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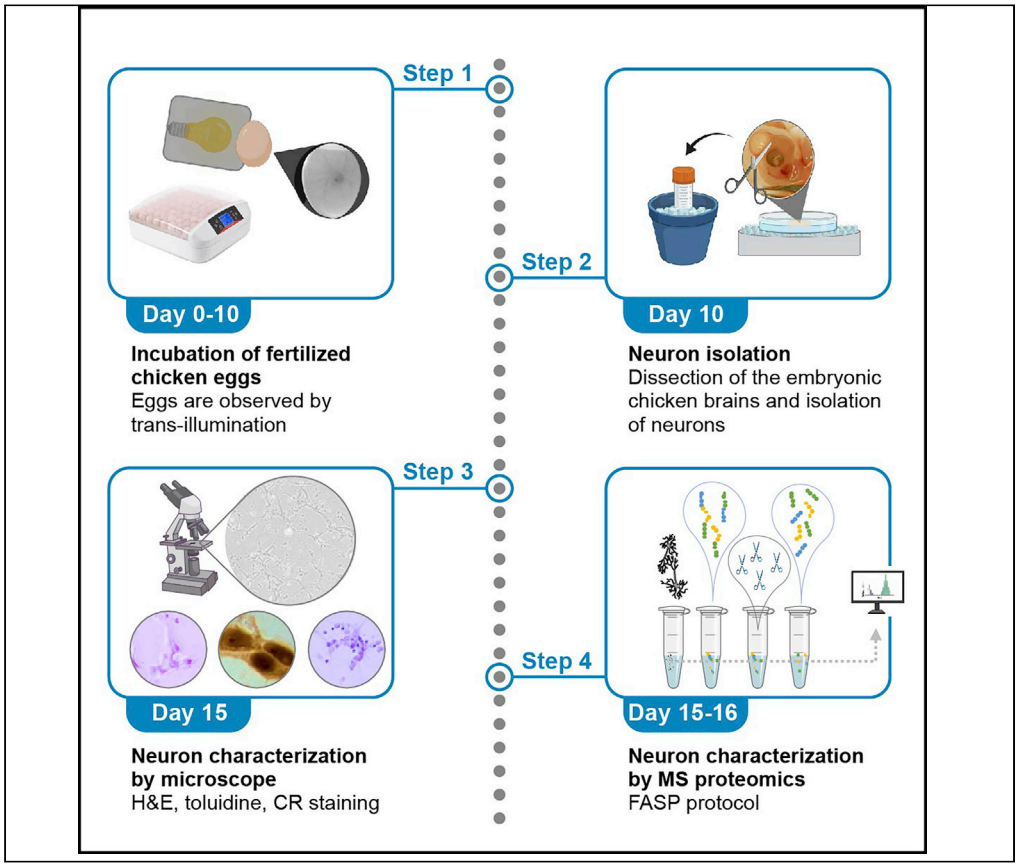
Protocol

Optimized protocol for obtaining and characterizing primary neuron-enriched cultures from embryonic chicken brains



We present here an optimized protocol to obtain primary neuron-enriched cultures from embryonic chicken brains with no need for an animal facility. The protocol details the steps to isolate a neuron-enriched cell fraction from chicken embryos, followed by characterization of the chicken neurons with mass spectrometry proteomics and cell staining. Because of the high homology between chicken and human amyloid precursor protein processing machinery, these chicken neurons can be used as an alternative to rodent models for studying Alzheimer disease.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Cátia F. Marques, Pedro F. Pinheiro, Gonçalo C. Justino

catiafmarques@tecnico.ulisboa.pt (C.F.M.)
pedro.pinheiro@tecnico.ulisboa.pt (P.F.P.)
goncalo.justino@tecnico.ulisboa.pt (G.C.J.)

Highlights

An *in vitro* model that can be used for Alzheimer disease studies

Fast and accessible protocol with no need for an animal facility

Isolation of a neuron-enriched cell fraction from chicken embryos

Characterization of the neuronal model using proteomics and cell staining approaches

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Optimized protocol for obtaining and characterizing primary neuron-enriched cultures from embryonic chicken brains

Cátia F. Marques,[1,2,*] Pedro F. Pinheiro,[1,*] and Gonçalo C. Justino[1,3,*]

[1]Centro de Química Estrutural, Institute of Molecular Sciences, Instituto Superior Técnico, Universidade de Lisboa, 1049-001 Lisboa, Portugal

[2]Technical contact

[3]Lead contact

*Correspondence: catiafmarques@tecnico.ulisboa.pt (C.F.M.), pedro.pinheiro@tecnico.ulisboa.pt (P.F.P.), goncalo.justino@tecnico.ulisboa.pt (G.C.J.)
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SUMMARY

We present here an optimized protocol to obtain primary neuron-enriched cultures from embryonic chicken brains with no need for an animal facility. The protocol details the steps to isolate a neuron-enriched cell fraction from chicken embryos, followed by characterization of the chicken neurons with mass spectrometry proteomics and cell staining. Because of the high homology between chicken and human amyloid precursor protein processing machinery, these chicken neurons can be used as an alternative to rodent models for studying Alzheimer disease.

BEFORE YOU BEGIN

Introduction

Although birds and mammals diverged about 270 million years ago, they share some gene sequences, which allow primary chicken neurons to be a better and non-transgenic model for amyloid precursor protein (APP) and APP-processing than mice (Sarasa and Pesini, 2009). Chicken APP has a close sequence homology with human APP (93% amino acid identity and 96% similarity) and the $A\beta_{1-42}$ sequence is identical (100% similarity) between chick and human, contrary to rodent $A\beta_{1-42}$, in which arginine, tyrosine, and histidine residues are mutated (Figure 1) (Carrodeguas et al., 2005; Czvitkovich et al., 2011; Mileusnic and Rose, 2010). The enzymatic machinery responsible for APP processing in humans is also present in the embryonic chicken brain and $A\beta_{1-42}$ is also the major Aβ peptide produced during chick embryogenesis. APP processing in chick involves the BACE-1/2, Psen1/2, nicastrin, neprilysin, and ADAM-10/17 homologous proteins (Carrodeguas et al., 2005; Hall and Erickson, 2003).

In addition to the APP and APP-processing machinery, the Tau protein has also been identified in chicken, where it is likewise linked to neurodegeneration (Beyrent and Gomez, 2020; Goldsbury et al., 2008; Rebhan et al., 1995; Rösner et al., 1994; Tucker et al., 1988; Yoshida and Goedert, 2002).

Despite the fact that the chicken lifespan may reach 30 years, these animals generally do not live that long, and thus no studies on whether chicken can suffer any Alzheimer-like pathology have been performed (Sarasa and Pesini, 2009). However, chicken neurons might be a very good model for high throughput screening of new drugs and to study drug interactions during the various APP-processing steps, allowing the identification of possible targets involved in the amyloid pathway, for example.

```
                1              10              20              30          40 42
Human           DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
Chicken         DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
Mouse           DAEFGHDSGFEVRHQKLVFFAEDVGSNKGAIIGLMVGGVVIA
                *       *   *   *           **
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Figure 1. Alignment of the amino acid sequences of human, chick, and mouse $A\beta_{42}$

The APP sequences from human, chicken and mouse were obtained from the Uniprot database (UniProt Consortium, 2021) (Uniprot IDs P05067, Q9DGJ7 and P12023, respectively). The residues in red and underlined differ from the human sequence. The asterisks indicate the residues involved in the amyloidogenic pathway (Carrodeguas et al., 2005).

Institution permission

According to the European directive 2010/63/EU regarding animal protection for scientific proposes, which was transposed to the Portuguese law 113/2013, chicken embryos at the 10th-day post-fertilization are not considered live animals, and no ethical approval is required. In the remaining aspects, best practices were followed throughout the work, particularly regarding cell manipulation and residue disposal, in agreement with national regulations and by-laws. Researchers are advised to consult their host institutions for adequate permissions in each case.

Chicken egg incubation

Timing: 10 days

This protocol was optimized for the isolation of primary neuron-enriched fraction from embryonic chicken (*Gallus gallus*) eggs at the 10th day post-fertilization (Kumar and Mallick, 2016; Pettmann et al., 1979; Rani et al., 1983; Roth et al., 2010; Saito et al., 2003; Sensenbrenner et al., 1978). From 48 eggs, *ca.* 80% of the eggs were viable, yielding a total of *ca.* 110 million viable cells. Culture maintenance conditions are optimized for a seven-day period using 6-, 24-, and 96-well plates; however, it does not invalidate longer periods or other cell culture vessels.

1. Acquire fertilized eggs (*Gallus gallus domesticus*) and incubate them at 37.5°C in an humidified egg incubator with automatic tilting.
2. On the 9th post-fertilization day, analyze the eggs by trans-illumination with a white 60 W light bulb to confirm the presence of a viable embryo (observation of a vascular network around the embryo) (Figure 2).
3. Keep the eggs on the egg incubator until the next day.

Note: The observation and identification of viable chicken embryos is an important step before cell isolation. Egg opening should start by the expected non-fertilized or non-viable

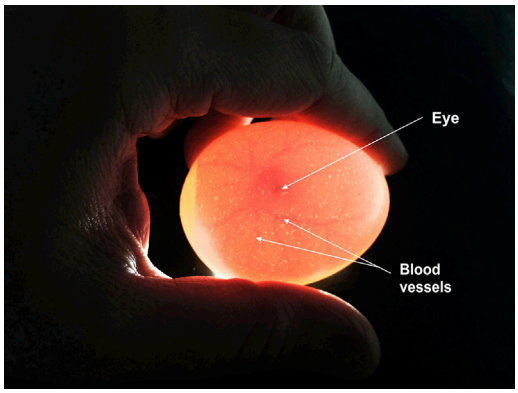

Figure 2. Trans-illumination of a fertilized egg

embryo eggs. Sometimes, eggs considered non-fertilized or non-viable by trans-illumination turn out to have a live embryo. Starting by the expected non-viable/non-fertilized eggs allows a smaller waiting time between brain collection from expected viable embryos and subsequent processing.

⚠ CRITICAL: The humidity in the egg incubator should be kept constant, ideally between 40%–50%.

Culture vessel coating

Timing: 3.5–4.5 h

Upon isolation of embryonic chicken neurons, cells are seeded in multiwell plates coated with poly-D-lysine (PDL). Multiwell plates should be coated in advance and stored at 4°C until use.

4. Prepare a 50 μg/mL poly-D-lysine (PDL) working solution in phosphate buffer solution.
   a. Dilute a 0.1 mg/mL PDL stock solution in sterile PBS to obtain a 50 μg/mL working solution.
5. Coat the surface of the vessel of choice with the working solution.
   a. Add the adjusted volume of working solution per well.
      i. In a 96-well plate, 50 μL working solution per well.
      ii. In a 24-well plate, 300 μL working solution per well.
      iii. In a 6-well plate, 1.5 mL working solution per well.
6. Incubate the vessel at room temperature for 1 h.
7. Remove the PDL solution and wash the wells.
   a. Rinse each well 3 times with a large volume of water (e.g., 100 μL/well for a 96-well plate).
8. After removing the final wash, leave the coated culture vessel uncovered in the laminar flow cabinet for 2 h.
9. After drying, seal the culture vessels with parafilm and store at 4°C. Stored culture vessels should be used within two weeks.

Note: Depending on the manufacturer, the PDL coating protocol can be adjusted. For the PDL used in this work, instructions are available online.

Note: Various steps are carried out at room temperature, about 22°C–24°C.

⚠ CRITICAL: Excess PDL can be toxic to the cells. Make sure all wells are well rinsed. Culture vessels should be placed at room temperature (*ca.* 22°C) before use.

Isolation procedure preparation

Timing: 30 min

10. Decontaminate the laminar flow cabinet (LFC) working surface using a 70% ethanol solution and prepare the working area. All other material (tubes, pipettes, etc. should be sterilized by autoclave beforehand).
    a. Place the microscope camera and screen inside the LFC (optional but recommended).
    b. Fill a tray with crushed ice and place it inside the LFC. This will serve as dissection platform.
    c. Place the additional material in your working area, as suggested in Figure 3.
11. Fill a 50 mL conical tube with *ca.* 10 mL of dissection medium. Keep the tube on ice during all the isolation procedure.

Note: We usually isolate neurons on Wednesday and perform experiments with the cells on the following Monday (5th day post-isolation).

Figure 3. Suggested setup for the working space

Suggested setup for an easy workflow during the brain isolation. 1, screen coupled to the microscope camera (optional but recommended); 2, microscope camera (optional but recommended); 3, ice box with crushed ice; 4 and 5, dissecting tweezers; 6, Petri dish (lid); 7, dissecting scissors; 8, waste bag; 9, aluminum foil working area; 10, sterile beaker with milli-Q water; 11, sterile beaker for egg waste; 12, Petri dish; 13, curved tweezers; 14, tweezers; 15, standard scissors; 16, rubber cone (or other support for the egg); 17, 1 mL pipette tips; 18, 1 mL pipette; 19, extra Petri dishes; 20, dissection medium; 21, 50 mL falcon with *ca.* 10 mL dissection medium on ice; 22, glass Pasteur pipettes.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
0.25% trypsin-EDTA solution	Gibco	Cat# 25200-072
0.9% sodium chloride solution	B. Braun	N/A
1,4-dithiothreitol (DTT)	Acros Organics	Cat# 327190010
10% Fetal Bovine Serum (FBS)	Gibco	Cat# 10500-064
Acetic acid, glacial	CARLO ERBA	Cat# 524521
Acetonitrile, Optima™ LC/MS grade	Fisher Scientific	Cat# A955-212
Ammonium bicarbonate, eluent additive for LC-MS	Fluka	Cat# 40867-50G-F
Ammonium chloride	Fluka	Cat# 09718
Amphotericin B solution (250 µg/mL)	Gibco	Cat# 15290-026
Bio-Rad Protein Assay Dye Reagent Concentrate	Bio-Rad	Cat# 500-0006
cOmplete™ Mini, EDTA-free Protease Inhibitor Cocktail	Roche	Cat# 11836170001
Congo red (CR)	Merck	Cat# 841L456540
Dextrose	Fisher Bioreagents	Cat# BP350-1
Dulbecco's Modified Eagle Medium (DMEM)	Gibco	Cat# 31600-083
EDTA (disodium salt)	Merck	Cat# 8418
Eosin Y	PanReac AppliChem	Cat# 251301.1211
Ethanol absolute	CARLO ERBA	N/A
Ficoll-Paque™ PLUS	Cytiva	Cat# 17144002
Formic acid, Optima™ LC/MS grade	Fisher Scientific	Cat# A117-50
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	PanReac AppliChem	Cat# A3724
Iodoacetamide	Sigma-Aldrich	Cat# I1149-5G
Lithium carbonate	Riedel-de-Haën	Cat# 13009
Mayer's hematoxylin solution	PanReac AppliChem	Cat# 254766.1610
Milli-Q water (sterile)	N/A	N/A
Nutrient Mixture F-12 (F12)	Sigma-Aldrich	Cat# N3520-1L
Paraformaldehyde	Sigma	Cat# 158127-500G

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Penicillin-streptomycin solution (10,000 units/mL of penicillin and 10,000 µg/mL of streptomycin)	Gibco	Cat# 15140-122
Phenol red	Sigma-Aldrich	Cat# P3532-5G
Poly-D-Lysine	MP Biomedicals	Cat# 150175
Sequencing-grade porcine trypsin	Roche	Cat# 11418033001
Sodium bicarbonate	Fisher Bioreagents	Cat# BP328-500
SDS solution (sodium dodecyl sulfate, 20% m/v)	Fisher Bioreagents	Cat# T-27069
Sodium pyruvate 100 mM solution	Gibco	Cat# 11360-039
Sodium selenite	Merck	Cat# 6606
Toluidine blue solution 0.5%	Servicebio	Cat# G1032
TRIS (tris(hydroxymethyl)aminomethane)	Sigma	Cat# 252859-500G
Trypan blue solution 0.4%	Gibco	Cat# 15250-061
Urea	Sigma-Aldrich	Cat# U5378-100G
Water, Optima™ LC/MS grade	Fisher Scientific	Cat# W6-212
Experimental models: Organisms/strains		
Fertilized eggs (*Gallus gallus domesticus*)	Sociedade Agrícola da Quinta da Freiria, S.A, (Bombarral, Portugal)	N/A
Deposited data		
Raw mass spectrometry data	This paper	https://doi.org/10.17632/44tg5cm93g.1
Software and algorithms		
GraphPad Prism v9.0.0	GraphPad Software, LLC	https://www.graphpad.com/scientific-software/prism/
MARS software v3.32	BMG LABTECH	N/A
MaxQuant software v. 2.0.3.0	Cox and Mann (2008), Cox et al. (2011), Tyanova et al. (2016a)	https://www.maxquant.org/
Motic Images Plus 3.0 ML software v3.9.18.92	Motic	N/A
Perseus v1.6.15.0	Tyanova et al. (2016b)	https://www.maxquant.org/perseus/
PHANTER v16.0	Mi et al. (2021)	http://www.pantherdb.org/
Other		
0.22 µm polyethersulfone (PES) membrane syringe filter	Branchia	Cat# SFPE-22E-050
15 mL conical tube (sterile)	Orange Scientific	Cat# 4440310
40 µm nylon cell strainer (sterile)	Corning	Cat# 431750
50 mL conical tube (sterile)	Abdos	Cat# P1040
60 W light bulb	N/A	N/A
Aluminum foil paper	N/A	N/A
Amicon® Ultra 0.5 mL centrifugal filters with 3 KDa molecular weight cutoff regenerated cellulose membrane	Merck Millipore	Cat#UFC500396
Beakers (sterile)	N/A	N/A
bioZen™ 2.6 µm Peptide XB-C18 (100 Å, 100 × 2.1 mm)	Phenomenex	Cat#00D-4768-AN
Centrifuge with swinging-bucket rotor	MPW MED. INSTRUMENTS	Cat# MPW-380
CO_2 Incubator	Heal Force	Cat# HF90
Dissecting scissors (sterile)	N/A	N/A
Dissecting tweezers (sterile)*	N/A	N/A
Egg incubator with automatic tilting	Nanchang Howard Technology Co., Ltd.	Cat# YZ-56S
Glass Pasteur pipette (sterile)	Normax	N/A
Hemocytometer	BRAND	N/A
High-resolution mass spectrometer	Bruker	Impact II
Ice box (Styrofoam)	N/A	N/A
Phase-contrast inverted microscope	OPTIKA	IM-3
Membrane, 0.2 µm, 47 mm, grid, individual sterile pack	Pall	Cat# 66234

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Microcentrifuge, refrigerated	Gyrozen	Cat# 1730R
Microscope USB camera	N/A	N/A
Moticam S1 camera	Motic	S1
Paper towels (optional)	N/A	N/A
Pencil	N/A	N/A
Petri dishes (plastic, sterile)	N/A	N/A
Pipettes, variable volume, 1–10 µL, 20–200 µL, 100–1,000 µL	Eppendorf	Research Plus
Pipette controller	Bellco Glass	Cat# BELC1225-80122
Rubber cone (or other support)	N/A	N/A
Scissor, standard (sterile)	N/A	N/A
Serological pipettes 10 mL (sterile)	Sarstedt	Cat# 86.1254.001
Serological pipettes 25 mL (sterile)	Sarstedt	Cat# 86.1685.001
Serological pipettes 5 mL (sterile)	Sarstedt	Cat# 86.1253.001
SPECTROstar BMG Labtech equipped with a multiwell plate reader and a low-volume microspot plate	BMG LABTECH	SpectrostarNano
24-well clear flat bottom polystyrene TC-treated plates (sterile)	Sarstedt	Cat# 83.3922
6-well clear flat bottom polystyrene TC-treated plates (sterile)	Sarstedt	Cat# 83.3920
96-well clear flat bottom polystyrene TC-treated plates (sterile)	Sarstedt	Cat# 83.3924
Sterile syringe (25 and 60 mL)	B. Braun	N/A
Tweezers, curved (sterile)	N/A	N/A
Tweezers, standard (sterile)	N/A	N/A
UHPLC system	Bruker	Elute LC
Ultrasonic bath	BANDELIN electronic	RK 52
Waste bag (autoclavable)	N/A	N/A
Water bath, thermostatized	LBX Instruments	N/A

Note: *Tweezers should preferentially have serrated tips, to enhance the grip. While several types were used, best results were obtained using common 130 mm stainless steel tweezers with serrated blunt ends with an opening angle of about 30°. A key factor in choosing the adequate tweezers is ensuring they have an opening angle as large as possible, facilitating tissue manipulation.

MATERIALS AND EQUIPMENT

Neuron isolation protocol

All culture media and solutions prepared were found to be stable for at least 6 months. Deterioration of liquid media may be recognized, among others, by color change, formation of precipitates or particulates, or a cloudy appearance.

Dissection medium

Reagent	Final concentration	Amount
Glucose	1 g/L	1 g
EDTA disodium salt	0.25% (m/v)	2.5 g
Phenol red	10 mg/L	10 mg
Penicillin-streptomycin solution (10 000 units/mL / 10 000 µg/mL)	100 units/mL / 100 µg/mL	10 mL
Amphotericin solution (250 µg/mL)	0.25 µg/mL	1 mL
PBS	N/A	Up to 1 L
Total	**N/A**	**1 L**

Note: Sterilize by filtration and store at 4°C; stable for at least 6 months. Preferably, add penicillin-streptomycin and amphotericin immediately before use.

DMEM with 4.5 g/L glucose		
Reagent	Final concentration	Amount
DMEM	N/A	9.99 g
Sodium bicarbonate	3.7 g/L	3.7 g
Glucose	4.5 g/L*	3.5 g*
Milli-Q water	N/A	Up to 1 L
Total	**N/A**	**1 L**

Note: Sterilize by filtration and store at 4°C; stable for at least 6 months. *The conventional DMEM culture medium is formulated as low glucose medium (1 g/L).

F12 with 4.5 g/L glucose		
Reagent	Final concentration	Amount
Nutrient Mixture F-12 Ham	N/A	11.1 g
Sodium bicarbonate	2.5 g/L	2.5 g
Glucose	4.5 g/L*	3.5 g*
Milli-Q water	N/A	Up to 1 L
Total	**N/A**	**1 L**

Note: Sterilize by filtration and store at 4°C; stable for at least 6 months. *The conventional F12 culture medium is formulated as low glucose medium (1 g/L).

Neuron culture medium		
Reagent	Final concentration	Amount
DMEM with 4.5 g/L glucose	N/A	217 mL
F12 with 4.5 g/L glucose	N/A	217 mL
FBS	10% (v/v)	50 mL
Sodium pyruvate solution (100 mM)	1 mM	5 mL
Penicillin-streptomycin solution (10 000 units/mL / 10 000 μg/mL)	100 units/mL / 100 μg/mL	5 mL
Amphotericin solution (250 μg/mL)	0.25 μg/mL	0.5 mL
Sodium selenite solution (83.3 μM)	83.3 nM	0.5 mL
HEPES solution (1 M)	10 mM	5 mL
Total	**N/A**	**500 mL**

Note: Prepared from sterile solutions under aseptic conditions; stable for at least 6 months at 4°C.

Red blood cells (RBC) lysis buffer		
Reagent	Final concentration	Amount
Ammonium chloride	155 mM	0.8291 g
Sodium bicarbonate	12 mM	0.10 g
EDTA disodium	0.1 mM EDTA	0.0037 g
Milli-Q water	N/A	100 mL
Total	**N/A**	**100 mL**

Note: Adjust pH to 7.4, sterilize by filtration and store at 4°C; stable for at least 6 months.

Ficoll-Paque working solution	
Reagent	Amount
Ficoll-Paque™ Plus	40 mL
PBS (sterile)	20 mL
Total	**60 mL**

Note: Prepared from sterile solutions under aseptic conditions; stable for at least 6 months at 4°C.

Phosphate-buffered saline (PBS) solution		
Reagent	Final concentration	Amount
Na_2HPO_4	10 mM	1.42 g
KH_2PO_4	1.8 mM	0.24 g
NaCl	137 mM	8.00 g
KCl	2.7 mM	0.20 g
Water	N/A	1 L
Total	**N/A**	**1 L**

Note: Adjust the pH to 7.4 with hydrochloric acid or sodium hydroxide, as necessary. Sterilize by autoclaving and store at room temperature. PBS solutions can be used indefinitely as long as they do not show signs of microbial contamination.

Other solutions	
Name	Reagents
Sodium selenite solution	83.3 µM sodium selenite (Na_2SeO_4) in milli-Q water. Add 1.4 mg of sodium selenite in 100 mL of milli-Q water. Sterilize by filtration and store at −20°C for up to 2 years.
HEPES solution	1 M HEPES in milli-Q water. Sterilize by filtration and store at −20°C in 10 mL aliquots for up to 2 years.
Ficoll-Paque working solution	Add 20 mL of sterile PBS to 40 mL Ficoll-Paque™ Plus. Store at 4°C, according to the manufacturer's instructions (available online).
Poly-D-Lysine stock solution	10 mg/mL PDL in milli-Q water. Sterilize by filtration and store at −20°C up to 1 year.
Poly-D-Lysine 1 mg/mL	Dilute the 10 mg/mL PDL stock solution to 1 mg/mL with sterile milli-Q water. Store at 4°C up to 1 year. Use this solution to prepare the working solution.
Poly-D-Lysine 50 µg/mL (working solution)	Dilute the 1 mg/mL PDL solution to 50 µg/mL with sterile PBS. Prepare fresh before use.

Proteomics protocol

Lysis base buffer: 0.1 M Tris-HCl pH 7.8 with 2% SDS		
Reagent	Final concentration	Amount
TRIS	0.1 M	1.211 g
SDS solution (20% m/v)	2%	10 mL
Water LC/MS grade	N/A	90 mL
Total	**N/A**	**100 mL**

Note: Adjust pH to 7.8 before the addition of the SDS and store at room temperature; stable for at least 3 months.

Lysis buffer: Lysis base buffer with 0.05 M DTT and protease inhibitor		
Reagent	Final concentration	Amount
Lysis base buffer	N/A	1 mL
DTT	0.05 M	0.0077 g
Protease inhibitor cOmplete™ Mini	7×	150 µL
Total	**N/A**	**1.15 mL**

Note: Prepare immediately before use. The protease inhibitor should be prepared and used according to the manufacturer instructions (available online), and 150 µL of the prepared solution are added to 1 mL of lysis base buffer.

Urea base buffer: 8 M urea and 25 mM ammonium bicarbonate solution		
Reagent	Final concentration	Amount
Urea	8 M	24 g
Ammonium bicarbonate LC/MS grade	25 mM	0.1 g
Water LC/MS grade	N/A	Up to 50 mL
Total	**N/A**	**50 mL**

Note: Store at room temperature; stable for at least 3 months.

3% acetonitrile with 0.1% formic acid solution		
Reagent	Final concentration	Amount
Acetonitrile LC/MS grade	3% (v/v)	1.5 mL
Formic acid LC/MS grade	0.1% (v/v)	50 µL
Water LC/MS grade	N/A	48.5 mL
Total	**N/A**	**50 mL**

Note: Store at room temperature; stable for at least 6 months.

Other solutions	
Name	Reagents
50 mM iodoacetamide in urea base buffer	50 mM iodoacetamide in urea base buffer (8 M urea and 25 mM ammonium bicarbonate solution). Add 9.2 mg of iodoacetamide in 1 mL of urea base buffer. Prepare fresh before use.
Trypsin stock solution 200 µg/mL	Prepare a 0.2 mg/mL trypsin solution in 1% acetic acid. Store at −80°C for up to 6 months. Preparation instructions depend on the manufacturer (available online).
25 mM ammonium bicarbonate	0.1 g ammonium bicarbonate (NH_4HCO_3, LC/MS grade) in 50 mL water (LC/MC grade). Store at room temperature for up to 6 months.
12.5 mM ammonium bicarbonate	0.05 g ammonium bicarbonate (LC/MS grade) in 50 mL water (LC/MC grade). Store at room temperature for up to 6 months.

Cell staining protocols

Solutions for cell staining	
Name	Reagents
4% paraformaldehyde	4% (m/v) in PBS solution. It can be stored at 4°C for two weeks but it is best to prepare fresh.
1% acid ethanol	1 mL HCl 37% in 400 mL ethanol 70%. Store at room temperature for up to 6 months.
Alkaline alcohol solution	1 mL NaOH 1% in 100 mL ethanol 50%. Store at room temperature for up to 2 months, tightly closed.

Note: Paraformaldehyde is a toxic reagent that should be manipulated exclusively in a fume hood.

Congo Red solution		
Reagent	Final concentration	Amount
Congo Red	0.3% (m/v)	30 mg
Sodium chloride	0.3% (m/v)	30 mg
Sodium hydroxide	0.01% (m/v)	1 mg
Ethanol (absolute)	N/A	8 mL
Total	**N/A**	**10 mL**

Note: Prepare fresh before use, and keep protected from light sources while working.

STEP-BY-STEP METHOD DETAILS

Isolation of a neuron-enriched cell fraction from chicken embryos

Timing: 5–6 h

This step describes the detailed protocol for the isolation of a neuron-enriched cell from 48 embryonic chicken eggs.

1. On the 9th day post- fertilization, analyze the eggs by trans-illumination with a white 60 W light bulb to confirm the presence of a viable embryo (Figure 2). Keep the eggs in the incubator until the next day.
2. On the 10th day, prepare the working area as well as the necessary material (see isolation procedure preparation and Figure 3).
3. Spray and wipe the eggs with 70% ethanol to minimize contamination.
4. Isolate the head and collect the brain from embryonic chicken eggs (Methods video S1).
   a. In the laminar flow cabinet, place the egg in the support with the air sack facing upward and, using a pair of scissors, make a hole through the air sack. Use tweezers to increase the size of the hole (Figure 4A).

   Note: Start with the eggs that are apparently not fertilized or possibly non-viable, to check that no viable embryos are present. This will minimize the waiting time of viable cells before the further steps of the isolation protocol.

   b. Locate the embryo and transfer it from the amniotic fluid to a clean Petri dish on ice, using curved tweezers (Figures 4B and 4C).

Note: This step requires some practice. In the beginning, transfer the egg content into a clean Petri dish and transfer the embryo to another clean Petri dish on ice.

c. Identify the head and dissect the embryo brain using a sterile pair of scissors (Figures 4D–4H).
d. With a 1 mL pipette tip and some dissection buffer, clean the dissected brain. Collect it and transfer it into a 50 mL tube containing approximately 10 mL of ice-cold dissection buffer.

5. Upon collection of all the brain tissue, grind them mechanically using a sterile glass Pasteur pipette.

Note: apply some pressure with a finger on top of the glass pipette to avoid the rise of dissection medium.

6. Pellet the tissues by centrifugation at 80 × *g* for 5 min, at room temperature.
7. Discard the supernatant and resuspend the pellet in 4–6 mL of 0.25% trypsin-EDTA solution at 37°C, in a water bath. See troubleshooting 1.
   a. Homogenize the cell suspension by gently swirling the tube.
   b. After 5 min of incubation, homogenize the cellular suspension by pipetting up and down with a 5 mL serological pipette.
   c. Return the 50 mL tube to the water bath.
8. After 10 min of incubation, add 3 volumes of neuron culture medium (at room temperature). See troubleshooting 1.
9. Filter the cell suspension through a 40 μm nylon cell strainer to remove clumps, flushing the strainer with 2–3 mL of medium. See troubleshooting 2.
10. Pellet the cells by centrifugation at 200 × *g* for 10 min. Discard the supernatant.
11. Lyse red blood cells via an osmotic shock.
    a. Add 6 mL of ice-cold RBC lysis buffer and incubate for 8 min on ice.
    b. Dilute the cell suspension with 34 mL of PBS.
    c. Pellet the cells by centrifugation at 270 × *g* for 10 min, at room temperature.
    d. Repeat once, with a 4 min-RBC buffer incubation.
12. After RBC lysis, resuspend cells in *ca.* 16 mL dissection buffer and pass them through a 40 μm nylon cell strainer.

Note: This volume can be adjusted based on the pellet size and the number of falcons that will be used in step 13.

13. Isolate a neuron-enriched cell fraction using a Ficoll density gradient for separation.
    a. Add 4 mL of a Ficoll-PAQUE:PBS (2:1) mixture to a 15 mL tube.

⚠ CRITICAL: The Ficoll-PAQUE:PBS (2:1) mixture should be at room temperature before use.

    b. Gently, add 4 mL of cell suspension on top of the Ficoll-PAQUE:PBS solution.
    c. Centrifuge at 1,500 × *g* for 30 min.

⚠ CRITICAL: The centrifuge acceleration and break should be turned off or, if not possible, set to minimum.

    d. Discard the upper phase and keep the bottom pellet (Figure 5).
    e. Wash the pellet (neuron-enriched cell fraction) with *ca.* 10 mL of neuron culture medium, twice, centrifuging at 270 × *g* for 10 min.

Note: If you want to characterize both isolate fractions by mass spectrometry before culturing, save the upper phase and the pellet, and skip to step 18 (Characterization of the neuron-enriched cell fraction by mass spectrometry proteomics).

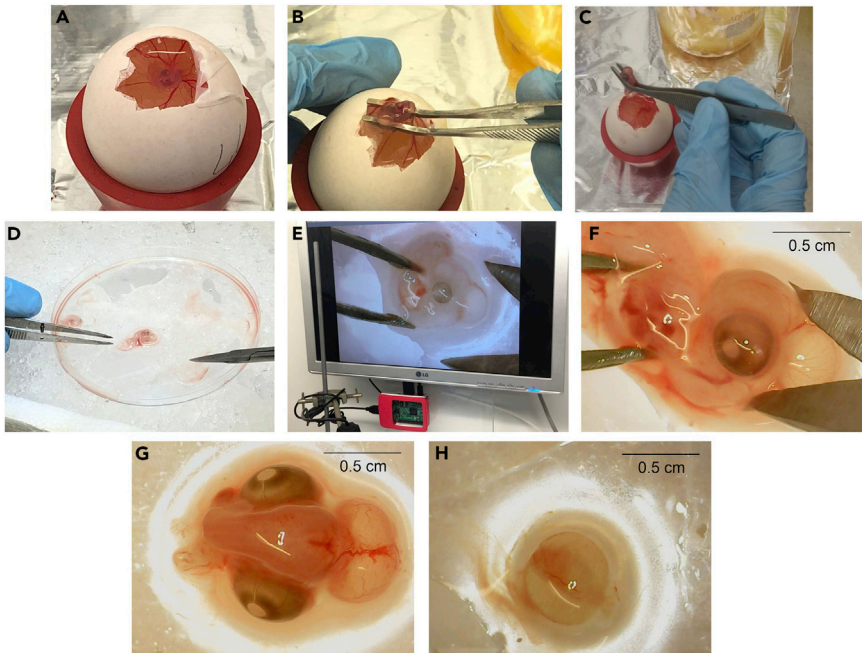

Figure 4. Isolation of the chicken embryo and dissection of its brain

A sequence of illustrative photos of the different steps of the process are presented.

(A–D) (A–C) After opening a hole in the eggshell and through the air sack, identify the embryo and transfer it using curved tweezers to a Petri dish placed on ice (D).

(E–H) Identify the head and dissect the brain. The eye is clearly visible as a large disproportionate darker circle.

(F) On top of the embryo, above the eye, the brain is transparent round organ.

14. Determine cell number and viability using 0.1% trypan blue using a hemocytometer. See troubleshooting 3.
15. Seed the cells at 200 000 cells/cm^2 with 1.8 mL, 400 µL or 50 µL of culture medium per well (6-, 24-, and 96-well PDL-coated plates, respectively; see culture vessel coating). See troubleshooting 4.
16. Maintain cultures at 37°C in a humidified atmosphere of 5% CO_2 in air.
17. On the 2nd and 5th day after seeding, change the culture medium.
    a. Pre-warm the culture medium to 37°C in a water bath.
    b. Remove 50% of the well volume and add equal volume of new culture medium.

⚠ CRITICAL: Since we are working with a neuron-enriched fraction, do not replace the total volume of culture medium, but only 50% of the volume per well, in order to facilitate cell adaptation to the fresh medium.

See troubleshooting 5.

Note: After 5 days, the neuronal network should be established (Figure 6) and the cells are ready for further assays, e.g., cell viability using the resazurin reduction assay, metabolomics, and proteomics.

Characterization of the neuron-enriched cell fraction by mass spectrometry proteomics

Timing: 2 days

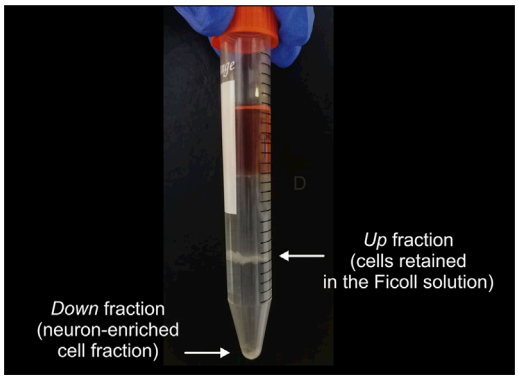

Figure 5. Isolation of a neuron-enriched cell fraction after a Ficoll-Paque:PBS density separation

The resulting pellet is the neuron-enriched cell fraction, while the white interface corresponds to a neuron-poor cell fraction.

After neuron isolation, the two fractions obtained after a density separation can be characterized by mass spectrometry proteomics. The following filter-aided sample preparation (FASP) protocol (Figure 7) was adapted from Carvalho et al. (2020); Wiśniewski (2016); Wiśniewski (2019); Wiśniewski et al. (2009).

18. Make sure all the solutions are ready, including those that must be prepared fresh (see proteomics protocol).
19. Before starting the proteomics work, wash the cells with 0.9% NaCl solution.
    a. Add 0.9% NaCl solution. Adjust the volume according to the pellet size. Using 3 pellet-volumes of NaCl is usually adequate; for example, use 600 µL of NaCl solution to wash a cell pellet with a size *ca.* 200 µL.
    b. Pellet the cells by centrifugation at 270 × *g* for 10 min. Discard the supernatant.

Pause point: If necessary, cells can be stored at this point. Upon the last centrifugation, discard the supernatant and store at −80°C. Samples are stable at −80°C for at least 2 years. Upon thawing, proceed to step 20.

20. Add 400 µL of lysis buffer (0.1 M Tris-HCl pH 7.8, 2% SDS, 0.05 M DTT, and protease inhibitor) to each sample. E.g., 400 µL of lysis buffer to a cell pellet with a size *ca.* 200 µL.

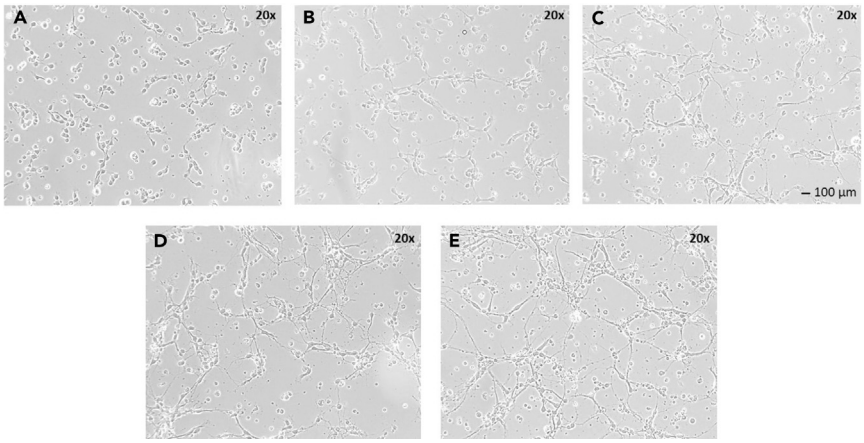

Figure 6. Development of neurons isolated from chick embryo brain

(A–E) Cultured neurons were observed daily under 20 × magnification, after A. 1 day, B. 2 days, C. 3 days, D. 4 days, and E. 5 days after plating. Morphological changes occurred during the growth and differentiation process. Scale bar 100 µm.

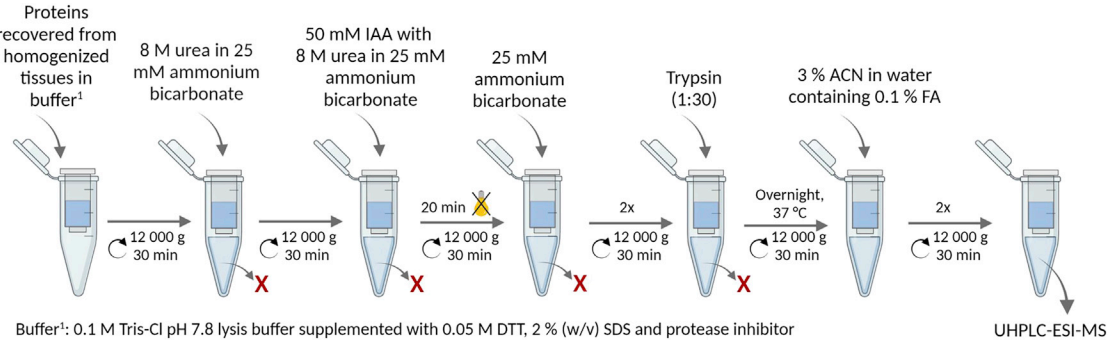

Figure 7. FASP protocol used to prepare samples for proteomic analyses

Samples were prepared according to an optimized FASP (Filter Aided Sample Preparation) protocol. Tissues were homogenized in a 0.1 M Tris-HCl pH 7.8 lysis buffer supplemented with 0.05 M DTT, 2% (w/v) SDS and a protease inhibitor cocktail. After sonication and incubation in a water bath, the supernatant protein content was quantified by a 96-well plate Bradford protein assay, and proteins were loaded on a 3, 10, or 30 KDa Amicon® ultra-centrifugation filter, and washed with an 8 M urea solution prepared in 25 mM ammonium bicarbonate. After centrifugation, the retained proteins were alkylated with iodoacetamide, washed, and digested with trypsin. Peptides were eluted and analyZed by high-resolution mass spectrometry. ACN, acetonitrile; DTT, 1,4-dithiothreitol; FA, formic acid; FASP, Filter Aided Sample Preparation; IAA, iodoacetamide; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride. Image created with BioRender.com.

Note: If necessary, adjust the volume of lysis buffer to avoid low protein concentration. As a general guideline, use 2 pellet-volumes of lysis buffer – for example, 200 µL of lysis buffer to a cell pellet with a size *ca*. 100 µL, or 100 µL of lysis buffer to a cell pellet with a size *ca*. 50 µL. If cells are being counted, use 200 µL of lysis buffer to 1.9 M cells.

21. Sonicate the samples for 2 min, and incubate them at 95°C for 5 min in a water bath.
22. After cooling to room temperature, centrifuge at 16 000 × *g* for 10 min.
23. Collect the supernatant and quantify their protein content using the Bradford quantification assay, using the Bio-Rad Protein Assay Dye Reagent, following the manufacturer's protocol, available online.

Note: It is not possible to use BCA (bicinchoninic acid) assay due to the DTT content of the lysis buffer.

24. Load the intended volume of protein solution into a regenerated cellulose membrane Amicon® ultra-centrifugal filter with a 3 kDa molecular weight cutoff (previously washed with water).

Note: The membrane molecular cut-off can be adjusted to the proteins of interest.

⚠ CRITICAL: Filter membranes should be of regenerated cellulose or equivalent to minimize losses due to protein adsorption.

25. Centrifuge the Amicon® at 12 000 × *g* for 30 min at 20°C.
26. Discard the eluate and wash the retained protein with 200 µL of an 8 M urea solution prepared in 25 mM ammonium bicarbonate (urea base buffer).
27. Centrifuge at 12 000 × *g* for 30 min at 20°C.
28. Discard the eluate and alkylate the retained protein with 100 µL of 50 mM iodoacetamide in 8 M urea and 25 mM ammonium bicarbonate solution.
29. Incubate for 20 min in the dark.
30. Centrifuge at 12 000 × *g* for 30 min at 20°C.
31. Discard the eluate and wash twice the retained proteins with 200 µL of a 25 mM ammonium bicarbonate solution.
32. Transfer the membrane to a new centrifuge microtube.

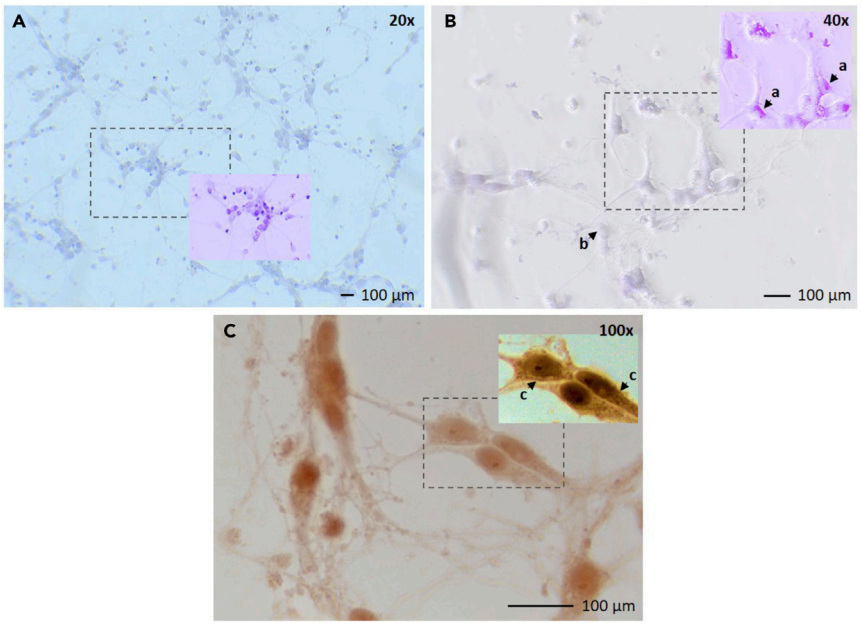

Figure 8. Microscopy images of neurons cultured for 5 days

Different staining dyes were used to identify cell components.

(A) Cells were stained with Mayer's Hematoxylin and Eosin Staining (H&E). Hematoxylin stains cell nuclei in blue, while eosin stains cytoplasm in pink.

(B) Toluidine blue staining has a high affinity for the acidic components of neuronal cells, especially the Nissl bodies. In figure B, neurons display a visible cytoplasm around the nucleus (a) and, in some large pyramidal neurons, two nucleoli (b).

(C) Cells were stained with Congo red, which has a high affinity to fibril proteins. In figure C, some amyloid aggregates (or neurofibrillary tangles) (c) became evident after staining. Cells were observed under 20 ×, 40 × or 100 × magnification, as indicated in each panel. The insets correspond to the dashed areas and were filtered for color enhancement.

33. Add 100 µL of a trypsin solution prepared in 12.5 mM ammonium bicarbonate buffer to each sample. Protease/total protein ratio should be 1 µg of trypsin per 30 µg of total protein content.

Note: Other peptidases can be used.

34. Digest the proteins, overnight (*ca.* 16 h), at 37°C.
35. Sonicate for 2 min, and centrifuge at 12 000 × *g* for 30 min at 20°C.

Note: Do not discard the eluate. Keep it in the collection tube for the following steps.

36. Add 50 µL of a 3% (v/v) acetonitrile in water containing 0.1% (v/v) formic acid to the membrane to elute the remaining peptides by centrifugation at 12 000 × *g* for 30 min at 20°C. Repeat this step once more.

Note: Do not discard the eluate.

37. Collect the eluates from the last two steps (steps 35 and 36) (following the overnight digestion) and analyze by UHPLC-ESI-HRMS or store at −80°C until analysis. Analysis parameters are detailed in SI. See troubleshooting 6.

Characterization of the neuron-enriched cell fraction by cell staining

Timing: 1 h

Neurons can also be characterized by conventional microscopy staining techniques. At least three different staining procedures can be applied: Hematoxylin and Eosin Staining, toluidine blue staining, and Congo red staining (Figure 8).

38. On the 5th day of culture maintenance, fix the cells with 4% paraformaldehyde (dissolved in PBS) for 30 min at 4°C.

Note: Despite having been performed on the 5th day of cell maintenance, staining can be applied at any time after complete cell adhesion. Cells became adherent around 4–8 h after being seeded.

39. Wash the cells with water 3 times.
40. Apply the intended staining.
    a. Mayer's Hematoxylin and Eosin Staining (H&E) (adapted from Patton et al. (2011)) (Figure 8A):
        i. Cover the cells with Mayer's hematoxylin solution for 8 min.
        ii. Differentiate the cells with 1% acid ethanol for 30 s.
        iii. Rinse with saturated lithium carbonate solution for 30 s.
        iv. Rinse with water.
        v. Dehydrate with 95% ethanol – dip the cells for 1 or 2 s in 95% ethanol and let all the ethanol drain and evaporate. Repeat the procedure 10 times.
        vi. Apply eosin Y for 5 min and wash with water.
    b. Toluidine blue staining (adapted from García-Cabezas et al. (2016) and Mohd Lazaldin et al. (2020)) (Figure 8B):
        i. Cover the cells with toluidine blue solution for 5 min.
        ii. Rinse with water.
        iii. Differentiate with 70% ethanol.
        iv. Rinse with water.
    c. Congo red staining (CR) (adapted from Song et al. (2018)) (Figure 8C):
        i. Cover the cells with Congo red solution for 20 min.
        ii. Rinse with water.
        iii. Differentiate with alkaline alcohol solution.
        iv. Rinse with water.
41. Observe the stained cells under the microscope.

EXPECTED OUTCOMES

The present protocol describes the isolation of a neuron-enriched cell fraction obtained from embryonic chicken eggs. The main advantages of this primary cell model include the affordable price, the easy access to the embryonic chicken eggs, with no need of an animal facility, and the small period between the isolation of the neurons and the performance of the experimental assays.

We used this protocol to perform drug toxicity and repurposing assays, in order to study the mechanisms affected by different drug treatments (results unpublished). These neurons were used in viability assays performed with resazurin, and different omics approaches (transcriptomics, metabolomics, and proteomics). Moreover, the performed assays used neurons after 5 days of maintenance (neurons with a well-established neuronal network), and developing neurons (neurons since day 0) in order to study the effect of drugs during the developing of neuronal networks.

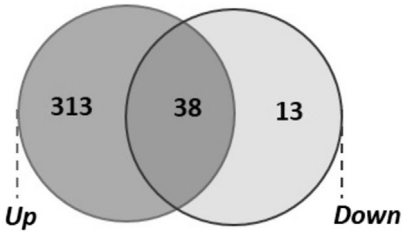

Figure 9. Venn diagram of proteins identified in both fractions recovered during the neuron-enriched cell model procedure

A total of 313 proteins were identified exclusively in the upper fraction (e.g., glial fibrillary acidic protein (GFAP), brain-derived neurotrophic factor (BDNF), and tenascin-R (TN-R)), while 13 were excusive of the lower fraction (e.g., neuropeptide Y receptor type 2 (NPY-2Y), netrin receptor 5 (UNC5), and microtubule-associated protein (MAPT)). A total of 38 proteins were present in both fractions.

Figure 6 shows the morphological changes during the five days that neurons take to establish a neuronal network.

Figure 8 shows microscopy images of stained neurons using Mayer's Hematoxylin and Eosin Staining, toluidine blue staining, and Congo red staining. Mayer's Hematoxylin and Eosin Staining (H&E) is used for differentiation of cellular components: while the nuclei stains blue, the cytoplasm is visible in pink. Toluidine blue staining allows visualizing Nissl bodies, a characteristic neuronal and dendritic presentation of the rough endoplasmic reticulum (García-Cabezas et al., 2016). Upon staining, neurons present a visible cytoplasm around the round or ovoid nucleus. The nucleolus stays purple and can be distinctly large (in larger neurons) or small and be hidden by thick perinucleolar heterochromatin clumps (in small neurons). Some large pyramidal neurons may have two nucleoli. Congo red (CR) is commonly used for amyloid detection due to its binding affinity toward fibril proteins. The existence of amyloid aggregation (or neurofibrillary tangles) inside neurons is consistent with the expected aging of our cell model, evident from the presence of CR-stained deposits in cells at the 5th day post-isolation. This fast aging and consequent apoptosis of cells can be explained by the absence of other neuronal cells with relevant support roles, such as microglia and astrocytes, which contribute to neuronal stability by providing trophic factors, regulating neuroinflammation, and promoting amyloid clearance (Tovar-y-Romo et al., 2014).

Figure 9 shows the number of proteins identified in both isolated cell-fractions. The identification of these proteins allowed concluding that the down fraction (pellet) is, as expected, enriched in neurons, while the up fraction (cells retained in the Ficoll solution) is enriched in microglia, astrocytes, among other cells.

Samples recovered from the cell fraction retained above the Ficoll layer were named fraction *up*, while the neuron-enriched fraction (pellet obtained after Ficoll density separation) was named fraction *down*. Upon analysis of each sample, 351 and 51 proteins were identified in fraction *up* and *down*, respectively. From these, 38 proteins were identified as common proteins between the two fractions.

In fraction *up*, the identified proteins included the glial fibrillary acidic protein (GFAP), the brain-derived neurotrophic factor (BDNF), and tenascin-R (TN-R), all of which are non-neuronal cell markers. GFAP is an intermediate cytoskeleton filament characteristic of astrocytes (Li et al., 2020), and BDNF is expressed by microglial and astroglial cells (Miranda et al., 2019). Tenascin-R is a large extracellular matrix glycoprotein expressed in oligodendrocytes (Probstmeier et al., 2001; Rathjen and Hodge, 2021).

Fraction *down* was characterized by the presence of proteins characteristically expressed in neurons, such as the neuropeptide Y receptor type 2 (NPY-2Y, also known as NPY7R); netrin receptor 5 (UNC5).

Some of the proteins common to both fractions were mainly characteristic of neurons, suggesting that some neurons were retained in fraction *up*. These proteins included, for example, the calmodulin-regulated spectrin-associated protein 2 (CAMSAP2).

QUANTIFICATION AND STATISTICAL ANALYSIS

Cell counting was performed manually.

Protein identification was performed employing a mass spectrometry-based shotgun proteomics approach. Following tryptic digestion, high-resolution mass spectrometry (HRMS) analyses were performed using an Elute UHPLC system coupled to an Impact II QqTOF mass spectrometer with an electrospray ion source. Peptides were separated on a reverse-phase column bioZen™ 2.6 μm Peptide XB-C18 (100 Å, 100 × 2.1 mm), at a constant temperature of 45°C, using a gradient elution at a flow rate of 300 μL/min (mobile phase A: 0.1% (v/v) formic acid in water; mobile phase B: 0.1% (v/v) formic acid in acetonitrile): 0–2 min 0% B, 2–5 min 1% B, 5–60 min 1%–50% B, 60–65 min 50% B, 65–70 min 50%–95% B, 70–78 min 95% B, 78–85 min 95 to 1% B, followed by a 5 min column re-equilibration step. After sample loading, a simple on-line desalting step was implemented in the first 2 min of elution (0% B) – using the mass spectrometer six-port valve, the flow was diverted to the waste, avoiding contamination of the MS instrument. MS acquisition parameters were set as follows: capillary voltage of 4.5 kV (ESI+), with an end plate offset of 500 V, a nebulizer pressure of 2.5 bar, a dry gas (N_2) flow of 8.0 L/min and a heater temperature of 200°C. The tune parameters were set as: transfer funnel 1/2 RF power (400/600 Vpp), hexapole RF power (400 Vpp), ion energy (5.0 eV), low mass (200 m/z), collision energy (7.0 eV), pre-pulse storage (5 μs), stepping (on, basic), collision RF power (200–1,200 Vpp), transfer time (50–110 μs), timing (50%), collision energy (100%–120%). Spectra acquisition was performed in auto MS/MS mode with a threshold of 28 counts per 1,000, cycle time of 3.0 s with exclusion after 1 spectra and release after 0.50 min. All acquisitions were performed with an *m/z* range from 150 to 2,200 and a 2 Hz spectra rate.

Proteomics data analysis was performed with the MaxQuant software v. 2.0.3.0 (Cox and Mann, 2008; Tyanova et al., 2016a) using the internal search engine Andromeda (Cox et al., 2011), followed by a statistical analysis on Perseus v1.6.15.0 with default settings (Tyanova et al., 2016b). Protein databases were retrieved from Uniprot (UniProt Consortium, 2021), restricted to specific groups of proteins from *Gallus gallus* (Proteome UP000000539). Carbamidomethylation of cysteine was set as a fixed modification, whereas oxidation of methionine, protein *N*-terminal acetylation, phosphorylation of serine, threonine and tyrosine, and ubiquitination of lysine were defined as variable modifications. Bruker Q-TOF was selected as instrument and its default parameters were used. Enzyme specificity was set to trypsin/P, allowing cleavages at the carboxyl side of arginine and lysine residues and before proline residues, with a maximum of 2 missed cleavages allowed. The false discovery rate for peptides and proteins was set to 1%, and the minimum score and delta score for modified peptides was set to 0. Match between runs was enabled with 0.7 min match time window and a 20 min alignment window. Dependent peptides were enabled with a fold discovery rate of 1%. Normalized spectral protein label-free quantification (LFQ) intensities were calculated using the MaxLFQ algorithm (Cox et al., 2014), with a minimum ratio count of 1. MaxQuant output data were processed using Perseus v1.6.15.0 with default settings (Tyanova et al., 2016b). After filtering, proteins only identified by site, contaminants, reverse hits were removed. Protein group LFQ intensities were log-2-transformed, and the quantitative profiles were filtered to remove missing values with a minimum valid number of (number of replicates/2)+1 in at least one group. Missing values were replaced with inferred ones, assuming a normal distribution (width 0.3 and down shift 1.8). Log ratios were calculated as the difference in average log 2 LFQ intensity values between the tested conditions (two-tailed, Student's *t* test, *p*-value 0.05 and S0 of 0).

LIMITATIONS

The major limitation to this protocol is the number of viable fertilized eggs.

This protocol was developed with a Ficoll-Paque:PBS solution, using Ficoll-Paque™ PLUS from Cytiva (Cat. 17144002). Other Ficoll preparations were tested; however, the yield of isolation decreased.

TROUBLESHOOTING

Problem 1

Formation of clumps after trypsinization procedure.

After trypsinization, there are clumps in solution (steps 7 and 8 of the step-by-step method details).

Potential solution

Some of the possible causes for this problem include a too long trypsinization step, the use of cold trypsin solution, and an inefficient trypsin inactivation.

Problem 2

Low flow during cell straining. During the filtration of cell suspension, there is a low flow of cell suspension (step 9 of the step-by-step method details).

Potential solution

The low flow could be due to the presence of clumps (troubleshooting 1) that do not cross the cell strainer, or due to the use of an incorrect mesh size.

Problem 3

Decreased isolation yield.

After density separation centrifugation, the yield of isolated neurons is low (step 14).

Potential solution

The low number of cells could be due to a wide range of factors, including a low number of fertilized eggs, different gestation ages, difficulties during the brain dissection step, and low trypsinization yield with consequent cell retention in the cell strainer. Moreover, incorrect preparation of the Ficoll:PBS solution or the use of a different Ficoll preparation can also be associated with low yield of the cell isolation protocol.

Make sure the centrifuge has a swinging-bucket rotor. Moreover, correct equilibration of the rotor is crucial for a correct separation.

Problem 4

Cells fail to adhere.

After seeding, cells take too long to adhere to the culture vessel, or they do not adhere (step 15).

Potential solution

This problem can occur as result of an incorrect coating procedure, as well as an incorrect washing step after the PDL coating.

Problem 5

Cells do not grow or detach easily.

After cell adherence, cells do not establish neuronal networks between them, or detach easily (step 17).

Potential solution

This problem can be caused by a lower number of seeded cells (lower than 200 000 cells/cm^2) that will influence the establishment of neuronal networks, decreasing their viability. In addition, problems during the coating procedure, namely an incorrect washing step after the PDL coating, could

also be involved in this problem. Any possible cell culture contamination by mycobacteria should not be discarded.

Problem 6

No peptides obtained after proteomics protocol.

After sample preparation protocol, no peptides were identified (step 37).

Potential solution

Make sure that the microtube membrane is a low protein binding membrane (e.g., regenerated cellulose or equivalent). In addition, the chromatographic and/or mass spectrometry parameters, as well as the MaxQuant parameters might to be adjusted to the experimental conditions.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Gonçalo Justino (goncalo.justino@tecnico.ulisboa.pt).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Mass spectrometry raw data, in mzXML format, have been deposited at Mendeley data repository and are publicly available as of the date of publication (Mendeley Data: https://doi.org/10.17632/44tg5cm93g.1). Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101753.

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AUTHOR CONTRIBUTIONS

Conceptualization, C.F.M.; methodology, C.F.M., P.F.P.; formal analysis, C.F.M.; investigation, C.F.M.; resources, P.F.P., G.C.J.; writing original draft preparation, C.F.M.; writing – review & editing, P.F.P., G.C.J.; visualization, C.F.M.; supervision, P.F.P, G.C.J.; funding acquisition, G.C.J.

All authors have read and agreed to the published version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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