leave to incubate 2-4 hrs at RT or overnight at 4°C.
12. Wash the primary antibody solution off three times in 1X PBS for 10 minutes at RT.
13. Add 150µL of the secondary antibody solution and leave to incubate for 1hr at RT.
14. Wash the secondary antibody off three times in 1X PBS for 10 minutes at RT.
15. Add 1µg/mL DAPI (working concentration) to the slides for 20 minutes.
16. Wash in 1X PBS for 5 minutes at RT.
17. Add 200µL of TrueBlack solution for 1 minute.

18. Quickly rinse the slides with 70% EtOH and then wash 3 times in 1X PBS for 10 minutes at RT
19. Add 10-20µL of Prolong Gold Anti-Fade solution and mount a 22mm x 22mm coverslip.
20. Leave the slides to dry in the dark overnight at RT before sealing the edges of the coverslip with nail varnish. Store them at 4C until use.

Note 1 In our experience, nuclear suspension autofluorescence can interfere with FISH signal detection, and so we have incorporated a quenching treatment step.

Note 2 For optimal acquisition, suspensions can be imaged within 2 weeks on any 4-colour fluorescence microscope with resolution to detect small FISH signals. We use 16 Z-stacks of 0.5uM to capture focal planes across the nucleus.

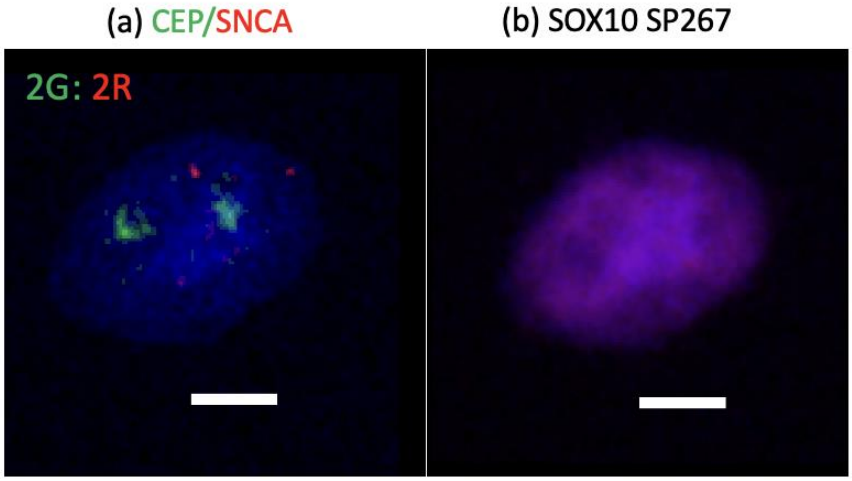

Figure 3. Examples showing (a) Chr 7 CEP and SNCA FISH signals and (b) a SOX10+ nucleus

Materials

Equipment

- Tissue culture hood for human sample handling
- PCR Laminar Flow Cabinet
- Refrigerated centrifuge for 1.5mL tubes capable of reaching 13,000xg
- Oven capable of maintaining 37°C for FISH hybridisation
- Water bath capable of reaching 72°C
- P1000, P200, P20, P2 Pipettes with filtered tips
- Fume hood
- Pair of forceps and scissors
- Haemocytometer
- Dounce tissue grinder set 2mL (Kimble via Sigma Aldrich – D8938)

Table 1. Specifications of reagents used for nuclei isolation method from human post-mortem brain tissue

Item	Supplier	Catalogue Ref.	Preparation prior use
UltraPure DNase/RNase-Free Distilled Water	Thermo Fisher	10977049	Aliquot and keep at RT
PBS (Phosphate Buffered Saline) 10X Solution (pH 7.4)	Thermo Fisher	15815418	Make 1x with dH_2O and store at 4°C
50x cOmplete Protease Inhibitor Cocktail EDTA-free	Roche via Sigma Aldrich	4693159001	Use 1 tablet in 1 ml dH_2O and store at -20°C
Triton-X100	Sigma Aldrich	T9287	Prepare 10% aliquot and store at RT
ODGM (Optiprep Density Gradient Medium)	Sigma Aldrich	D1556	Aliquot and keep at 4°C
Dithiothreitol (DTT)			Prepare 1mM and keep aliquots at -20°C
Sucrose			Prepare 1M and keep at -20°C

Table 2: Specifications of the consumables used for immuno-FISH protocol

Item	Supplier	Catalogue Ref.
EasyDip ™ slide staining system	Simport	M905-12DGY
SuperFrost Ultra Plus™ GOLD Adhesion Slides	Epredia™	11976299
Glass coverslips 22mm x 22mm	VWR	631-0124
Glass coverslips 22mm x 50mm	VWR	631-0137
FixoGum Rubber Cement	Marabu	29010017000
Nail Varnish		
1.5mL Polypropylene DNA LoBind Microcentrifuge Tubes	Eppendorf™	0030108418
0.2mL PCR Tubes	Eppendorf™	951010006

Table 3. Specifications of reagents used for immuno-FISH protocol

Reagent Name	Supplier	Catalogue Ref.	Preparation prior to use
Methanol >99.5% Pure	Thermo Fisher	M/4000/21	No
Glacial Acetic Acid	Thermo Fisher	BP1185	No
Magnesium Chloride Hexahydrate, BioXtra, ≥99.0%	Sigma-Aldrich	M2670	Dissolve 1M in dH_2O and store at RT

Pepsin 1g from porcine gastric mucosa	Sigma	D1000	Prepare 10% solution and store in aliquots at -20°C
1M Hydrochloric acid (HCl)	Thermo Fisher	124210025	No
UltraPure™ Formamide	Thermo Fisher	15515026	No
20X SSC Buffer, Molecular Grade	Promega	V4261	2X solution in dH_2O and stored at RT
UltraPure™ DNase/RNase-Free Distilled Water	Thermo Fisher	10977049	No
Molecular Grade 100% Ethanol (EtOH)	Thermo Fisher	BP2818	Prepare solutions of 70%, 90% and 100% EtOH and store one at RT and one at -20°C
SureFISH hybridisation buffer	Agilent	G9400A	No
SureFISH custom-designed probe 50kb SNCA 4q22.1	Agilent	G110902G-8	No
SureFISH Wash Buffer 1	Agilent	G9401A	No
SureFISH Wash Buffer 2	Agilent	G9402A	No
SureFISH Chr7 CEP probe 767kb P20 GR	Agilent	G110899G-8	No
Goat Serum	Sigma Aldrich	G9023	Store in aliquots at -20°C
Triton™ X-100, BioXtra	Merck	T9284	Prepare 0.2% Solution in 1X PBS stored at RT
PBS Tablets	Life Technologies	18912014	Prepare 1X with dH_2O stored at RT
DAPI (4', 6-diamidino-2-phenylindole, Dihydrochloride)	Sigma-Aldrich	D9542	Prepare 1mg/mL aliquots stored at -20°C
TrueBlack® Lipofuscin Autofluorescence Quencher	Biotium	23007	No
Prolong ™ Gold Anti-Fade Mountant	Thermo Fisher	P36930	No

Table 4. Specifications of antibodies used in this immuno-FISH protocol

Antibody	**Species**	**Supplier**	**Catalogue Ref.**
Primary antibodies			
SOX10 (SP267)	Rabbit	Abcam	Ab227680
a-Syn (Syn 211)	Mouse	Santa-Cruz	sc-12767
Secondary antibodies			
Anti-Rabbit Alexa Fluorophore 647	Goat	Thermo-Fisher	A21245
Anti-Mouse Alexa Fluorophore 488	Goat	Thermo-Fisher	A11001

Table 5. FISH pre-treatment solutions

Solution Name	**Reagents**	**Volume**	**Final Concentration**
Pepsin solution	10% Pepsin aliquot	50µL	0.005%
	dH_2O	100mL	
	1M HCl	1000µL	10mM
PBS/$MgCl_2$ solution	1X PBS	100mL	
	1M $MgCl_2$	100µL	1mM
Formamide solution	99.5% Formamide	70mL	70%
	2M SSC	30mL	0.6M

Table 6. FISH probe mixture per 22 x 22 mm reaction area / slide.

Reagent	**Volume (µL)**	**Final % concentration**
Custom-designed SureFISH probe 50kb SNCA 4q22.1 - Fluorophore 568	1	10
SureFISH Chr7 CEP probe 767kb P20 GR – Fluorophore 488	1	10

SureFISH Hybridisation buffer	7	70
Nuclease-free H_2O	1	10
Total	**10**	

Table 7. Immunofluorescence solutions

Solution Name	**Reagent**	**Volume (µL)**	**Final concentration**
Blocking Solution	Goat serum	30	10%
	0.2% Triton-X in 1X PBS	270	
Primary Antibody solution	Rabbit anti-SOX10	3	0.5µg/mL
	Mouse anti-Syn 211	0.75	1µg/mL
	Goat serum	3	2%
	0.2% Triton-X in 1X PBS	Adjust to 150µL	
Secondary Antibody solution	Goat Anti-Rabbit Fluorophore 648	0.3	2µg/mL
	Goat Anti-Mouse Fluorophore 488	0.3	2µg/mL
	Goat serum	3	2%
	0.2% Triton-X in 1X PBS	Adjust to 150µL	
TrueBlack solution	20X TrueBlack Lipofuscin quencher	10	1X
	70% EtOH	190	