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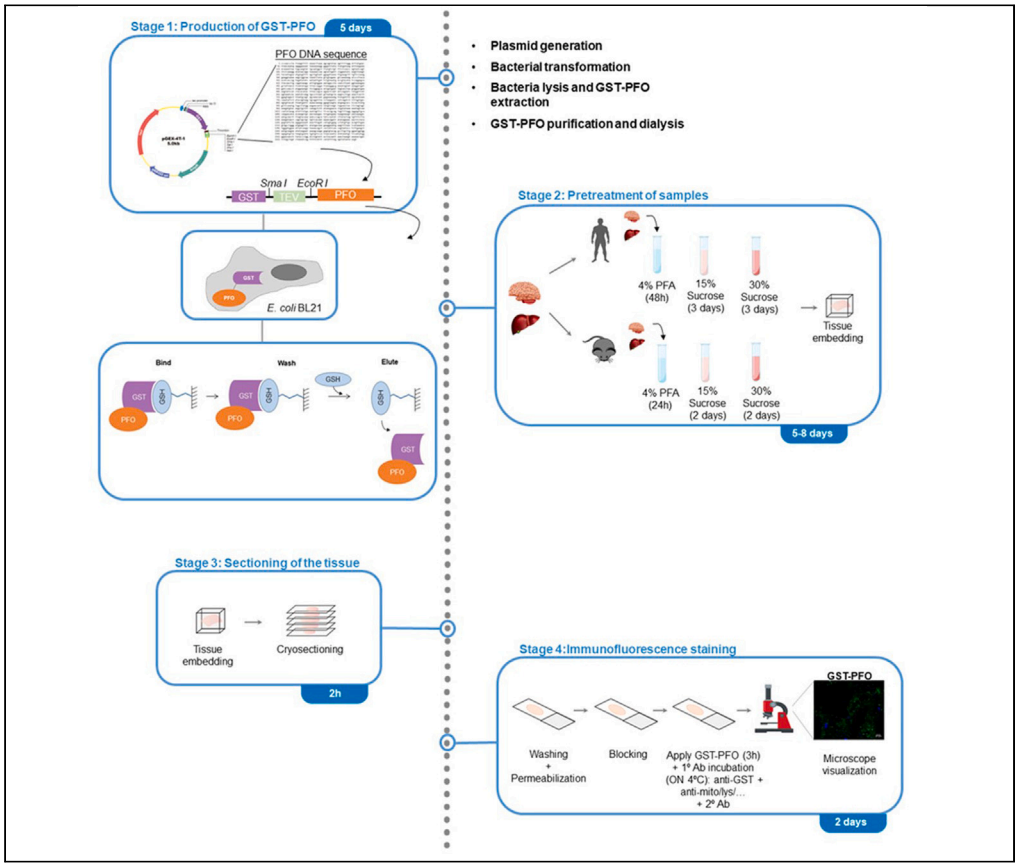
Protocol

GST-Perfringolysin O production for the localization and quantification of membrane cholesterol in human and mouse brain and liver



Abnormal cholesterol metabolism is linked to many neurodegenerative disorders. Here, we present a protocol for the production of a recombinant protein consisting of a Glutathione-S-Transferase tag fused with the Perfringolysin O (PFO). The GST-PFO tag enables analysis of the localization of cholesterol in subcellular membranes of human and mice brain and liver tissues. We have used this approach for samples from Niemann-Pick type C disease and non-alcoholic steatohepatitis models. The construct may also have applications for the diagnosis of cholesterol-accumulating disorders.

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Highlights

Protocol for producing a recombinant protein for tracing the localization of cholesterol

Recombinant protein can be combined with co-immunofluorescence experiments

Optimized approach for membrane cholesterol labeling in brain and liver tissue

Potential applications for the diagnosis of cholesterol-accumulating disorders

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SUMMARY

Abnormal cholesterol metabolism is linked to many neurodegenerative disorders. Here, we present a protocol for the production of a recombinant protein consisting of a Glutathione-S-Transferase tag fused with the Perfringolysin O (PFO). The GST-PFO tag enables analysis of the localization of cholesterol in subcellular membranes of human and mice brain and liver tissues. We have used this approach for samples from Niemann-Pick type C disease and non-alcoholic steatohepatitis models. The construct may also have applications for the diagnosis of cholesterol-accumulating disorders.

For complete details on the use and execution of this protocol, please refer to Kwiatkowska et al. (2014).

BEFORE YOU BEGIN

Neural cholesterol homeostasis has been studied over the last decades as one of the risk factors of diseases with such an impact as Alzheimer's Disease (AD) and a link between brain cholesterol levels and the production of one of the most important AD pathological markers, the amyloid peptide-ß (Aß), has been established (Arenas et al., 2020; Camponova et al., 2017; Fernández et al., 2009; Tabas, 2002). Besides AD, increased cholesterol is also a key driving factor for other diseases, including lysosomal storage disorders, such as Niemann-Pick type C (NPC) disease, in which increased cholesterol in lysosomes and mitochondria contribute to the progression of the disease (Torres et al., 2017; 2019). The accumulation of cholesterol in NPC disease is not limited to the brain but also occurs in visceral organs, including the liver, underlying the liver failure present in some patients with NPC disease (Patterson et al., 2012). Similarly, increased cholesterol in the liver has emerged as a key player in non-alcoholic steatohepatitis (NASH) (Conde de la Rosa et al., 2021; Ribas et al., 2021), an advanced stage of non-alcoholic fatty liver disease (NAFLD). Moreover, NASH can progress to hepatocellular carcinoma (HCC) (Park et al., 2010; El-Serag and Kanwal, 2014; Forner et al., 2018), one

of the most important causes of cancer-related deaths. In order to understand the role of cholesterol in these human diseases (AD, NPC and NASH), it is critical to develop a valuable tool for its detection and quantification.

Studies in brain cholesterol have been developed mainly with filipin, a naturally fluorescent sterol-binding polyene antibiotic that emits fluorescence when bound to sterols (Bittman and Fischkoff, 1972; Maxfield and Wüstner, 2012; Sezgin et al., 2016). However, the use of filipin presents some drawbacks: it requires UV excitation , it is easily scattered and undergoes rapid photobleaching, thus limiting the observation of samples over an extended period of time (Sezgin et al., 2016). In addition, filipin binding perturbs the bilayer structure, hence it cannot be used on living cells, and can also bind to GM1 ganglioside (Arthur et al., 2011). To overcome these limitations, biosensors have been developed by making use of the non-toxic full length of PFO or the domain 4 of the toxin (D4, D4H) employing different strategies, including the addition radioisotopes (^{125}I-PFO*), biotin (BCϴ), or fluorophores (EGFP, mCherry) (Maekawa et al., 2016; Maekawa, 2017).

GST-PFO makes fluorescent imaging easy and reliable, as its behavior depends on the secondary antibody used for tagging. The probe can be detected in a wide range of wavelengths, and the fluorochromes available are fairly stable. One of the greatest advantages of the recombinant protein over other PFO-derived biosensors, is that the GST tag allows the detection of the probe with a wide array of anti-GST antibodies in various detection techniques, such as immunofluorescence, ELISA or flow cytometry. Moreover, while biotinylated derivative of PFO, BCϴ, has a molecular mass of 57 KDa, which together with streptavidin makes a molecule of almost 120 KDa, GST-PFO is 78 KDa length (Iwamoto et al., 1997). This is an important fact regarding the entry of proteins across the plasma membrane, and particularly across the endosomal/lysosomal membranes. Further, GST-PFO can easily be produced in large quantities in bacteria, meaning that once generated, limitation in the quantities of the probe will not be a drawback. In regards to the disadvantages of GST-PFO, it should be mentioned that its production is a homemade and time-consuming process. In addition, the requirement of the incubation with primary and secondary antibodies that will recognize the GST tag requires extra time compared with filipin. For the aforementioned reasons, we strongly believe that GST-PFO is a versatile tool for the detection of subcellular membrane cholesterol compared to the techniques used so far and will help in the diagnosis of cholesterol-accumulating disorders.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse IgG_1 GST (A-6) (Immunofluorescence: 1:200 for human samples; 1:250 for mouse samples. Protein-lipid assay: 1:1000)	Santa Cruz	Cat#374171
Anti-IgG (1:5000)	Sigma-Aldrich	Cat#A9044
Rabbit Anti-ß amyloid (1:200)	Cell Signaling Technology	Cat#8243
Rabbit Anti-cytochrome c (1:200)	Cell Signaling Technology	Cat#4272S
Rat Anti-GFAP (1:200)	Calbiochem	Cat#345860
Rabbit Anti-TOM20 (1:200)	Cell Signaling Technology	Cat#11415
Rabbit Anti-LAMP1 (1:200)	Cell Signaling Technology	Cat#5570
Rat Anti- LAMP2 (1:250)	Abcam	Cat#ab13524
Anti-rabbit Alexa fluor (532) (1:200)	Molecular probes	Cat#A-11009
Anti-rat Alexa fluor (647) (1:200)	Molecular probes	Cat#A-21247
Anti-rat Alexa fluor (488) (1:200)	Molecular probes	Cat#A-11006
Anti-mouse Alexa fluor (647) (1:200)	Molecular probes	Cat#A32728

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Anti-rabbit Alexa fluor (647) (1:200)	Molecular probes	Cat#A32733
Bacterial and virus strains		
Escherichia coli BL21 (DE3)	New England BioLabs	N/A
Biological samples		
Healthy adult human brain tissue. Mean age (years) ± SEM: 69.7 ± 4.79. Males/females: 4/2.	Biobank of Hospital Clinic/ IDIBAPS of Barcelona	HCB/2015/0595
AD adult human brain tissue. Mean age (years) ± SEM: 80.7 ± 3. Males/females: 2/3.	Biobank of Hospital Clinic/ IDIBAPS of Barcelona	HCB/2015/0595
DS adult human brain tissue. Mean age (years) ± SEM: 56 ± 4.31. Males/females: 3/2.	Biobank of Hospital Clinic/ IDIBAPS of Barcelona	HCB/2015/0595
Healthy adult human liver samples. Mean age (years) ± SEM: 58.9 ± 17.7. Males/females: 9/2.	Biobank of Hospital Clinic/ IDIBAPS of Barcelona	HCB/2012/8011
NASH adult human liver samples. Mean age (years) ± SEM: 57.3 ± 6.4. Males/females: 7/5.	Biobank of Hospital Clinic/ IDIBAPS of Barcelona	HCB/2012/8011
Chemicals, peptides, and recombinant proteins		
Reduced glutathione (GSH)	Sigma-Aldrich	Cat#G6529
Lysogeny Broth medium	Sigma-Aldrich	Cat#L3022
Glycerol	Sigma-Aldrich	Cat# G5516
Ampicillin	Merck Millipore	Cat#10835242001
High cholesterol diet (2% cholesterol)	Research Diets	Cat#C15050R
Isopropyl β-d-1-thiogalactopyranoside	Sigma-Aldrich	Cat#I-6758
cOmplete™ EDTA-free Protease Inhibitor Cocktail	Roche	Cat#04693132001
PMSF	Sigma-Aldrich	Cat#P7626
DTT	Sigma-Aldrich	Cat#D0632
EDTA	Sigma-Aldrich	Cat#E7889
Tris-HCl (1M)	In-house generated	N/A
Lysozyme (100 mg/mL)	Sigma-Aldrich	Cat#L7651
DNase- Pierce™ Universal Nuclease for Cell Lysis	Thermo Scientific™	Cat#88700
Sucrose	Sigma-Aldrich	Cat#S9378
Sodium azide*	Merck Millipore	Cat#106687
M-1 Embedding Matrix	Epredia™	Cat#1310
Gelatin from cold-water fish skin	Sigma-Aldrich	Cat#G7041
Triton X-100	Sigma-Aldrich	Cat#T9284
Goat Serum	Sigma-Aldrich	Cat#500621Z
Phosphate buffered saline (PBS)	Sigma-Aldrich	Cat#P3813-10PAK
Sudan Black	Sigma-Aldrich	Cat#199664
ProLong® Diamond Antifade Mountant	Life Technologies	Cat#P36962
Formaldehyde solution 16%*	Thermo Scientific™	Cat# 28908
Fixation solution	In-house generated	N/A
Cryoprotectant solution	In-house generated	N/A
Immunofluorescence solution	In-house generated	N/A
Glutathione Sepharose 4B	Merck Millipore	Cat#GE17-0756-01
Critical commercial assays		
NucleoSpin® Plasmid	Macherey Nagel	Cat#740588.50
SuperSignal™ West Pico PLUS Chemiluminescent Substrate	Thermo Scientific™	Cat#34580
Sepharose™ GST bulk kit	Thermo Scientific™	Cat#27-4570-01

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
PFO DNA sequence	NCBI	NCBI: M36704
Experimental models: Organisms/strains		
Mouse: C57bl/6J (males and females, 48 weeks)	Charles River	Cat#63
Mouse: Stard1^{ff} and Stard1$^{\Delta Hep}$ (males and females, 30 weeks)	Conde de la Rosa et al. (2021)	N/A
Mouse: BALB/cNctr-Npc1m1N/J (males and females, 6 weeks)	The Jackson Laboratory	Cat#003092
Mouse: B6;C3-Tg(APPswe,PSEN1dE9)85Dbo/Mmjax (males and females, 48 weeks)	The Jackson Laboratory	Cat#34829-JAX
Software and algorithms		
ImageJ	Schneider, 2012	https://imagej.nih.gov/ij/
Graphpad Prism 5.0 Software	Graphpad Software, Inc	https://www.graphpad.com/scientificsoftware/p
Nbits colocalization plugin for Image	Confocal Microscopy Unit, Facultad de Medicina, Universidad de Barcelona	N/A
Oligonucleotides		
TEV-PFO construct	GenScript	N/A
Recombinant DNA		
pGEX4T	GenScript	N/A
Other		
Cryostat	Leica Biosystems	Cat#CM1950
Orbital Shaker	ELMI	Cat#DOS – 20L
Spectrophotometer	Beckman Coulter	Cat#DU®800
Confocal microscope	Leica Microsystems	Cat#DMI6000
Rotatory shaker	Lawson®Scientific	Cat#TM-80
Centrifuge	Sigma-Aldrich	Cat#3-15-3K15
Nanodrop 1000	Thermo Scientific™	Cat#Nanodrop 1000
Amicon Ultra 15 mL 50 K centrifugal filters	Merck Millipore	Cat#UFC910008
Disposable columns, prepacked, Sepharose™ GST bulk kit	Thermo Scientific™	Cat#27-4570-01
Incubator	Thermo Scientific™	Cat#MaxQ 4000

*Toxic

MATERIALS AND EQUIPMENT

Alternatives: Other cloning vectors different to pGEX4T could be used if desired.

Alternatives: Different image analysis software can be employed instead of ImageJ for cholesterol staining quantification.

Alternatives: Dialysis cassettes of the same pore size could be used as an alternative to Amicon Ultra 15 mL 50 K centrifugal filters. However, Amicon centrifugal filters provide fast ultrafiltration with capability for high concentration recoveries with short spin times (as fast as 10–15 min) compared to dialysis cassettes, which need longer incubation times (normally overnight dialysis is required).

Note: For human brain samples, twenty-one cases from the Biobank of Hospital Clinic/IDIBAPS of Barcelona were collected following approval of the Clinical Research Ethics Committee of the Hospital Clinic of Barcelona (HCB/2015/0595). Human liver samples were obtained from donors and recipients undergoing liver transplantation at the Liver Transplantation Unit of the Hospital Clinic, Barcelona, following the HCB/2012/8011 protocol approved by the

HCB/UB Ethics Committee of the Hospital Clinic of Barcelona, Spain. All procedures involving animals and their care were approved by the Ethics Committee of the University of Barcelona following national and European guidelines for the maintenance and husbandry of research animals.

Reagent setup

Fixation solution: to make 40 mL, add 10 mL of 16% (w/v) formaldehyde solution (PFA) to 30 mL of 1× PBS to obtain 4% (w/v) PFA. Prepare before use. Can be stored overnight (12–24 h) at 4°C or frozen at −20°C up to 12 months.

Alternatives: a ready for use 4% formaldehyde solution could be also purchased if desired. We recommend the Image-iT™ Fixative Solution (4% formaldehyde, methanol-free) (ThermoFisher: FB002).

Cryoprotectant solution: 15% (w/v) sucrose or 30% (w/v) sucrose in 0.02% (w/v) of sodium azide diluted in phosphate buffered saline (PBS). Prepare before use. Can be stored at 4°C for 1 month.

⚠ CRITICAL: Sodium azide is toxic. Use it in the gas chamber. Wear gloves.

Immunofluorescence solution: 1% of goat serum and 0.2% of Triton X-100 diluted in PBS. Prepare before use. Can be stored at 4°C for 6 months.

Additional solutions

Lysis solution		
Reagent	Final concentration	Amount
Triton X-100	1.25×	250 µL
PBS	1×	19.75 mL
DNase	3 U/mL	2.4 µL (60 U)
Protease Inhibitor	1×	2 tablets
PMSF (0.2 M)	1 mM	100 µL
DTT (1 M)	1 mM	20 µL
EDTA (0.5 M)	1 mM	40 µL
Lysozyme (100 mg/mL)	1 mg/mL	200 µL
Total		**20 mL**
Store at 4°C for a short term (1 month).		

Binding buffer, pH 7.5		
Reagent	Final concentration	Amount
PBS	0.01 M	19.78 mL
DTT (1M)	1 mM	20 µL
EDTA (0.5M)	1 mM	40 µL
Total		**20 mL**
Store at 4°C for a short term (1 month).		

Elution buffer, pH 8.4		
Reagent	Final concentration	Amount
Tris-HCl (1 M)*	50 mM	100 µL
Reduced Glutathione - PBS	20 mM	0.012 g
	40 mM	0.024 g

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Continued		
Reagent	Final concentration	Amount
DTT (1 M)	1 mM	20 μL
Final volume (V_f)		**2 mL**

Store at 4°C for a short term (1 month).
*To prepare 1M Tris-HCl: dissolve 121.1 g of Tris base in 800 mL of deionized water. Adjust the pH to 8.4 value by slowly adding concentrated HCl. Bring the final volume to 1 L with deionized water.

Note: Prepare two separate tubes, of 2 mL each, for each concentration of reduced glutathione.

Dialysis buffer, pH 8.4		
Reagent	Final concentration	Amount
PBS	0.01 M	47.25 mL
Tris-HCl (1 M)*	50 mM	2.5 mL
DTT (1 M)	5 mM	250 μL
Total		**50 mL**

Store at 4°C for a short term (1 month). Never use it at room temperature.
*To prepare 1M Tris-HCl: dissolve 121.1 g of Tris base in 800 mL of deionized water. Adjust the pH to 8.4 value by slowly adding concentrated HCl. Bring the final volume to 1 L with deionized water.

STEP-BY-STEP METHOD DETAILS

This section lists the major steps and provides step-by-step details and timing for the generation of the GST-PFO probe. The protocol is divided into 4 stages: (**Stage 1**) Plasmid generation and production of recombinant fusion protein GST-PFO, (**Stage 2**) Pretreatment of frozen brain and liver samples for immunological staining, (**Stage 3**) Sectioning of the tissue and (**Stage 4**) Immunofluorescence colocalization with GST-PFO. Additionally, (**Stage 1**) is divided into 8 consecutive steps (**1–8**). The detailed protocol for the correct realization of the procedure is described below.

Stage 1: Plasmid generation and production of recombinant fusion protein GST-PFO

Timing: 2–3 weeks, depending on the time required for plasmid generation step

This major step describes how the plasmid expressing GST-PFO protein was designed (based on the protocol described in Kwiatkowska et al. (2014)) and the protocol followed for the production of the fusion protein. Upon completion of the following steps, a high-purity GST-PFO recombinant protein should be obtained.

Plasmid generation

Timing: 1–2 weeks depending on the shipping/delivering time

1. Obtain the DNA sequence of the PFO gene available at the NCBI database (NCBI: M36704).
2. Leader sequence coding for 28 N-terminal amino acids should be deleted to enable the intracellular localization of the protein (Figure 1). In addition, modify the construct by introducing the sequence 5′ GAA AAC CTG TAT TTT CAG GGC 3′ encoding the ENLYFQG motif recognized by Tobacco Etch Virus (TEV) protease (Kapust et al., 2001) to allow potential removal of the GST tag if needed in further experiments.

```
        10         20         30         40         50
MIRFKKTKLI ASIAMALCLF SQPVISFSKD ITDKNQSIDS GISSLSYNRN
        60         70         80         90        100
EVLASNGDKI ESFVPKEGKK TGNKFIVVER QKRSLTTSPV DISIIDSVND
       110        120        130        140        150
RTYPGALQLA DKAFVENRPT ILMVKRKPIN INIDLPGLKG ENSIKVDDPT
       160        170        180        190        200
YGKVSGAIDE LVSKWNEKYS STHTLPARTQ YSESMVYSKS QISSALNVNA
       210        220        230        240        250
KVLENSLGVD FNAVANNEKK VMILAYKQIF YTVSADLPKN PSDLFDDSVT
       260        270        280        290        300
FNDLKQKGVS NEAPPLMVSN VAYGRTIYVK LETTSSSKDV QAAFKALIKN
       310        320        330        340        350
TDIKNSQQYK DIYENSSFTA VVLGGDAQEH NKVVTKDFDE IRKVIKDNAT
       360        370        380        390        400
FSTKNPAYPI SYTSVFLKDN SVAAVHNKTD YIETTSTEYS KGKINLDHSG
       410        420        430        440        450
AYVAQFEVAW DEVSYDKEGN EVLTHKTWDG NYQDKTAHYS TVIPLEANAR
       460        470        480        490        500
NIRIKARECT GLAWEWWRDV ISEYDVPLTN NINVSIWGTT LYPGSSITYN
```

Figure 1. Amino acid sequence corresponding to Perfringolysin O protein obtained in UniProtKB database (UniprotKB: P0C2E9)

Residues highlighted in yellow correspond to the signaling peptide, which is 28 residues long.

3. To obtain the recombinant PFO fused with a GST tag at the N-terminus, insert the construct into the pGEX-4T-1 vector (GenScript) using SmaI (5′ CCCGGG 3′) and EcoRI (5′ GAATTC 3′) restriction sites, after adding SmaI and EcoRI recognition sites to the construct.

   Note: In our case, the TEV-PFO construct (Figure 2) and pGEX-4T-1 vector expressing TEV-PFO were purchased from GenScript®.

   Alternatives: Alternatively, cloning experiments can be carried out in-house if preferred.

4. Transform *Escherichia coli* (*E. coli*) BL21 (DE3) with the GST-PFO expressing pGEX 4T-1 vector. Cryopreserve the transformed bacteria in Lysogeny Broth (LB) medium and 15% (v/v) glycerol and store at −80 °C.

Production of recombinant fusion protein GST-PFO

Timing: 5 days total

Timing: 2 days for protein production: sterile procedure

Timing: 4 h for bacteria lysis and GST-PFO purification: non-sterile process

Timing: 6 h for GST-PFO purification: non-sterile process

Timing: 1 day, 7 h for GST-PFO dialysis: non-sterile process

5. Protein production: sterile procedure;
   a. Mix the following reagents to pre-grow the transformed bacteria in a 100 mL flask:

Component	Final concentration	Amount (mL)
LB medium	1×	20
Ampicillin (100 mg/mL)	100 μg/mL	0.02
E. coli BL21 (DE3) pGEX 4T-1 GST-PFO (glycerol stock)	N/A	0.02

   b. Incubate the flask at 220 rpm and 37°C overnight (12–24 h).

Gene name: TEV-PFO
Length: 1581 bp
Additional 5' sequence: GAATTC (EcoRI restriction site)
Additional 3' sequence: CCCGGG (SmaI restriction site)
TEV protease recognition site: 5′ GAA AAC CTG TAT TTT CAG GGC 3′

> Sequence:

GAATTCGAAAACCTGTATTTTCAGGGCATGAAGGACATTACTGATAAAAACCAGTCCATTGATTCAGGAATCTCTAGTT
TATCCTATAATAGAAACGAGGTCTTGGCGTCAAACGGCGACAAGATAGAAAGCTTCGTTCCTAAAGAGGGAAAGAAA
GCGGGTAATAAATTTATAGTGGTTGAACGCCAAAAAAGAAGCCTGACAACTAGTCCCGTTGACATATCAATCATTGACT
CCGTTAATGATCGTACGTATCCAGGGGCCCTGCAATTAGCGGATAAAGCTTTTGTAGAGAATCGGCCCACGATCTTGAT
GGTCAAAGAGGCTATAAATATCAATATTGATCTTCCGGGGTTAAAAGGCGAGAATTCGATTAAAGTTGACGACCCCAC
TTACGGCAAAGTGTCTGGTGCAATAGACGAATTGGTCTCTAAGTGGAATGAGAAATATTCTTCGACACATACCTTGCCC
GCCCGGACTCAATACAGCGAGTCAATGGTTTACTCGAAGTCCCAGATATCCTCCGCGCTGAACGTGAACGCGAAAGTG
CTTGAGAACTCTTTAGGTGTGGATTTCAATGCCGTTGCGAACAACGAGAAAAAGGTCATGATCTTAGCCTATAAGCAGA
TCTTCTATACGGTTTCGGCAGATTTACCGAAGAATCCCTCCGACCTTTTTGACGACAGTGTTACGTTTAATGATCTGAAA
CAGAAAGGTGTGAGTAACGAAGCCCCACCGCTTATGGTGTCGAATGTGGCGTACGGGAGAACGATCTACGTGAAGTT
AGAAACCACGTCTTCGTCCAAAGACGTACAGGCAGCTTTTAAGGCTTTAATAAAAAATACCGATATAAAGAATTCCCAG
CAGTATAAAGATATTTACGAGAATTCATCGTTCACAGCCGTAGTTCTTGGTGGGGATGCTCAAGAACATAATAAGGTCG
TTACAAAAGATTTTGATGAAATACGCAAGGTGATAAAAGATAATGCAACATTCTCGACAAAAAACCCGGCCTACCCGAT
TTCGTACACCTCTGTATTCCTTAAGGATAACAGCGTCGCAGCTGTCCACAATAAAACAGACTACATAGAAACGACCAGT
ACCGAATACTCAAAGGGAAAGATTAACCTGGATCACAGTGGCGCGTACGTAGCCCAGTTTGAGGTCGCGTGGGACGA
AGTGAGTTACGATAAAGAAGGTAATGAAGTTCTGACGCACAAGACTTGGGATGGCAATTACCAAGATAAGACCGCTCA
CTACTCGACGGTGATACCACTTGAGGCAAATGCCCGCAACATACGGATTAAGGCCCGCGAATGCACTGGCCTTGCCTG
GGAGTGGTGGCGCGACGTCATTAGTGAATATGACGTTCCGCTTACCAATAATATCAACGTATCCATCTGGGGAACCACT
CTTTACCCTGGCAGTTCTATTACGTATAACTAACCAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTT
TCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCTACTAGAGTCACACTGGCTCACCTTCGGGTGGGCCTTTCTGCGTTTA
TACCCGGG

Figure 2. Nucleotide sequence of TEV-PFO gene construct (1581 bp)
EcoRI (5′ GAATTC 3′) (red) and SmaI (5′ CCCGGG 3′) (blue) recognition sites are included, which will allow the insertion of the construct in the plasmid. Green: Tobacco Etch Virus (TEV) protease recognition site.

c. Mix the following reagents in a 1 L flask to grow the culture of transformed bacteria:

Component	Final concentration	Amount (mL)
LB medium	1×	200
Ampicillin (100 mg/mL)	100 µg/mL	0.2
Pre-grown *E. coli* BL21 (DE3) pGEX 4T-1 GST-PFO	10×	~ 20

⚠ CRITICAL: Before adding the pre-grown bacteria, take 1mL of the LB medium and ampicillin mix sample in a spectrophotometer bucket and mark it as "blank".

d. Take a sample of the flask (once pre-grown bacteria have been added) in a spectrophotometer bucket and mark it as "St_0".
e. Incubate the flask at 220 rpm and 37°C.
f. Set up the spectrophotometer at 600 nm.
g. Adjust the spectrophotometer to rest the blank and read the "St_0" sample. Values are usually around 0.2 OD. Troubleshooting 1
h. Take aliquots (approximately every 30 min) and measure their absorbance until the values reach 0.6 OD.
i. Add 0.5 mM of IPTG to induce GST-PFO production.
j. Incubate the flask 20 h at 220 rpm and 18°C. Troubleshooting 2
k. Centrifuge the culture in four 50 mL falcons at 1600 *g* over 15 min at 4°C.
l. Discard the supernatant and keep the pellets at −80°C.
m. To continue with the lysis immediately freeze pellets at −80°C for a minimum of 30 min to help mechanical lysis.

⏸ **Pause point:** pellets can be stored over 6 months at −80°C.

6. Bacteria lysis and GST-PFO purification: non-sterile process;

Note: Use all reagents previously cooled and perform all the processes on ice to avoid protein degradation (except when the requirement of room temperature is specifically stated).

a. Add 5 mL of "**Lysis Solution**" to each frozen pellet and mix it with a pasteur pipette without adding oxygen to the sample. Keep mixing until solid traces of the pellet disappear from the liquid part. Perform this step on ice.

⚠ CRITICAL: Degradation can occur if lysis is not performed on ice.

Do not add oxygen to the sample to avoid oxidation processes.

b. Mix the four pellets in a 50 mL falcon and incubate them over 30 min at room temperature (20°C–22°C) in a rotatory shaker at 30 rpm.
c. Add the following reagents to the lysate.

Component	Final concentration	Amount (μL)
DTT (1M)	10 mM	180
EDTA (0.5 M)	10 mM	360

Note: The reagents added in this step are not part of the "Lysis solution", even if they are also included in its composition.

d. Centrifuge the lysate at 9000 *g* for 45 min at 4°C.
e. Collect the supernatant, label it as "**Total Protein Solution**" and proceed with purification.

7. GST-PFO purification: non-sterile process;

Note: Use all reagents previously cooled and filtered with a 0.2 μm filter.
GST-PFO purification was carried out by column affinity chromatography using prepacked Sepharose™ GST kit disposable columns and Glutathione Sepharose™ 4B medium (or resin), which shows high affinity to GST-tagged proteins, thus allowing rapid purification of the proteins applying gravity flow. For every 200 mL of initial induced culture, use 200 μL of the resin.

a. Equilibrate the column by adding 1 mL of "**Binding buffer**" and following the manufacturer's instructions (https://es.vwr.com/store/product/10674189/disposable-columns-prepacked-sepharosetm-gst-bulk-kit). This will provide the optimal conditions to ensure that the target molecule, GST-PFO, interacts effectively with the ligand and is retained by the affinity medium as all other molecules wash through the column. Centrifuge at 500 *g* at 4°C for 5 min. Discard supernatant by careful pipetting. Repeat the process other two times to allow complete column equilibration.
b. Pipette 200 μL of the resin in an eppendorf. Add the "**Total Protein Solution**" sample and incubate it for 3 h at 4°C in a rotatory shaker at 30 rpm.
c. Mount the elution column following the manufacturer's instructions and prepare your work area to work with the gravity flow technique.
d. Add the "**Total Protein Solution**" sample to the column and collect the flow-through. Keep the flow through in an eppendorf or falcon on ice during the process. Label them as "**Non-Bound**".
e. Wash the column with 3 mL of "**Binding buffer**" and collect the flow through. Collect and label as "**Wash**" and its respective numeration. Repeat the process 2 more times.
f. Elute with 500 μL of "**Elution buffer**" and incubate it for 10 min before letting it flow. Repeat 3 more times. Label as "**Elution**" and its corresponding number. Troubleshooting 3

Note: It is important to take into account that the GST-PFO yield depends on the recuperation of the protein from this step. The reduced glutathione concentrations described in this protocol were optimized to obtain the largest concentration of the recombinant protein without compromising its purity. You may decide which situation suits your assay better. After the

last wash in the previous step, it is always recommended to do a first elution with a low GSH concentration as 20 mM to displace nonspecific proteins without losing the protein of interest. If your production is mostly uncontaminated you can start eluting with larger concentrations of GSH to recuperate more concentrated GST-PFO. In this assay, three elutions with 20 mM and two with 40 mM were done.

g. Take aliquots of each sample for SDS-PAGE gels, needed volume: 15 μL.
h. Store all samples at −20°C until use.

Pause point: samples can be stored over weeks. Nevertheless, it is strongly recommended to do dialysis before long storage times to avoid protein loss by degradation or crystallization.

8. GST-PFO dialysis: non-sterile process;

Note: Use all reagents previously cooled and filtered with a 0.2 μm filter. The process described here is for 200 mL of initial induced culture.

a. Run an SDS-PAGE to analyze the collected samples from the purification step and decide which elution fractions dialyze together.
b. Select the eluted fractions with the purest and highest amounts of GST-PFO, pool them together in the same Amicon Ultra 15 mL centrifugal filters and dialyze them slowly with a "**Dialysis Buffer**".
c. Centrifuge the Amicon Ultra 15 mL centrifugal filters at 5000 *g* over 10 min. Troubleshooting 4
d. Calculate the concentration of glutathione per Amicon Ultra 15 mL centrifugal filter once eluted in the "**Dialysis Buffer**". GSH concentration was determined by the "recycling method" based on the protocol previously described in Tietze (1969).

Alternatives: Alternatively, GSH levels can be determined by high-performance liquid chromatography. The protocol we routinely follow in the lab is based on the one published by Fariss and Reed (1987).

e. Repeat elutions until the concentration of glutathione in protein samples is 0.01 mM or less.

⚠ CRITICAL: Glutathione can interfere in other assays if it is not washed off from samples.

f. Aliquot the concentrated protein with low glutathione concentration and store 10 μL apart for Nanodrop quantification. Add 20% (v/v) sucrose to the protein sample to prevent crystal formation.
g. Quantify protein concentration with 1 μL of the stored solution. Correct the concentration given by the Nanodrop with the following formula:

GST-PFO protein concentration (mg/mL) ÷ GST-PFO Extinction coefficient (*E*) = Corrected GST-PFO protein concentration

*Extinction coefficient (E) for GST-PFO = 1.384

h. Adjust the concentration obtained by the formula to the volume of sucrose added per sample.
i. Aliquot the protein in adequate volumes for your assays. Label each sample with its corresponding concentration. Store samples at −80°C.

⚠ CRITICAL: Thaw samples only once.

Stage 2: Pretreatment of frozen brain and liver samples for immunological staining

Timing: 5–8 days total

Timing: 8 days for human samples

Timing: 5 days for mouse samples

This step helps to preserve the tissue in optimal conditions and maintains cellular and subcellular structures intact for histological techniques. This protocol is a guide to the process.

9. Pretreatment of frozen brain and liver samples for immunological staining;
   The pretreatment described here is for tissues that have been frozen at −80°C. The first part of the process consists in the perfusion of the sample. Afterwards, cryopreservation is done to keep the internal structures intact. Do all incubations at 4°C. Take into account that the reagents are the same for the processing of human and mouse samples, only incubation times will differ.
   a. Human samples,
      i. Immerse the tissue sample in "**Fixation Solution**" for 48 h.

   ⚠ CRITICAL: PFA is toxic. Wash the sample with "Cryoprotectant solution" to remove residual PFA traces that may remain.

   Sodium azide is toxic.
      ii. Transfer the samples to "**Cryoprotectant solution**" (15% (w/v) sucrose) until the samples sink, approximately 3 days.
      iii. Transfer the samples to "**Cryoprotectant solution**" (30% (w/v) sucrose) until the samples sink, approximately 3 days.
      iv. Transfer the sample to a histology cassette and swamp it with a M-1 Embedding Matrix. Freeze it in dry ice. Wrap the blocks in aluminum foil and label them.
      v. Keep the histology cassette at −80°C until sectioning.
   b. Mouse samples,
      i. Immerse the tissue sample in "**Fixation Solution**" for 24 h.

   ⚠ CRITICAL: PFA is toxic. Wash the sample with "Cryoprotectant solution" to remove residual PFA traces that may remain.

   Sodium azide is toxic.
      ii. Transfer the samples to "**Cryoprotectant solution**" (15% (w/v) sucrose) until the samples sink, approximately 2 days.
      iii. Transfer the samples to "**Cryoprotectant solution**" (30% (w/v) sucrose) until the samples sink, approximately 2 days.
      iv. Transfer the sample to a histology cassette and swamp it with a M-1 Embedding Matrix. Freeze it in dry ice. Wrap the blocks in aluminum foil and label them.
      v. Keep at −80°C freezer until sectioning.

   Pause point: tissues can be stored at −80°C over months before sectioning.

Stage 3: Sectioning of the tissue

Timing: 2 h

Here we describe the protocol and the details we took into consideration for getting samples that were suitable for the cellular and subcellular localization of cholesterol in human and mouse brain and liver tissue by the recombinant protein detailed above.

Table 1. List of the antibodies and dilutions employed in this protocol

Organism	Primary antibody	Host	Reference	Dilution
Human	Anti-GST (A-6)	Mouse	Cat#374171	(1:200)
	Anti-ß amyloid	Rabbit	Cat#8243	(1:200)
	Anti-cytochrome c	Rabbit	Cat# 4272S	(1:200)
	Anti-GFAP	Rat	Cat# 345860	(1:200)
	Anti-TOM20	Rabbit	Cat#11415	(1:200)
	Anti-LAMP1	Rabbit	Cat#5570	(1:200)
Mouse	Anti-GST (A-6)	Mouse	Cat#374171	(1:250)
	Anti- LAMP2	Rat	Cat#ab13524	(1:250)
	Anti-cytochrome c	Rabbit	Cat# 4272S	(1:200)
	Anti-TOM20	Rabbit	Cat#11415	(1:200)

10. Sectioning of the tissue;

The conditions described here were tested in areas of the entorhinal cortex and the hippocampus, in human and mouse brains respectively.

a. Adjust the camera of the cryostat to −19°C and the sample holder head to −22°C.
b. Adjust the thickness of the slide to 30 μm and cut (for both human and mouse tissue).
c. Place the cut section on the slide.
d. Label the slide.
e. Store slides at −80°C.

Pause point: slides can be stored at −80°C for months.

Stage 4: Immunofluorescence of postmortem human and mouse brain tissues and liver

Timing: 2 days

This step describes the optimized protocol for the effective localization of cholesterol using the recombinant protein we detailed above in human and mouse brain tissue, and the combination of the probe with immunofluorescence assays for localizing cholesterol in target organelles and cell-types. Once the methodology was established, the same protocol was applied to detect cholesterol in human and mouse liver tissue.

11. Co-Immunofluorescence with GST-PFO;

Apply all reagents in volumes of 200 μL for sections of human samples and 100 μL for sections of mouse samples. Incubate the samples in an orbital shaker at 10 rpm and 4°C.

a. Surround the cut sections with a water-repellent pen.
b. Wash cut sections with PBS, 3 times, for 5 min to remove traces of PFA.
c. Apply NH_4Cl-PBS (50 mM) over the cut sections and incubate for 40 min to quench fluorescence coming from PFA.
d. Wash with Triton X-100 1% over 30 min.
e. Block non-specific union sites with 10% goat serum containing Triton X-100 1% for 2 h and 30 min.
f. Apply GST-PFO, 30 μg/mL for human tissue sections and 20 μg/mL for mouse tissue sections, diluted in "**Immunofluorescence Solution**". Incubate for 3 h.
g. Wash cut sections with PBS for 10 min.
h. Incubate cut sections with primary antibodies diluted in "**Immunofluorescence Solution**" overnight (12–24 h) at 4°C. The primary antibodies employed in this protocol are listed in the key resources table. Dilutions employed for each primary antibody are specified in Table 1.

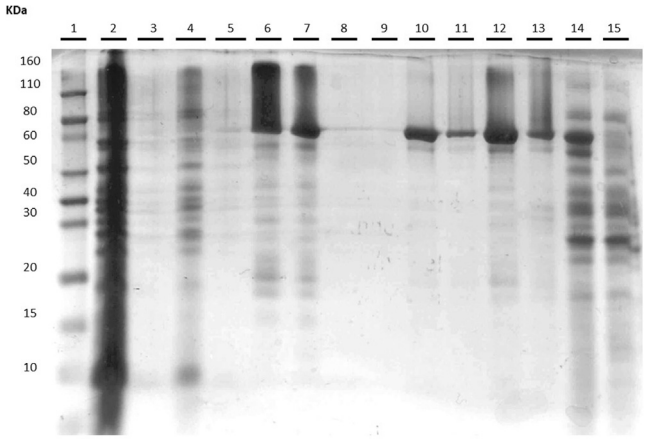

Figure 3. SDS-PAGE analysis of the purification of the recombinant GST-PFO protein

Lane 1: Molecular weight marker -Novex® Sharp Pre-stained Protein Standard, Lane 2: Fraction of non-resin bound proteins, Lane 3: Wash 1, Lane 4: Wash 2, Lane 5: Wash 3, Lane 6: Elution 1 (20 mM GSH), Lane 7: Elution 2 (20 mM GSH), Lane 8 and 9: empty, Lane 10: Elution 3 (20 mM GSH), Lane 11: Elution 4 (40 mM GSH), Lane 12: Elution 5 (40 mM GSH), Lane 13: Final resin sample, Lane 14: Total cells after induction, Lane 15: Total cells before induction. Acrylamide percentage 4–12%. Induction was performed for 20 h at 220 rpm and 18 °C. GSH: reduced glutathione.

i. Wash cut sections with PBS for 10 min.
j. Apply all the recommended secondary antibodies diluted 1:200 in "**Immunofluorescence Solution**" for 4 h. The secondary antibodies employed in this protocol are listed in the key resources table.
k. Add Sudan black 0.1% in 70% ethanol for 10 min to reduce autofluorescence.
l. Wash carefully with PBS until the sample is freed from precipitates.
m. Remove carefully the excess liquid from the sample with a paper towel.
n. Coverslip the samples with 8 μL of mounting media. Let them dry.

⚠ CRITICAL: Avoid air bubbles while mounting the samples.

o. Visualize the mounted samples in the confocal microscope.

⚠ CRITICAL: Samples must be stored in darkness to avoid photobleaching.

⏸ **Pause point:** the mounted samples can be visualized up to 2 days after mounting if kept at 4°C.

EXPECTED OUTCOMES

Successful completion of this protocol results in the generation of the GST-PFO recombinant protein. As a first approach, it was monitored whether the process of induction, extraction and purification of GST-PFO protein was effective and efficient using SDS-PAGE gels. Figure 3 shows that not-induced bacteria (Lane 15) do not possess the band expected for GST-PFO (78 kDa), however, once protein production is induced (addition of 0.5 mM IPTG) a band is observed (Lane 14). Likewise, from the visualization of the recovered fractions in the washing steps, it is concluded that the displacement of non-specific proteins is efficient and that only a minimal fraction of GST-PFO is lost during the process (Lanes 3–5). Observing the bands corresponding to the eluted fractions, it can be noted that the protein is satisfactorily recovered, increasing its purity in the third elution of 20 mM GSH (Lane 10) and the 40 mM GSH elutions (Lanes 11, 12 and 13). Fractions with the highest purity were chosen (Lanes 10, 11 and 12) for the purification step, and 700 μL of 0.83 mg/mL GST-PFO were recovered. However, the yield of the obtained probe may differ depending on the success

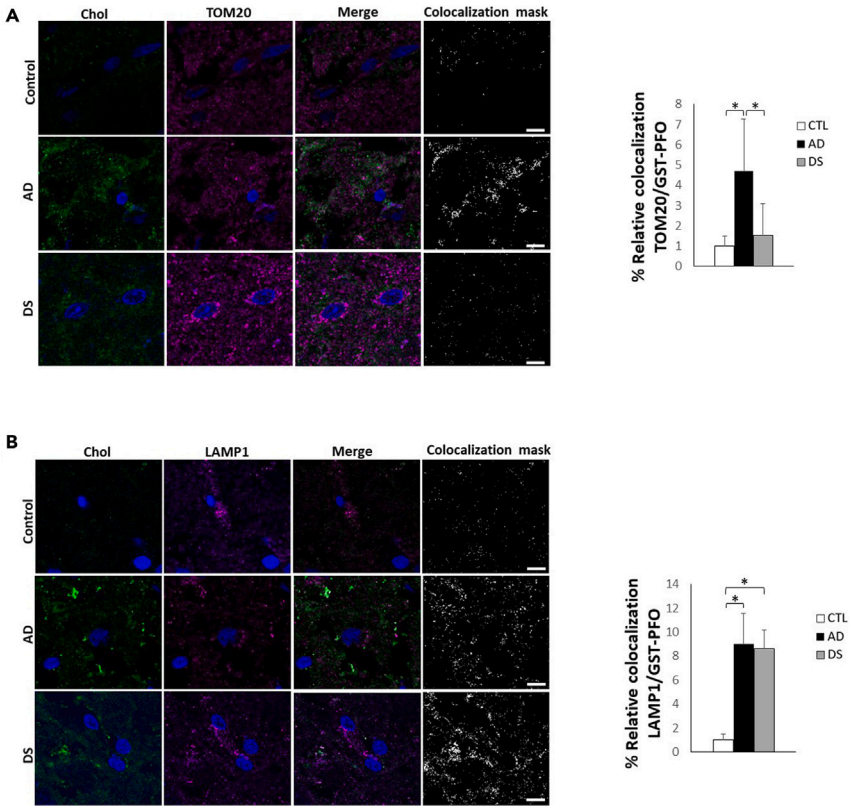

Figure 4. Localization of intracellular cholesterol in human brain samples

Representative immunostaining images of brain sections from healthy donors, AD and DS subjects stained with GST-PFO (green) and (A) TOM20 or (B) LAMP1 (both cyan), showing their colocalization as merge and mask (white). Nuclei are stained with Dapi (blue). Scale bar: 10 μm. Data presented as mean ± SD. Statistical analysis done with One-way ANOVA (*$p \leq 0.05$).

of the protein induction and purification steps. Finally, despite the fact that the resin loses a fraction of non-eluted protein, this is not a very significant fact considering that the purity of the recombinant protein is the main objective of the presented method.

This fusion protein can be used as a cholesterol-detecting probe and has shown effective localization of intracellular cholesterol in human and mouse neuronal tissues (Figures 4 and 5). The contribution of any other lipid class to the fluorescence is avoided as the probe shows no binding to any of the tested lipid classes, except for cholesterol (Figure 6). Besides, the probe is prescribed for co-immunofluorescence techniques, as the simultaneous incubation of three different antibodies resulted in the localization of cholesterol in mitochondria and lysosomes from AD/DS's (Down syndrome) astrocytic cells (Figure 7). Further, the implementation of the probe in our works has permitted us to identify the StARD1, steroidogenic acute regulatory protein, cholesterol transporter as a pathological marker in post-mortem brains from AD and DS patients (Arenas et al., 2020).

Compared with other cholesterol visualizing probes, the combination of PFO with the GST tag provides an attractive tool that can be employed in a large variety of molecular techniques. This, together with its high affinity with cholesterol, reduced cost and easy generation, makes GST-PFO one of the most suitable options among those available on the market. This protocol puts within reach of researchers a powerful tool in the quantitative determination of the increased cerebral cholesterol and neurodegeneration in patients suffering from AD and DS. This holds also true for other cholesterol-accumulating disorders, such as NPC or NASH-driven HCC, as the probe

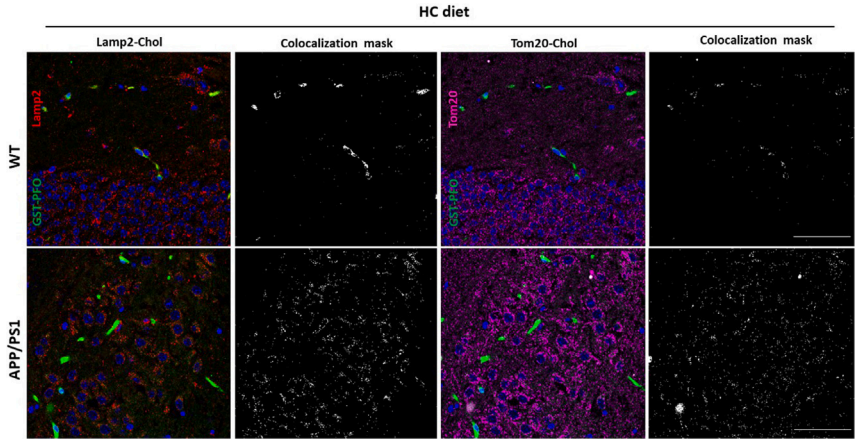

Figure 5. Immunofluorescence images of hippocampal sections from WT and APP/PS1 mice stained with GST-PFO (green), Lamp2 (red), and Tom20 (cyan), showing their colocalization as merge and mask (white)

Nuclei are stained with Dapi (blue). Scale bar: 50 μm. HC: high-cholesterol, APP/PS1: AD mouse model, mutant for amyloid precursor and presenilin 1 proteins.

also allows the identification of the increased levels of mitochondrial cholesterol in brain and liver sections of NPC1-deficient mice (Figure 8), and human NASH-derived HCC specimens (Figure 9) and liver sections from experimental NASH-driven HCC mouse models (Figure 10). The protocol presented here was optimized for its use in brain tissue from mice and humans based on the one published by Kwiatkowska et al. (2014), who described the use of the recombinant protein for the visualization of cholesterol deposits in cells. There are other publications in which the GST-PFO probe has been employed in NPC cells (Contreras et al., 2020) or brain hippocampal samples from patients with AD (Arenas et al., 2020). However, to the best of our knowledge, we report for the first time the use of GST-PFO to monitor hepatic cholesterol in NPC disease and NASH-HCC.

Therefore, GST-PFO is a powerful tool for the diagnosis of cholesterol-accumulating neurological disorders. Studies focused on the elucidation of the contribution of the disrupted mitochondrial cholesterol transport to the pathophysiology of these diseases will be crucial for the identification of new therapeutic targets in the early stages of these and many other devastating neurodegenerative diseases and will pave the way for pharmacological actions for these patients.

QUANTIFICATION AND STATISTICAL ANALYSIS

Percentage of total GST-PFO colocalizing with mitochondria and lysosomes was analyzed with the "Colocalization nBits nimages" plugin (Confocal Microscopy Unit, Medicine Faculty, University of Barcelona) in the Image J software. This software plugin highlights the colocalized points of two 32-bits images. Two points are considered as colocalized if their respective intensities are strictly higher than the threshold of their channels and if their ratio of intensity is strictly higher than the ratio setting value, which has been defined at 50%. Colocalization index is calculated as the ratio of co-localized points between total threshold-passed. The colocalization mask displays the pixels that show colocalization using this parameter.

In the protein-lipid assay, quantification of the immunoreactivity was accomplished by calculating the percentage of intensity of each dot with ImageJ Software (National Institutes of Health): intensity density of the positive staining surface from each dot corresponding to the different concentrations of the different lipid species/(total intensity density of the total positive staining surface × 100%). Integrated density is defined as the sum of the values of the pixels in the image or for a given selection.

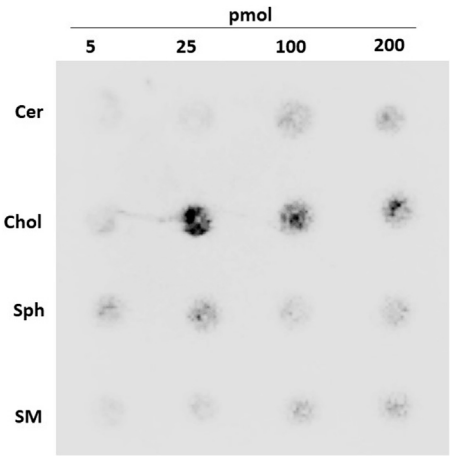

Figure 6. Selective recognition of cholesterol by GST-PFO
Protein-lipid overlay assay. Indicated amounts of lipids were spotted on nitrocellulose membrane and incubated with 5 μg/mL GST-PFO. Immunoreactive spots were revealed by chemiluminescence. Cer: ceramide, Chol: cholesterol, Sph: sphingosine and SM: sphingomyelin.

LIMITATIONS

The generation of this probe is completely homemade, so its quality, specificity and sensitivity are linked to possible human error throughout the entire protocol. Nevertheless, the probe is highly selective for cholesterol, as we demonstrated in a protein-lipid overlay assay (Figure 6; Table 2). For the protein-lipid assay, we followed the protocol described by Kwiatkowska et al. (2007). Troubleshooting 5

Although GST-PFO production requires time and human-handling it can easily be produced in large quantities in bacteria and purified by simple one-step column affinity chromatography, meaning that once generated, limitation in the quantities of the probe will not be a bottleneck.

GST-PFO is able to track membrane cholesterol as long as a membrane contains more than 30% mol of cholesterol, meaning that it cannot be applied to tissues with less cholesterol than this amount. The tissues employed in this work, brain and liver, are major cholesterol-containing sites in the organism, as the brain contains the highest level of cholesterol in the body and cholesterol degradation occurs in the liver. Thus, the applicability of the probe for the detection in these tissues is fairly convenient.

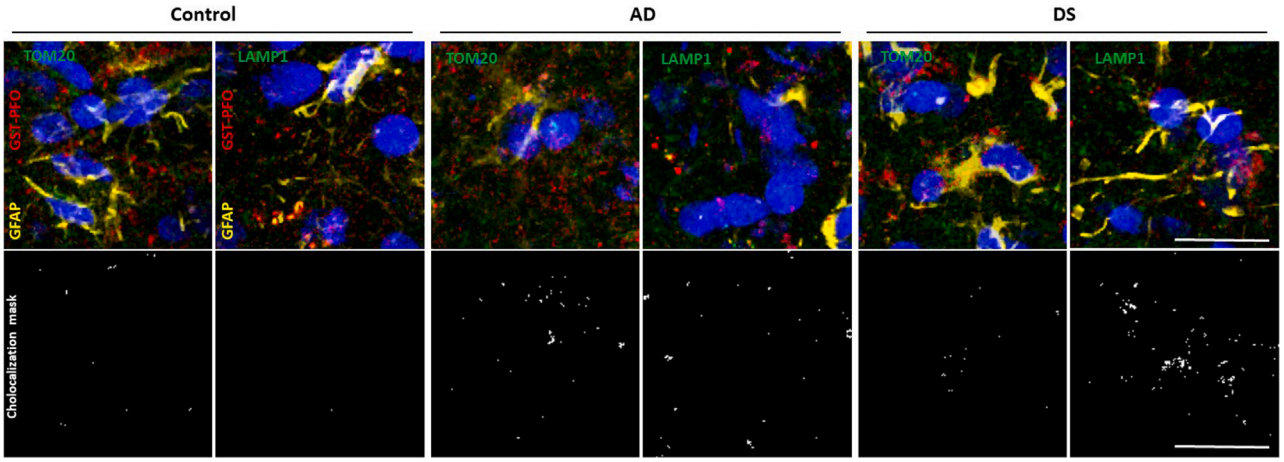

Figure 7. Representative confocal images of cryopreserved brains from healthy donors, AD and DS subjects immunolabeled with GST-PFO (red), GFAP (yellow), and TOM20 or LAMP1 (both green)
Nuclei are stained with Dapi (blue). Lower panels show a colocalization mask (white) between GST-PFO and Tom20 or Lamp1, respectively. Scale bar: 10 μm.

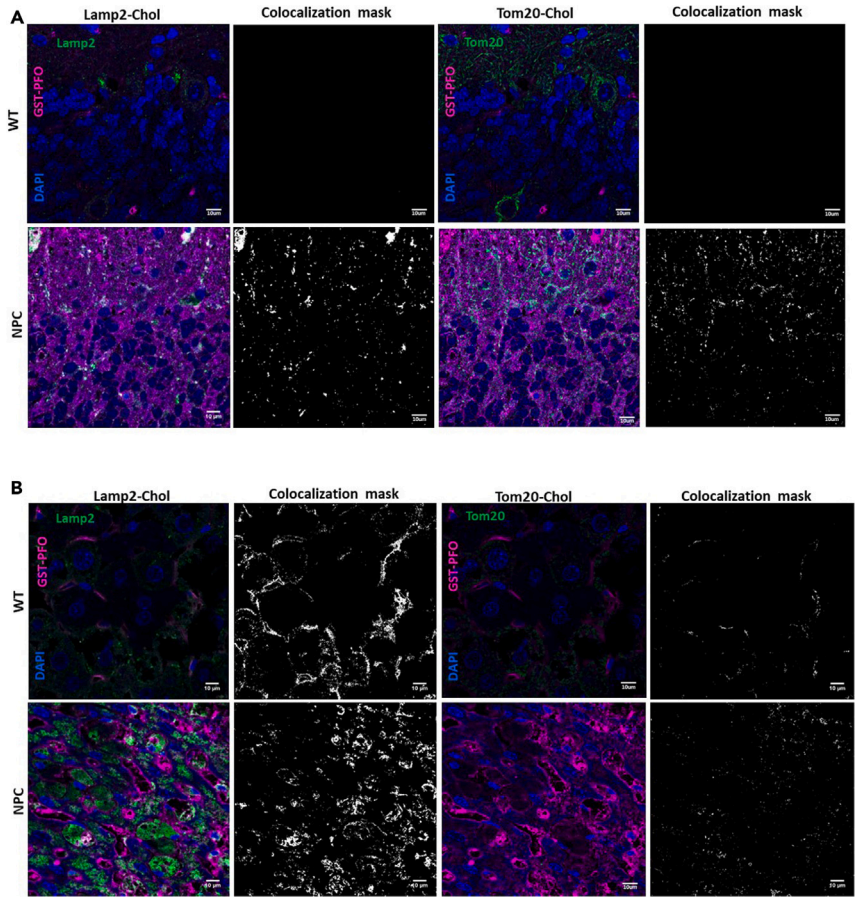

Figure 8. Localization of intracellular cholesterol in mouse brain and liver samples

Immunostaining of (A) cerebellum and (B) liver sections from WT and NPC1-deficient mice stained with GST-PFO (cyan), Lamp2 (green) and Tom20 (green), showing their colocalization as merge and mask (white) Nuclei are stained with Dapi (blue). Scale bar: 10 μm.

TROUBLESHOOTING

Problem 1

Low optical density (OD) value during bacterial production. (Stage 1, Step 5, g).

Potential solution

Low optical density means low bacterial concentration. This could be attributed to (i) the initial amount of bacterial inoculation from the glycerol stock and/or (ii) the quality of the bacterial glycerol stock employed during bacterial production. Consider first (i) increasing x2 the amount of bacterial inoculation in Stage 1 and if the problem persists (ii) replacing glycerol stock with newly transformed cells. If you have an extra bacterial glycerol stock stored at −80°C, check whether it has greater OD values. Correct OD values range around 0.6. If it is not your case, proceed to generate a new recombinant protein to try to get a more efficient bacterial production. Also, we recommend making sure that correct bacterial incubation conditions are employed (18°C for 20 h) and bacterial stress is avoided during bacterial growth.

Problem 2

Contaminated GST-PFO protein production. (Stage 1, Step 5, j).

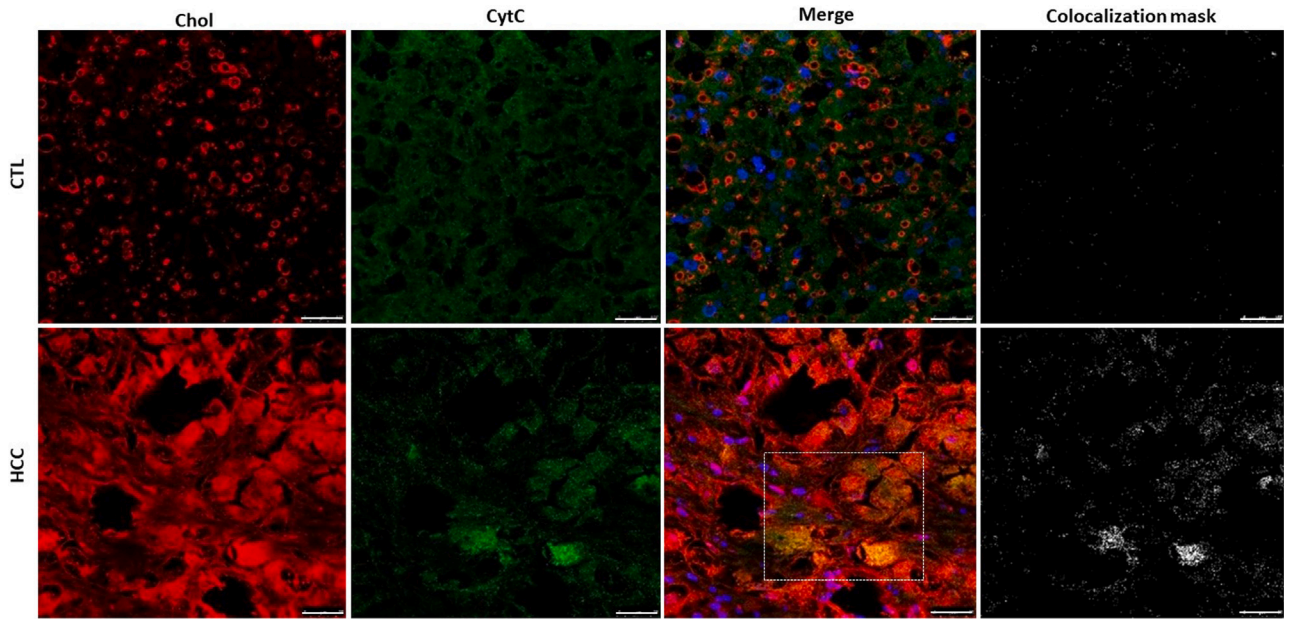

Figure 9. Immunofluorescence images of liver sections from healthy donors and HCC subjects stained with GST-PFO (red) and Cyt C (green), showing their colocalization as merge (area delimited by dotted line) and mask (white)

Nuclei are stained with Dapi (blue). Scale bar: 100 μm for control and 25 μm for HCC samples. CytC: cytochrome c (mitochondrial marker).

Potential solution

Not maintaining correct bacterial growth conditions could lead to contaminations. Make sure correct bacterial incubation conditions are employed (18°C for 20 h) and bacterial stress is avoided during bacterial growth.

Problem 3

GSH degradation. (**Stage 1, Step 7, f**).

Potential solution

GSH degradation can occur when the "**Elution buffer**" is not fresh enough or when pH is not correct. In order to avoid GSH degradation, use all reagents previously cooled and filtered with a 0.2 μm filter, and make sure that the pH is correct (pH 8.4). If not, adjust it. Finally, remember that the "**Elution buffer**" must be fresh every time to avoid GSH degradation.

Problem 4

GST-PFO recombinant protein degradation. (**Stage 1, Step 8, c**).

Potential solution

Centrifugation time may extend during dialysis if the Amicon Ultra 15 mL centrifugal filters employed are obstructed. Consider using new Amicon Ultra 15 mL centrifugal filters every time if this happens.

Problem 5

Presence of dots in other lipid classes not corresponding to cholesterol. (Protein-lipid assay, limitations section).

Potential solution

One potential reason for the presence of dots in other lipid classes not corresponding to cholesterol is due to mutations in the PFO DNA sequence. To verify this, sequence the constructed plasmid to corroborate the plasmid was correctly synthesized and identify possible mutations in the PFO DNA sequence.

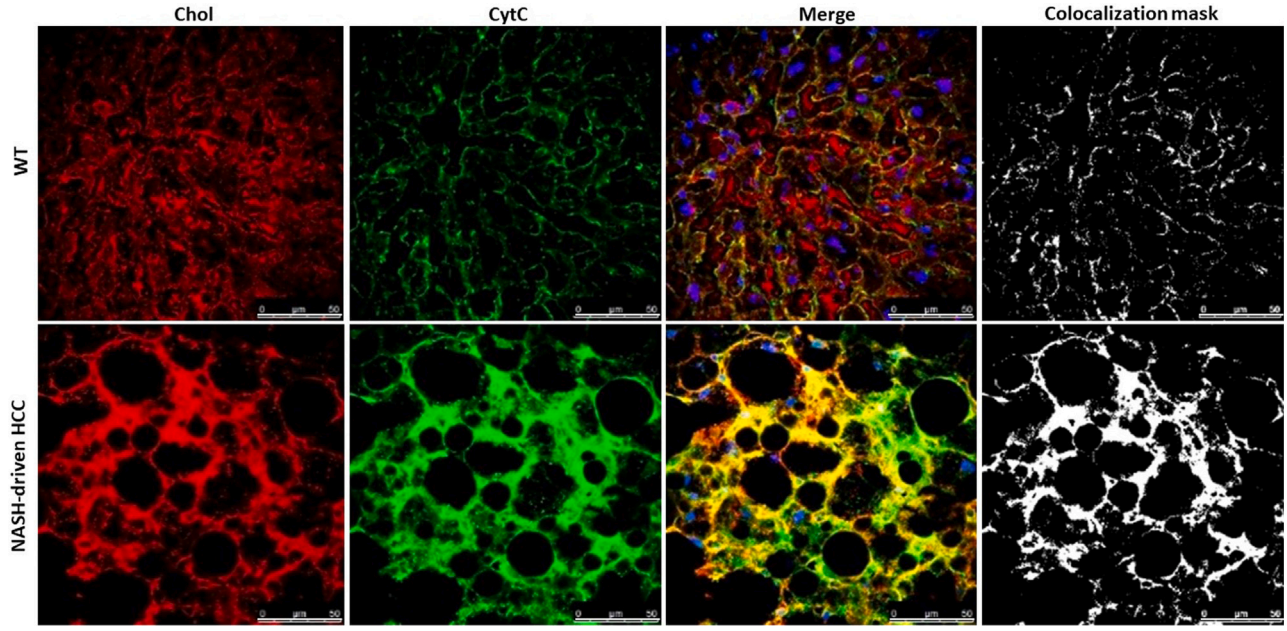

Figure 10. Immunofluorescence images of liver sections from WT and NASH-driven HCC mice stained with GST-PFO (red) and Cyt C (green), showing their colocalization as merge and mask (white)

Nuclei are stained with Dapi (blue). Scale bar: 50 μm. CytC: cytochrome c (mitochondrial marker).

If mutations in the DNA sequence are discarded, presence of unspecific lipids could be due to incorrect protein folding during the GST-PFO synthesis. To ensure correct protein folding, make sure correct bacterial incubation conditions are employed (18°C for 20 h) during bacterial growth.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, J.C. Fernandez-Checa (checa229@yahoo.com).

Materials availability

Successful completion of this protocol results in the generation of the GST-PFO recombinant protein. Additional information regarding the generation of the probe should be requested to the lead contact.

Data and code availability

This study did not generate new unique data or code. The DNA sequence of the PFO gene employed to design the plasmid is available at the NCBI database (NCBI: M36704).

Table 2. Table summarizing quantification of the protein-lipid overlay assay

Lipid species (pmol)	5	25	100	200
Cer	13.81%	1.31%	6.48%	3.38%
Chol	**5.42%**	**19.59%**	**15.43%**	**12.40%**
Sph	6.60%	6.63%	0.55%	1.39%
SM	2.93%	0.63%	2.74%	0.72%

Indicated amounts (pmol) of different lipid species were spotted in a nitrocellulose membrane and incubated with 5 μg/mL GST-PFO. Intensity percentage of each dot was quantified with Image J. Cer: ceramide, Chol: cholesterol, Sph: sphingosine and SM: sphingomyelin.

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AUTHOR CONTRIBUTIONS

Each author contributed to the planning, performance, and analyses of the data. F.C. and F.A. developed and optimized the protocol. S.N. and S.T. contributed to the experiments. F.C. and L.G. prepared the manuscript and discussed data. L.G., F.A., C.G.-R., and J.C.F.-C. planned and designed the study, participated in data analyses and discussions, and assisted in manuscript text preparation.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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