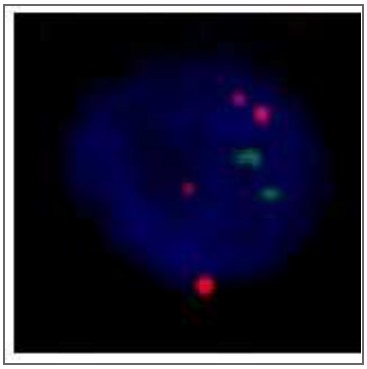

OCT 25, 2023

DOI:
dx.doi.org/10.17504/protocols.io.5jyl8p6r7g2w/v1

Protocol Citation: Caoimhe Morley, Diego-Perez Rodriguez, Monica Emili Garcia-Segura, Christos Proukakis, Ester Kalef-Ezra 2023. Targeted detection of SNCA CNVs in SOX10+ nuclei from oligodendrocytes containing alpha-synuclein inclusions isolated from human post-mortem brain. **protocols.io** https://dx.doi.org/10.17504/protocols.io.5jyl8p6r7g2w/v1

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

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University College London

DISCLAIMER

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).

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

Created: Oct 09, 2023

Last Modified: Oct 25, 2023

PROTOCOL integer ID: 89876

Keywords: ASAPCRN

Funders Acknowledgement:
Multiple System Atrophy Trust
Grant ID: 2022/93020 (572015)
The Michael J. Fox Foundation for Parkinson's Research (MJFF) and the Aligning Science Across Parkinson's (ASAP)
Grant ID: 000430

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.

ATTACHMENTS

862-2224.pdf

GUIDELINES

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.

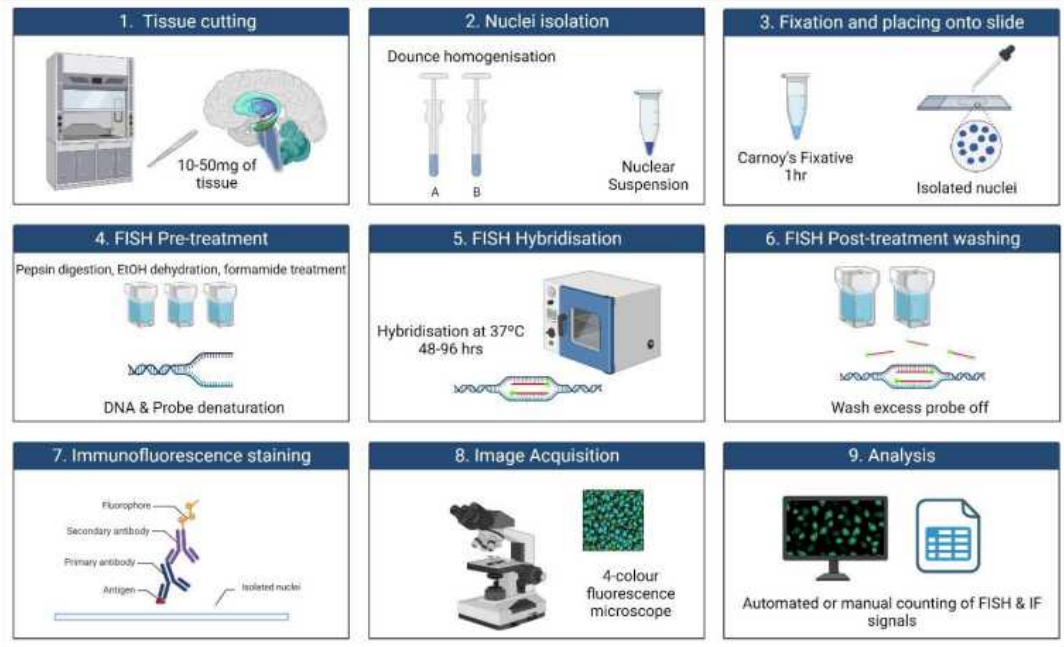

Figure 1. Overview of nuclei isolation and immuno-FISH protocol (created using BioRender).

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.

	A	B	C	D
	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 1 mM and keep aliquots at -20°C
	Sucrose			Prepare 1 M and keep at -20°C

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

	A	B	C
	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.

	A	B	C	D

	A	B	C	D
	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 1 M 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 1 mg/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.

	A	B	C	D
	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.

	A	B	C	D
	Solution Name	**Reagents**	**Volume**	**Final Concentration**
	Pepsin solution	10% Pepsin aliquot	50 µL	0.01%
		dH_2O	100 mL	
		1M HCl	1000 µL	10 mM
	PBS/$MgCl_2$ solution	1X PBS	100 mL	
		1M $MgCl_2$	100 µL	1 mM
	Formamide solution	99.5% Formamide	70 mL	70%
		2M SSC	30 mL	0.6 M

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

	A	B	C
	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.

	A	B	C	D
	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	0.5 µg/mL
		Mouse anti-Syn 211	0.75	1 µg/mL
		Goat serum	3	2%
		0.2% Triton-X in 1X PBS	Adjust to 150 µL	
	Secondary Antibody solution	Goat Anti-Rabbit Fluorophore 648	0.3	2 µg/mL
		Goat Anti-Mouse Fluorophore 488	0.3	2 µg/mL
		Goat serum	3	2%
		0.2% Triton-X in 1X PBS	Adjust to 150 µL	
	TrueBlack solution	20X TrueBlack Lipofuscin quencher	10	1X
		70% EtOH	190	

UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Catalog #10977049**

PBS (Phosphate Buffered Saline) 10X Solution (pH 7.4) **Fisher Scientific Catalog #15815418**

cOmplete mini EDTA free protease inhibitor cocktail **Merck MilliporeSigma (Sigma-Aldrich) Catalog #4693159001**

Triton™ X-100 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #X100-5ML**

OptiPrep™ Density Gradient Medium **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D1556)**

Methanol, Certified AR for Analysis **Thermo Fisher Scientific Catalog #M-4000-21**

Acetic Acid, Glacial (Aldehyde-Free/Sequencing), Fisher BioReagents™™ **Thermo Fisher Scientific Catalog #BP1185-500**

Magnesium chloride hexahydrate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M2670**

Pepsin from porcine gastric mucosa **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P7000**

Hydrochloric acid, 1N standard solution **Thermo Fisher Scientific Catalog #124210025**

UltraPure™ Formamide **Thermo Fisher Catalog #15515026**

SSC Buffer, 20X, 1L **Promega Catalog #V4261**

UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Catalog #10977049**

Ethanol, Absolute, Molecular Biology Grade **Thermo Fisher Scientific Catalog #BP2818500**

FISH Hybridization Buffer **Agilent Technologies Catalog #G9400A**

FISH Wash Buffer 1 **Agilent Technologies Catalog #G9401A**

FISH Wash Buffer 2 **Agilent Technologies Catalog #G9402A**

Goat serum **Merck MilliporeSigma (Sigma-Aldrich) Catalog #G9023**

Triton™ X-100 **Contributed by users Catalog #T9284**

PBS Tablets **Thermo Fisher Catalog #18912014**

4',6-Diamidino-2-phenylindole **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D9542**

TrueBlack® Lipofuscin Autofluorescence Quencher **Biotium Catalog #23007**

ProLong™ Gold Antifade Mountant **Thermo Fisher Catalog #P36930**

Recombinant Anti-SOX10 antibody **Abcam Catalog #ab227680**

Anti-α-synuclein Antibody (211) **Santa Cruz Biotechnology Catalog #sc-12767**

Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 647 **Thermo Fisher Scientific Catalog #A-21245**

Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488 **Thermo Fisher Scientific Catalog #A-11001**

SAFETY WARNINGS

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.

Nuclei isolation from human post-mortem brain tissue using i...

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:

3.1 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.

3.2 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

Notes

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

Nuclear yield check and visualization with DAPI (optional)

30m

4 Resuspend the pellet containing the isolated nuclei in 500 µL of DAPI (1 undetermined in 1x PBS working concentration).

5 Leave the tube on a rotator disk for 00:20:00 at 4 °C. 20m

6 Centrifuge at 800 x g, 4°C, 00:05:00 and remove the supernatant. 5m

7 Resuspend in 100-200 µL of 1X PBS.

8 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).

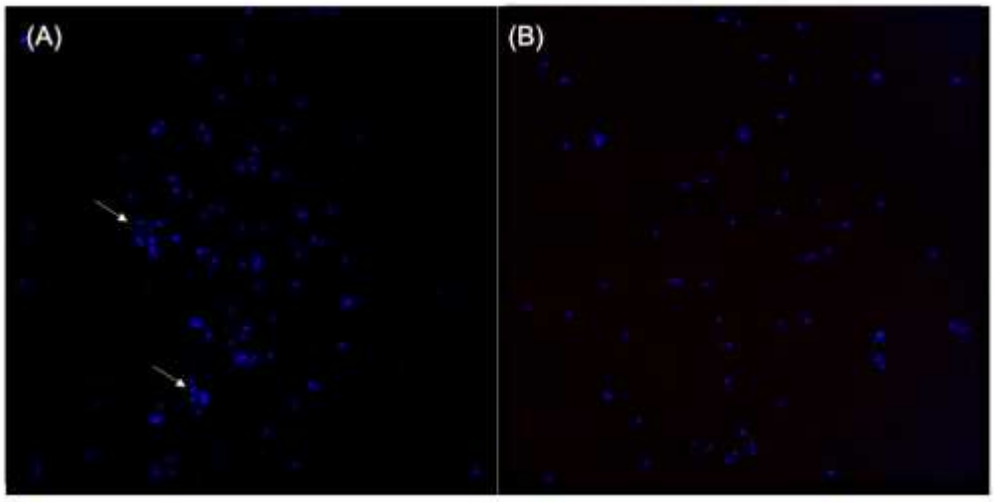

Figure 2. Examples of isolated nuclei stained with DAPI illustrating (A) areas of nuclei clumping and (B) evenly distributed, single nuclei.

9 Centrifuge at 800 x g, 00:05:00 to pellet nuclei and remove the supernatant. 5m

Nuclei fixation and preparation onto slide 1h 15m

10 Resuspend pellet containing the isolated nuclei in 1mL of pre-chilled Carnoy's fixative and leave to fix on a rotator disk for 01:00:00 at 4 °C. 1h

11 Centrifuge 800 x g, 00:05:00 and remove the supernatant. 5m

12 Resuspend pellet in 100-200 µL of Carnoy's fixative by pipetting up and down.

12.1 Optional: A 70 µm Flowmi cell strainer can be used to filter large clumps and debris.

13 Using a dropper or a pipette, place nuclear suspension onto an EprediaTM SuperFrost Plus Gold Adhesion slide and leave to evaporate for 20-60 min Room temperature.

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.
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.

14 Wash slides.

14.1 Wash slides for 00:05:00 at Room temperature in an EasyDip slide staining jar containing 1X PBS. (1/2) 5m

14.2 Wash slides for 00:05:00 at Room temperature in an EasyDip slide staining jar containing 1X PBS. (2/2) 5m

15 Check under microscope to assess the spread of nuclei before proceeding with immuno-FISH.

FISH Pre-treatment 1h 25m

16 Prepare slide staining jars for FISH Pre-treatment according to Table 5 of Materials.

17 Place the water bath in a fume hood and set it to 72 °C.

18 Submerge the staining jar containing formamide solution into the water bath.

19 Set the oven to 37 °C and place the jar with dH_2O inside (to which pepsin will be added afterwards), allow at least 00:30:00 for solutions to reach the desired temperature. 30m

20 Add HCl and pepsin to dH_2O jar (according to Table 5 of Materials), then immediately place the slides in the pepsin solution for 00:05:00 in the oven. 5m

21 Transfer the slides to the PBS/$MgCl_2$ solution and leave for 00:05:00 at Room temperature. 5m

22 Wash with 1X PBS once for 00:05:00 at Room temperature. 5m

23 Dehydrate the isolated nuclei in increasing concentrations of EtOH.

23.1 Dehydrate the isolated nuclei in 70% EtOH stored at Room temperature for 00:02:00. 2m

23.2 Dehydrate the isolated nuclei in 90% EtOH stored at Room temperature for 00:02:00. 2m

23.3 Dehydrate the isolated nuclei in 100% EtOH stored at Room temperature for 00:02:00. 2m

24 Allow the slides to air-dry for 00:10:00 on the bench at Room temperature. 10m

Note

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

25 Incubate the slides in the formamide solution for 00:03:00 at 72 °C. 3m

26 Dehydrate the nuclei in EtOH (pre-chilled at -20 °C).

26.1 Dehydrate the nuclei in 70% EtOH (pre-chilled at -20 °C) for 00:02:00 at Room temperature. 2m

26.2 Dehydrate the nuclei in 90% EtOH (pre-chilled at -20 °C) for 00:02:00 at Room temperature. 2m

26.3 Dehydrate the nuclei in 100% EtOH (pre-chilled at -20 °C) for 00:02:00 at 2m

Room temperature .

27 Allow the slides to air-dry for 00:10:00 on the bench at Room temperature . 10m

Note

Notes

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

28 Denature the FISH probe mixture for 00:05:00 at 72 °C in the water bath. 5m

29 Add 10 µL of the probe mixture to the slide, evenly distributing small droplets onto the nuclear suspension.

30 Place a 22mm x 22mm coverslip and seal the edges with rubber cement.

31 The FISH probes can be left to hybridise to DNA in a humidified box kept in the dark at 37 °C for 48-96 hrs.

FISH Post-hybridisation treatment & immunofluorescence sta... 30m

32

Note

Prepare immunofluorescence solutions according to Table 7 of Materials.

Place the water bath in a fume hood, then set temperature to 72 °C.

33 Add Wash Buffer 1 at least 00:30:00 in the water bath. 30m

34 Take out an aliquot of goat serum from -20 °C and leave to thaw at Room temperature.

35 Peel off the rubber cement manually, soak the slides in 2X SSC for 00:10:00 and then remove the coverslips from the slides. 10m

36 Wash the slides in FISH Wash Buffer 1 for 00:02:00 at 72 °C in the water bath. 2m

37 Wash the slides in FISH Wash Buffer 2 for 00:01:00 at Room temperature. 1m

38 Wash the slides.

38.1 Wash the slides 00:10:00 in 1X PBS at Room temperature. (1/3) 10m

38.2 Wash the slides 00:10:00 in 1X PBS at Room temperature. (2/3) 10m

38.3 Wash the slides 00:10:00 in 1X PBS at Room temperature. (3/3) 10m

39 Hand-dry sections with tissue to remove PBS excess and create a hydrophobic barrier around the section using a Super PAP pen.

Note

Notes

1. Be careful not to damage the nuclei on the slides.
2. If the barrier pen was previously used for containing the nuclear suspension, apply more in the same area.

40 Add 300 µL of the blocking solution and leave the slides in a humidified chamber for 01:00:00 at Room temperature or Overnight at 4 °C. 2h

41 Remove the blocking solution excess and apply 150 µL of the primary antibody solution.

42 Leave to incubate 2-4 hrs at Room temperature or Overnight at 4 °C. 1h

43 Wash the primary antibody solution off.

43.1 Wash the primary antibody solution off in 1X PBS for 00:10:00 at Room temperature. (1/3) 10m

43.2 Wash the primary antibody solution off in 1X PBS for 00:10:00 at Room temperature. (2/3) 10m

43.3 Wash the primary antibody solution off in 1X PBS for 00:10:00 at Room temperature. (3/3) 10m

44 Add 150 µL of the secondary antibody solution and leave to incubate for 01:00:00 at Room temperature. 1h

45 Wash the secondary antibody off.

45.1 Wash the secondary antibody off in 1X PBS for 00:10:00 at Room temperature. (1/3) 10m

45.2 Wash the secondary antibody off in 1X PBS for 00:10:00 at Room temperature. (2/3) 10m

45.3 Wash the secondary antibody off in 1X PBS for 00:10:00 at Room temperature. (3/3) 10m

46 Add 1 undetermined DAPI (working concentration) to the slides for 00:20:00. 20m

47 Wash in 1X PBS for 00:05:00 at Room temperature. 5m

48 Add 200 µL of TrueBlack solution for 00:01:00. 1m

49 Quickly rinse the slides with 70% EtOH and then wash.

49.1 Wash the slides in 1X PBS for 00:10:00 at Room temperature. (1/3) 10m

49.2 Wash the slides in 1X PBS for 00:10:00 at Room temperature. (2/3) 10m

49.3 Wash the slides in 1X PBS for 00:10:00 at Room temperature. (3/3) 10m

50 Add 10-20 µL of Prolong Gold Anti-Fade solution and mount a 22mm x 22mm coverslip.

51 Leave the slides to dry in the dark Overnight at Room temperature before sealing the edges of the coverslip with nail varnish. Store them at 4 °C until use. 10m

Note

Notes

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

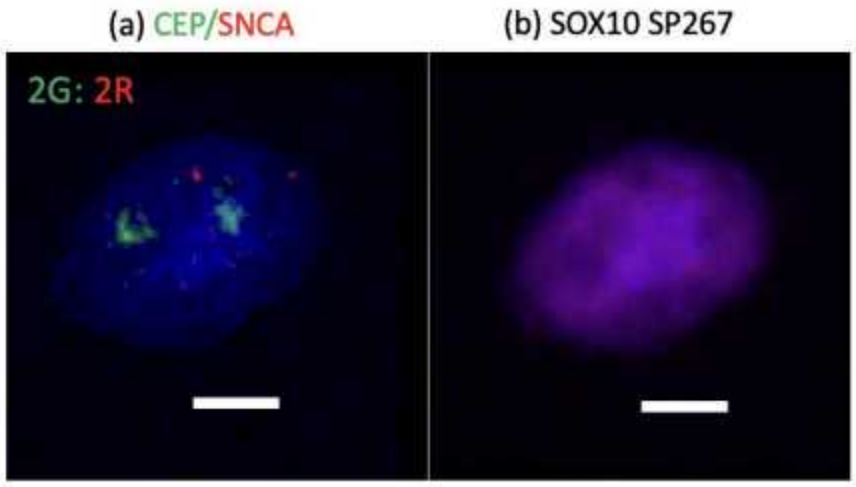

Figure 3. Examples showing (a) Chr 7 CEP and SNCA FISH signals and (b) a SOX10+ nucleus.