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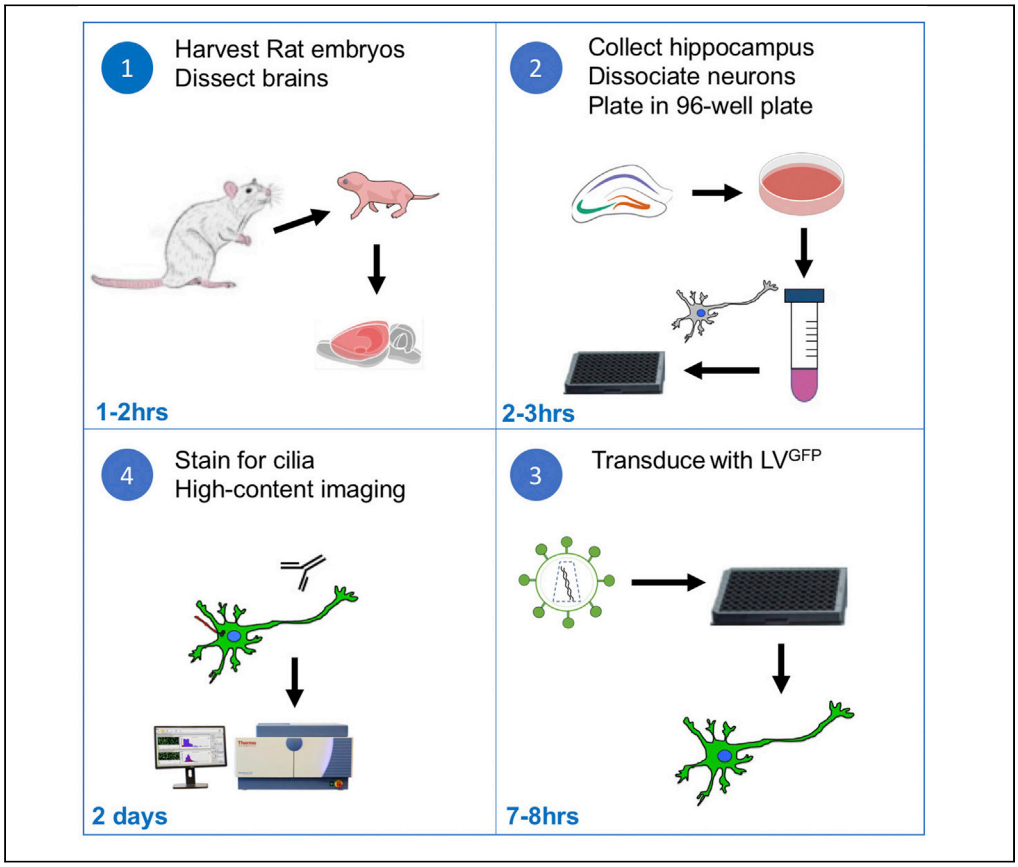
A Cell-Based Assay Optimized for High-Content Cilia Imaging with Primary Rat Hippocampal Neurons



Genetic manipulations of dissociated rodent neurons provide translatable *in vitro* models for disease-driven phenotypes. Cilia are cellular antenna with a role in neuronal maturation and function often perturbed in neurodevelopmental disorders. Efforts for automated imaging of these microscopic protrusions are crucial given the role of cilia in the brain. We developed a cell-based assay to monitor cilia in rat hippocampal neurons using lentiviral-mediated shRNA-based gene editing. This optimized platform can be used for high-throughput cilia imaging, disease modeling, and drug screening.

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HIGHLIGHTS

A protocol for the isolation of rat hippocampal neurons

Lentiviral transduction for gene expression and/or silencing in neurons

Cell-based assay optimized for high-content imaging of neuronal cilia

Adaptable for other assay endpoints (e.g., cell number, size)

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A Cell-Based Assay Optimized for High-Content Cilia Imaging with Primary Rat Hippocampal Neurons

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SUMMARY

Genetic manipulations of dissociated rodent neurons provide translatable *in vitro* models for disease-driven phenotypes. Cilia are cellular antenna with a role in neuronal maturation and function often perturbed in neurodevelopmental disorders. Efforts for automated imaging of these microscopic protrusions are crucial given the role of cilia in the brain. We developed a cell-based assay to monitor cilia in rat hippocampal neurons using lentiviral-mediated shRNA-based gene silencing. This optimized platform can be used for high-throughput cilia imaging, disease modeling, and drug screening.

For complete details on the use and execution of this protocol, please refer to Di Nardo et al. (2020).

BEFORE YOU BEGIN

Day before Procedure

Timing: 15–30 min

1. Plate coating: Coat 96-well plate with poly-D-lysine (PDL) at 20 μg/mL in borate buffer (80 μL/well). Place plate in a 37°C, 5% CO_2 humidified incubator for 16–24 h.

Note: Reagents are dispensed to 96-well plate using filtered tips and multichannel pipettes. Buffers are prepared in sterile reservoirs, consider some extra volume for reservoir dead volume (~0.5 mL) (Figure 1A).

Note: The 96-well plate is placed in the incubator within a chamber to allow CO_2 exchange and with a reservoir filled with sterile water to avoid media evaporation (Figures 1B and 1C).

⚠ CRITICAL: PDL-borate buffer is made fresh before use. Plates are coated with PDL-borate buffer for a minimum of 16–24 h and for up to 2 days.

Note: PDL stocks are made at 1 mg/mL in sterile Milli-Q H_2O, filter sterilized with 0.2 μm membrane filter, aliquoted and stored in small aliquots (1 mL) at −20°C.

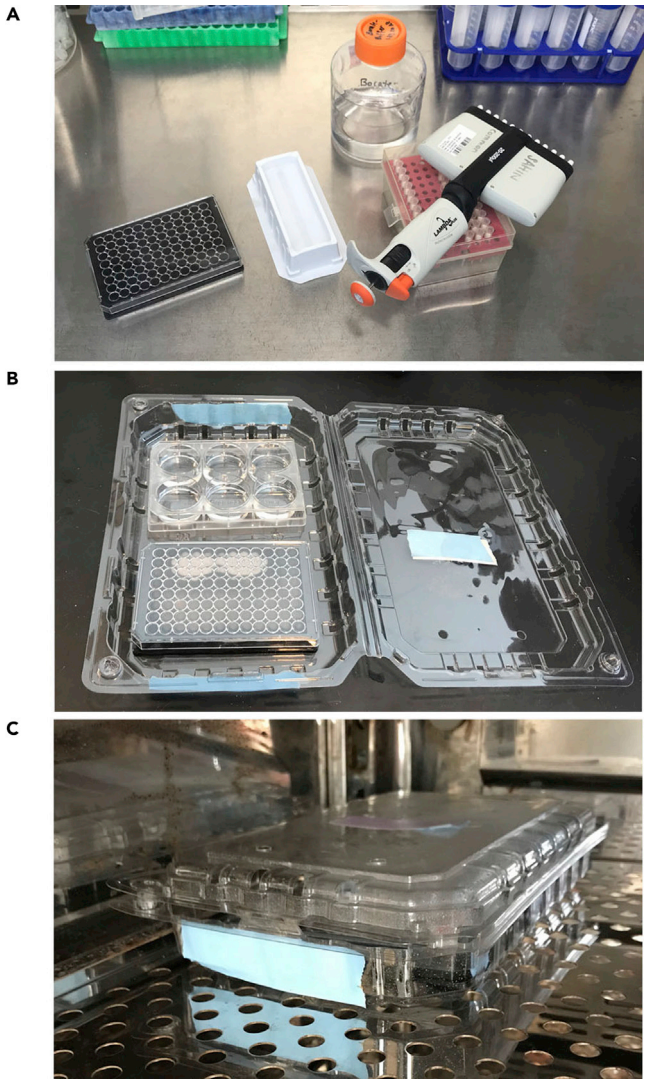

Figure 1. Plate Coating

(A) Image showing material needed for plate coating.

(B) Example of humidified chamber containing a 96-well plate coated with PDL and a 6-well plate filled with sterile water to avoid media evaporation.

(C) Coated plate is placed in a 37°C, 5% CO_2 tissue culture incubator within the humidified chamber containing holes to allow CO_2 exchange.

Borate Buffer

Reagent	Final Concentration	Amount
Boric acid	100 mM	3.09 g
NaCl	75 mM	2.19 g
Sodium tetraborate	25 mM	2.5 g
Sterile Milli-Q water	n/a	Up to 500mL

Note: Dissolve ingredients in sterile Milli-Q H_2O. Use a stir bar and heat (~90°C) to ensure that the boric acid dissolves fully. Adjust pH to 8.4 and autoclave. Filter sterilize by vacuum-filtration with a 0.22 μm membrane filter and store at 4°C (stable for 2–3 months).

Day of Procedure:

Timing: 1–2 h

2. Plate washing: Wash plate four times with sterile Milli-Q H_2O (100 µL/well). Replace last wash with 80 µL/well of plating medium (PM), keep plate in a 37°C, 5% CO_2 humidified incubator until further use. Refer to Key Resources Table/Materials and equipment for complete list of reagents.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-ACIII	Proteintech	Cat# 19492-1-AP; RRID: AB10638445
Chicken polyclonal anti-GFP	Thermo Fisher Scientific	Cat# A10262; RRID: AB2534023
Goat anti-chicken Alexa Fluor-488	Molecular Probes	Cat# A11039; RRID: AB142924
Goat anti-rabbit Alexa Fluor-647	Molecular Probes	Cat# A21244; RRID: AB141663
Bacterial and Virus Strains		
Stbl3 competent cells	Thermo Fisher Scientific	Cat# C737303
Lentivirus	Previously reported (see text for details)	N/A
Experimental Models: Organisms/Strains		
Long Evans rat	Charles River Laboratories	N/A
Experimental Models: Cell Lines		
HEK293T	ATCC	Cat# CRL-3216; RRID: CVCL_0063
Chemicals, Peptides, and Recombinant Proteins		
NaCl	American Bioanalytical	Cat# AB01915
Boric acid	American Bioanalytical	Cat# AB00250
TWEEN 20	Sigma	Cat# P1379
Triton X-100	Sigma	Cat# X100-500ML
BSA, Fraction V	American Bioanalytical	Cat# AB00440
Glycine	Sigma	Cat# G8790
Sodium tetraborate	Sigma	Cat# 221732
Hoechst	Invitrogen	Cat# H3570
Hank's balanced salt solution (w/o Ca^{2+})	Thermo Fisher Scientific	Cat# 14170-112
Neurobasal medium	Thermo Fisher Scientific	Cat# 21103-49
B27 Supplement (50×)	Thermo Fisher Scientific	Cat# 17504-044
Penicillin-streptomycin (10,000 U/mL)	Thermo Fisher Scientific	Cat# 15140-122
L-Glutamine (200 mM)	Thermo Fisher Scientific	Cat# 25030-081
Primocin (50 mg/mL)	Invivo Gen	Cat# ant-pm2
Trypan blue solution	Sigma	Cat# T8154
Hexadimethrine bromide (Polybrene)	Sigma	Cat# H9268
Paraformaldehyde (PFA)	Sigma	Cat# P6148
Papain	Worthington Biochemical	Cat# LS003126
L-Cysteine	Sigma	Cat# C7352

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Poly-D-lysine hydrobromide	MP Biomedicals	Cat# 0210269450
Trypsin inhibitor (MW 14 kDa)	Sigma	Cat# T9253
Methanol	Thermo Fisher Scientific	Cat# A412P-4
Sodium azide (NaN_3)	Sigma	Cat# S2002
$MgCl_2$	American Bioanalytical	Cat# AB01310
Kynurenic acid	Sigma	Cat# K3375
HEPES	Calbiochem	Cat# 391338
KCl	Sigma	Cat# P1767
Na_2HPO_4	Sigma	Cat# S5136
KH_2PO_4	American Bioanalytical	Cat# AB01660
Critical Commercial Assays		
Pure Link Plasmid Kit	Thermo Fisher Scientific	Cat# K21007
Recombinant DNA		
Luciferase-ctrl-sh-GFP	Previously reported (see text for details)	N/A
Other		
96-Well plate	Greiner Bio-One	Cat# 655090
Cell strainer 40 μm	CellTreat	Cat# 229481
Fisherbrand Isotemp Water Bath	Fisher Scientific	N/A
Hemacytometer	Hausser Scientific	Cat# 3200
Arrayscan XTI	Thermo Fisher Scientific	N/A
Reagent reservoir	VistaLab Technologies	Cat# 3054-1006
Dissecting scope	Nikon SMZ645	N/A
Humidified incubator	SANYO MCO-18 AIC (UV) CO_2 Incubator	N/A
0.2 μm membrane filter	EMD Millipore	Cat# SLGPR33RS
0.45 μm membrane filter	EMD Millipore	Cat# SLHVR33RS
6 cm dish	Corning	Cat# 354401
Software and Algorithms		
GraphPad PRISM v8.2.1	GraphPad Software	https://www.graphpad.com/scientific-software/prism/ RRID:SCR_002798
Photoshop CS5.1 v12.1	Adobe	https://www.adobe.com/products/photoshop.html
HCS Studio Cell Analysis Software v6.6.0	Thermo Fisher Scientific	N/A

MATERIALS AND EQUIPMENT

Here is a list of reagents needed.

Plating Medium (PM)

Reagent	Final Concentration	Amount
B27 supplement (50×)	n/a	10 mL
L-Glutamine (200 mM)	2 mM	5 mL
Penicillin-streptomycin (10,000 U/mL)	100 U/mL	5 mL
Primocin (50 mg/mL)	0.1 mg/mL	1 mL
Neurobasal medium	n/a	Up to 500 mL

Note: Filter sterilize by vacuum-filtration with a 0.22-μm filter. PM is warmed up for 15–20 min before use. Store at 4°C (stable for 2 months).

10× Dissociation Medium (10× DM)

Reagent	Final Concentration	Amount
$MgCl_2$ (MW203)	100 mM	10 g
Kynurenic acid (MW189)	10 mM	0.9 g
HEPES (238)	100 mM	12 g
Hank's Balanced Salt Solution (w/o Ca^{2+})	n/a	Up to 500 mL

Note: Adjust pH to 7.2. Filter sterilize by vacuum-filtration with a 0.22-μm filter, make 50 mL aliquots. Store at −20°C (stable for 4–5 months).

1× Dissociation Medium (1× DM)

Reagent	Final Concentration	Amount
10× DM	1×	50 mL
Penicillin-streptomycin (10,000 U/mL)	100U/mL	5 mL
Hank's balanced salt solution	n/a	Up to 500 mL

Note: Filter sterilize by vacuum-filtration with a 0.22-μm filter. Prepare 1× DM fresh the day of Procedure, precool on ice.

3 mM L-Cysteine Solution

Prepare fresh the day of procedure. Add a few grains (~3.5 mg) of L-Cysteine to 10 mL of 1× DM in a 15 mL conical tube. Adjust pH by color adding 1 N NaOH (until solution turns pink, same color as 1× DM). Place in water bath at 37°C until further use.

0.9 M Trypsin Inhibitor

Prepare fresh the day of procedure. Dissolve 0.25 g of trypsin inhibitor to 20 mL of 1× DM in a 50 mL conical tube. Adjust pH by color by adding 1 N NaOH (until solution turns pink, same color as 1× DM). Place in water bath at 37°C until further use.

NB-Wash

Reagent	Final Concentration	Amount
Penicillin-streptomycin (10,000 U/mL)	100 U/mL	5 mL
Neurobasal medium	n/a	Up to 500 mL

Note: Filter sterilize by vacuum-filtration with a 0.22-μm filter. NB-wash medium is warmed up for 15–20 min before use and it is stored at 4°C (stable for 4 months).

10× PBS

Reagent	Final Concentration	Amount
NaCl (MW 58.4)	1.4 M	80 g
KCl (MW 74,5)	27 mM	2 g
Na_2HPO_4 (MW 142)	101 mM	14.4 g
KH_2PO_4 (MW 136)	18 mM	2.4 g
Milli-Q H_2O	n/a	Up to 1 L

Note: Adjust to pH 7.4. Autoclave and store at 20°C–25°C. Stable for 2–3 months.

1× PBS

Reagent	Final Concentration	Amount
10× PBS	n/a	100 mL
Milli-Q H_2O	n/a	Up to 1 L

Note: Store at 20°C–25°C, stable for 2–3 months.

PBT

Reagent	Final Concentration	Amount
Tween 20	0.05%	0.5 mL
10× PBS	n/a	100 mL
Milli-Q H_2O	n/a	Up to 1 L

Note: Store at 20°C–25°C stable for 2–3 months.

PBT-Glycine

Reagent	Final Concentration	Amount
Glycine (MW 75)	50 mM	0.16 g
1× PBT	n/a	Up to 40 mL

Note: Make fresh on the day of immunofluorescent staining (prepare 40 mL/plate). Filter sterilize with 0.2 μm membrane filter.

PBT-Block Solution

Reagent	Final Concentration	Amount
BSA	2%	0.4 g
PBT-glycine	n/a	Up to 20 mL

Note: Make fresh on the day of immunofluorescent staining (prepare 20 mL/plate). Filter sterilize with 0.45 μm membrane filter.

Paraformaldehyde (PFA)

Reagent	Final Concentration	Amount
PFA	4%	4 g
10× PBS	n/a	10 mL
Milli-Q H_2O	n/a	Up to 100 mL

Note: Dissolve PFA in 90 mL of sterile Milli-Q H_2O. Stir and heat to ~50°C, add 1–2 drops of 1 N NaOH until solution clears. Add 10 mL of 10× PBS, filter sterilize by vacuum-filtration with a 0.22 μm membrane. Cool and dispense in 10 mL aliquots. Store at −20°C (stable for 3–4 months).

⚠ CRITICAL: PFA is toxic and corrosive. Prepare under a chemical hood and dispose in appropriate biohazard waste.

STEP-BY-STEP METHOD DETAILS

Preparation of Rat Hippocampal Cultures

Timing: 3–4 h

This step describes isolation and dissociation of hippocampi from rat brains at embryonic day 18 (E18). All experimental procedures were done in agreement with animal protocols approved by the Institutional Animal Care and Use Committee at Boston Children's Hospital.

Note: This protocol is an optimization of a previous protocol (Di Nardo et al., 2014)

1. Collect rat embryos to 10 cm dish containing 1× DM (Figures 2A and 2B). Peel away skin and skull using forceps under dissecting scope and collect brain. Transfer brain to fresh 1× DM in 6 cm dish on ice, collect brain for each embryo.
2. Transfer brain to fresh 1× DM in 10 cm dish (Figure 2C). Under dissecting scope grasp meninges from brain with sterile forceps and gently pull it away (see Troubleshooting 1). Separate hemispheres from midbrain and hindbrain. Isolate hippocampi and transfer to a 6 cm dish on ice containing 5 mL of fresh 1× DM in (Figure 2D). Repeat for each brain.
3. Add 25–30 units/mL papain to 10 mL solution of 3 mM L-Cysteine, mix and place in water bath at 37°C for a few minutes.
4. Transfer 5 mL of hippocampi to a 15 mL conical tube using a 10 mL pipette and let them settle to the bottom of the tube (Figure 2E). Filter sterilize papain/L-Cysteine with 0.2 μm membrane into a 50 mL conical tube.
5. Remove 4 mL of 1× DM from hippocampi, add 5 mL papain/L-Cysteine, mix by inverting tube and place in water bath at 37°C for 5 min.
6. Filter sterilize 20 mL of trypsin inhibitor with 0.2 μm membrane into fresh 50 mL conical tube and place in water bath. Remove papain/L-Cysteine from hippocampi using a 10 mL pipette, add 5 mL trypsin inhibitor. Mix by inverting the tube, remove trypsin inhibitor. Add 5 mL fresh trypsin inhibitor. Mix and place in water bath at 37°C for 5 min. Repeat three times.
7. Remove last trypsin inhibitor wash, add 5 mL warm PM and gently triturate with a 10 mL pipette until all clumps have disappeared. Add 5 mL warm PM for a total volume of 10 mL (Figure 2F). Filter the hippocampal culture through a cell strainer with pore size of 40 μm.
8. Neuron counting: Dilute culture 1:5 in PM (make 50 μL). Aliquot 10 μL of 0.4% Trypan Blue solution to an Eppendorf tube, add 10 μL of diluted neurons, mix by pipetting up/down and incubate for 3 min. Transfer 10 μL to hemocytometer and count viable (clear) neurons under light microscope. Do not count unhealthy (blue) neurons. Count 4 squares and calculate the number of viable cells using the following formula: [4 squares × 2 × 5]/400 = $n \times 10^6$ neurons/mL. For accurate estimate of neuronal counts make three different neuronal dilutions.
9. Neuronal plating: Dilute culture to 5×10^5 cells/mL with PM (make 6 mL). Add 50 μL neuron culture to wells of 96 plate containing 80 μL of PM/well, gently mix by pipetting up/down. Final neuronal density is 25,000 neurons/well. Tap plate to homogenously disperse neurons in the wells and place in a 37°C, 5% CO_2 humidified incubator. Dissociation/plating day is considered DIV0.

⚠ CRITICAL: Sterility is critical when preparing primary neuronal cultures. Sterilize dissecting scissors and forceps with ethanol before use, dry, and keep aside. Steps 1 and 2 are performed on the bench/dissecting microscope wearing a mask to prevent contamination, ensure sterile environmental conditions. Steps 3–9 are performed under a laminar flow tissue culture hood.

⚠ CRITICAL: Trypan blue is a cell-membrane impermeable dye used as exclusion test to determine cell viability. It is important not to leave neurons in trypan blue staining solution for prolonged times as it might result to cell death and inaccurate cell counting.

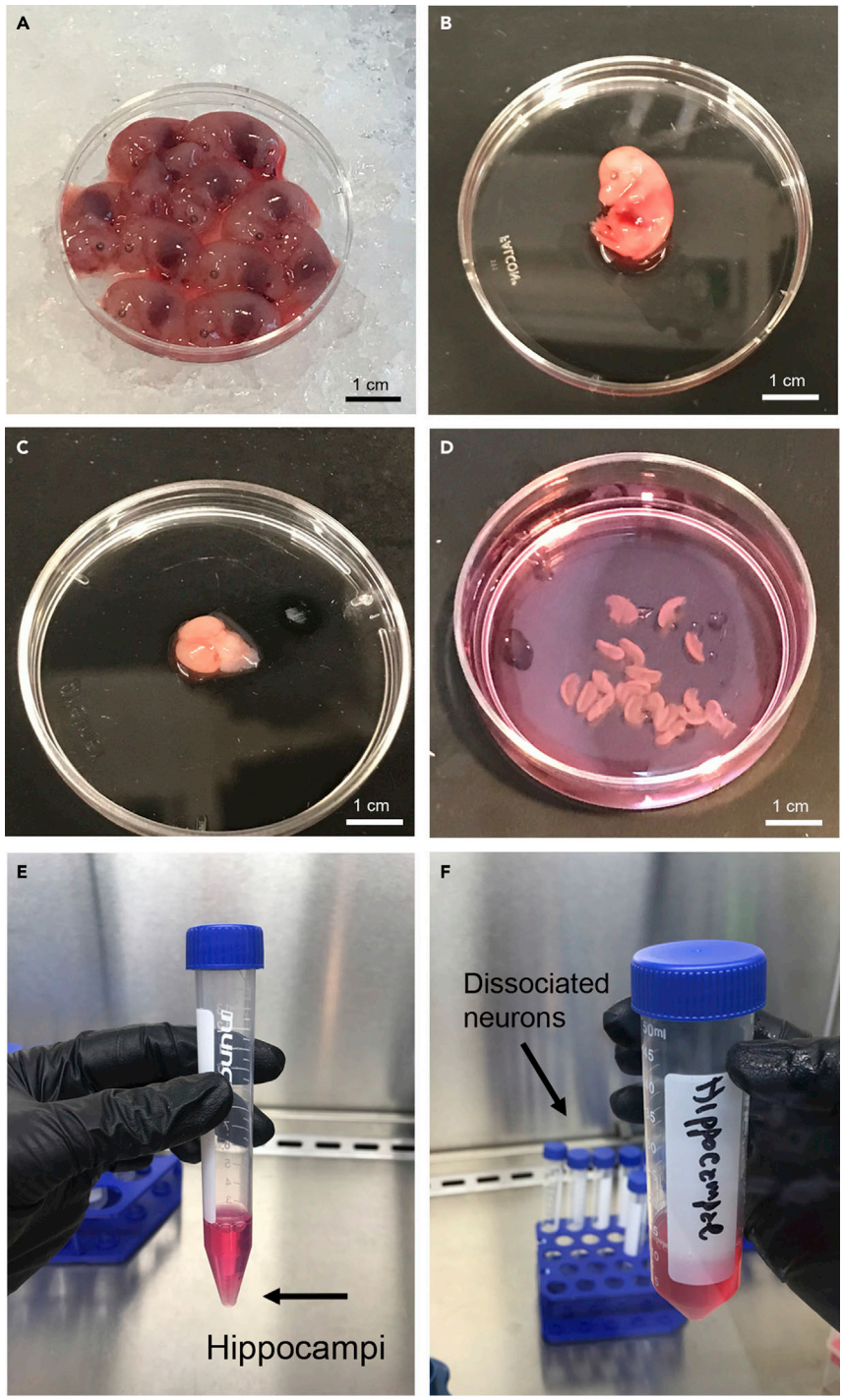

Figure 2. Overview of Hippocampal Dissociation

Rat embryos collected at E18 (A). Each embryo is transferred to a fresh dish (B), brain is removed (C). Hippocampi are collected in a 6 cm dish (D), transferred to a conical tube (E), dissociated by papain and resuspended in plating medium (F).

⚠ CRITICAL: For cilia imaging, neurons are plated at 25,000/well in a 96 well plate (Figures 3A and 3B). However, neuronal density can range between 10,000–30,000 neurons/well. Densities lower than 10,000 neurons/well are not recommended as sparse neuronal

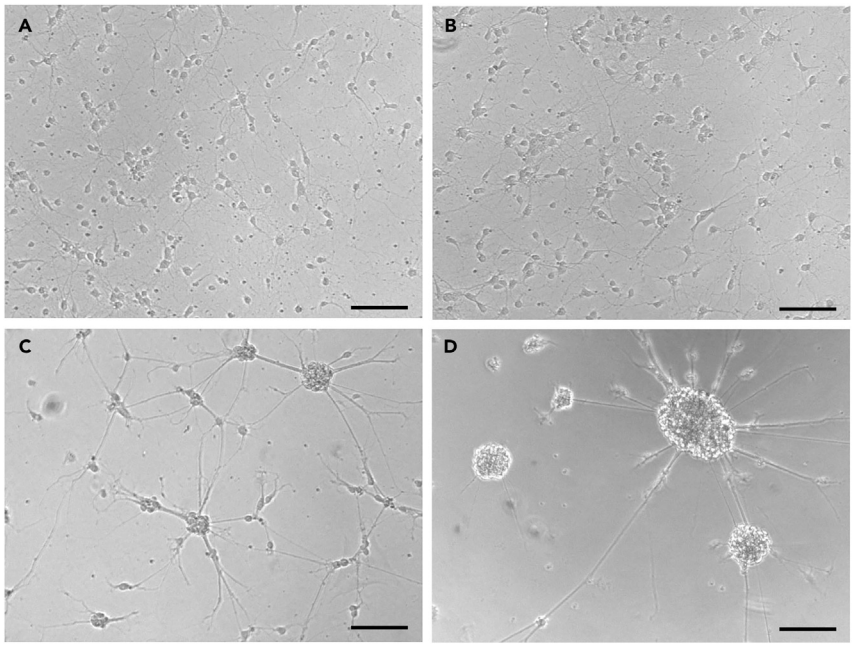

Figure 3. Neuronal Plating for Cilia Imaging

Representative phase contrast images showing DIV13 cultures of healthy neurons plated at a single-cell density optimal for cilia imaging (A and B), sparse neurons with several clumps (C) and dead neurons with high cell clumping (D). Scale bar, 100 μm.

cultures will result to clump formation and cell death (Figures 3C and 3D; see Troubleshooting 2 for tips on how to monitor healthy neuronal cultures).

Note: When plating the neurons use gentle pipetting and do not generate aerosol as it will cause cell damage.

Pause Point: Dissociated neuronal cultures can be stored on ice for up to 4–5 h before plating. Longer pause is not recommended as it may decrease neuronal viability.

Lentiviral Transduction of Neurons

Timing: 7–8 h

This step describes how to transduce DIV1 rat hippocampal neurons with lentivirus expressing Luciferase- control shRNA tagged with green fluorescent protein (LV^{GFP}) (Di Nardo et al., 2020).

10. Thaw LV particles in water bath, prepare 1:1 dilution of LV (Vol/Vol) in NB medium in the presence of polybrene at 0.6 μg/mL. Transduce neurons by replacing PM medium with diluted virus at 70 μL/well in a 37°C, 5% CO_2 humidified incubator.
11. After 6 h, wash neurons twice with NB-wash medium at 100 μL/well. Replace last wash with 130 μL of PM medium.
12. Change PM medium every 3–4 days; keep neurons in culture until DIV13.

⚠ CRITICAL: Primary neuronal cultures are extremely delicate. Limit the time outside the incubator to af minimum and make sure to have all reagents/equipment ready under the sterile laminar tissue culture hood before procedure.

⚠ CRITICAL: LV are biosafety level 2 material and need to be handled with safety precautions. De-contaminate any labware that touched LV (tips, Eppendorf tubes, conical vials) by soaking in 30% bleach. Incubate for at least 20 min before discarding in biohazardous waste. After infection, LV particles are recovered from neurons with multichannel pipette and soaked in reservoir containing 30% bleach before discarding in biohazardous waste.

Optional: LV-transductions can also be performed at later neuronal ages in culture (DIV2–5) and/or extended for prolonger times (up to DIV20).

Note: LV particles are stored in small aliquots at −80°C. When preparing dilutions avoid thawing too much virus, repeated thawing/freezing is not recommended as it will decrease transduction efficiency.

Pause Point: LV-transductions should be performed for a minimum of 6 h and can last for up to ~10 h. Longer incubation times are not recommended as they might result in neuronal cell death.

Immunofluorescent Staining Protocol

Timing: 2 days

This step describes fixation and immunofluorescent staining to monitor cilia by high-content image-based assay (cilia^{HCA}) in neuronal cultures transduced with LV^{GFP} (Di Nardo et al., 2020).

13. Day 1 (timing 3–4 h): Before Procedure, set up a reservoir filled with 6 mL of 100% ice-cold methanol (MeOH) on ice. Under chemical hood prepare a reservoir containing 6 mL of 1× PBS and a reservoir with 6 mL of freshly thawed 4% PFA at 20°C–25°C.
    a. Transfer plate with neurons to chemical hood, replace PM medium with 4% PFA and incubate for 10 min at 20°C–25°C. Replace fixative with 1× PBS.

Note: Unless otherwise specified, all reagents for immunofluorescent staining are aliquoted to the neurons at 50 µL/well using filtered tips with a multichannel pipette.

Note: 100% MeOH is stored at −20°C and precooled on ice before use.

⚠ CRITICAL: PFA is toxic and corrosive, it needs to be disposed as hazardous waste in an appropriate waste container.

⚠ CRITICAL: Incubations of plates with neurons expressing fluorescent proteins are performed in a dark container.

Pause Point: Once fixed, neurons can be kept for up to 2 weeks in 200 µL of 1× PBS with 0.05% Sodium Azide (NaN_3). Wrap plate with parafilm and store at 4°C.

Optional: For cilia imaging we typically fix the neurons at DIV13 however, assay endpoint can vary anywhere between DIV5–20 (Di Nardo et al., 2020).

    b. Transfer plate on ice, replace 1× PBS with ice-cold 100% MeOH, incubate for 10 min. Invert plate to quickly remove MeOH, dry plate by tapping on clean tissue, and incubate with PBT-glycine for 5 min at 20°C–25°C. Wash with PBT-glycine two times.
    c. Replace PBT-glycine with PBT-block solution, incubate for 90 min at 20°C–25°C. Prepare primary antibody solution with anti-rabbit-adenylyl cyclase III [(ACIII) cilia marker antibody, diluted 1:700] and anti-chicken-GFP (LV-expressing neurons marker, diluted 1: 1,000) in

PBT-block (make 6 mL). Replace PBT-block with antibody solution, wrap plate with parafilm and store at 4°C for 16–24 h.

14. Day 2 (timing 3 h)
    a. Wash neurons with PBT buffer three times for 5 min at 20°C–25°C. Prepare secondary antibody solution by diluting anti-rabbit Alexa-647 and anti-chicken Alexa-488 fluorescently labeled secondary antibodies diluted at 1:1,000 in PBT-Block (make 6 mL). Incubate neurons with secondary antibody solution for 90 min at 20°C–25°C (see Troubleshooting 3, Figure 4).
    b. Replace secondary antibody mix with Hoechst dye diluted 1:5,000 in PBS, incubate for 5 min at 20°C–25°C. Wash neurons three times with PBT buffer and two times with 1× PBS. Replace last wash with 200 µL of 1× PBS with 0.02% sodium azide (NaN_3).

Pause Point: Plates can be imaged right away or within to 2–3 weeks after staining. Store plate in a dark container at 4°C wrapped in parafilm.

Note: Unless otherwise specified, reagents are always aliquoted at 50 µL/well with filtered tips using a multichannel pipette.

Note: Keep track of antibody lot numbers as staining efficiency can vary from batch to batch (see Troubleshooting 3; Figure 4).

Note: Fluorescently labeled secondary antibodies are stored in small aliquots at −20°C. Once thawed, they are kept at 4°C.

High-Content Cilia Imaging

Timing: 14–18 h

This step describes the setup for cilia imaging using the high-content platform Cellomics Arrayscan XTI (Thermo Fischer Sci).

15. Cilia are imaged on 80 fields of view per well (20% of the well, average scan time: 12–14 h/96-well plate) using a 40× air objective (Numerical aperture = 0.75). Nuclei are detected by Hoechst staining at 386 nm emission for 30 ms, the LV-transduced neurons are detected using GFP staining at 485 nm emission for 20 ms at a 1.9 µm Z offset above the focal plane of the nuclei, and cilia are detected using ACIII staining at 647 nm emission for 130 ms. Cilia are imaged using the "image projection tool" which allows the acquisition of a stack of images at three different focal planes with a step size of 1.9 µm/step for a total of 5.7 µm (Figure 5A).
16. Objects are identified using the spot detector algorithm of the HCS Studio Cell Analysis Software. Hoechst staining is used to draw a circular mask for nuclei identification and for the region of interest (ROI) for the subsequent channels. GFP^+ spots are identified using a circular mask of the size of the nuclei. $ACIII^+$ spots are identified by a circular mask with a diameter of 32 µm drawn from the nuclei ROI. Border objects at the border of the well are excluded (Figure 5B).

⚠ CRITICAL: Gene knockdown by LV-mediated shRNA in primary neurons is more efficient than transient transfection methods (Karra and Dahm, 2010). However, when monitoring ciliation after gene knockdown it is critical to analyze the cilia in the shRNA transduced neurons (LV-GFP^+ neurons). The Spot detector algorithm available with the HCS Studio Cell Analysis Software allows the detection of subcellular structures and their co-localization. A positive event should be considered a spot detection in both the GFP and ACIII channel. The Spot detector algorithm allows the quantification of cilia number, but it does not quantify cilia length.

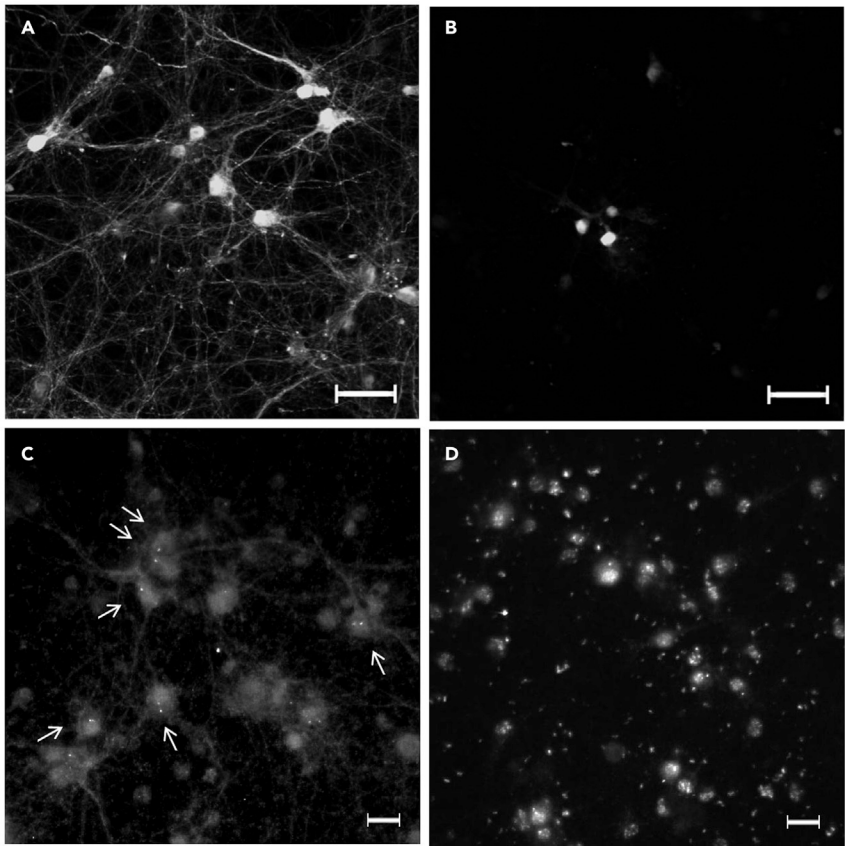

Figure 4. Immunofluorescent Staining of the Neurons

Representative images showing a culture with good (A) and bad (B) detection of GFP positive neurons and good (C) and bad (D) detection of cilia stained with ACIII antibody. White arrows in (C) indicate cilia. Scale bars: 40 μm in (A) and (B) and 25 μm in (C) and (D).

Note: Other than fluorescent tags, alternative markers that could be used for the identification of the shRNA transduced neurons include the use of lentiviral particles co-expressing non-fluorescent tags followed by antibody staining of the tag (e.g., HA, Myc).

Note: The reason for choosing a 32 μm circular mask is that it allows the detection of spots in the perinuclear region of the cell body which is where the $ACIII^{+}$ cilia are typically observed. The 32 μm diameter is drawn from the nuclei ROI to ensure the inclusion of cilia in certain disease models with enlarge some size (Di Nardo et al., 2020).

Note: Use Hoechst staining for focal plane acquisition of the well, then adjust Z offset for optimal resolution of neurons and cilia at 485 nm and 647 nm, respectively (see Troubleshooting 4 for tips on how to overcome out of focus imaging).

EXPECTED OUTCOMES

A litter of 8–10 rat E18 embryos usually yields ~ 35 M of a relatively pure population of hippocampal neurons. The use of Neurobasal medium supplemented with B27 supports neuronal survival, and it minimizes the presence of other contaminating brain cell types. Neurons appear round and sparse at DIV1; by DIV4–5 they show enlarged cell bodies and neurites begin to be visible. Plate coating with 20 μg/mL PDL in borate buffer provides an optimal substrate for

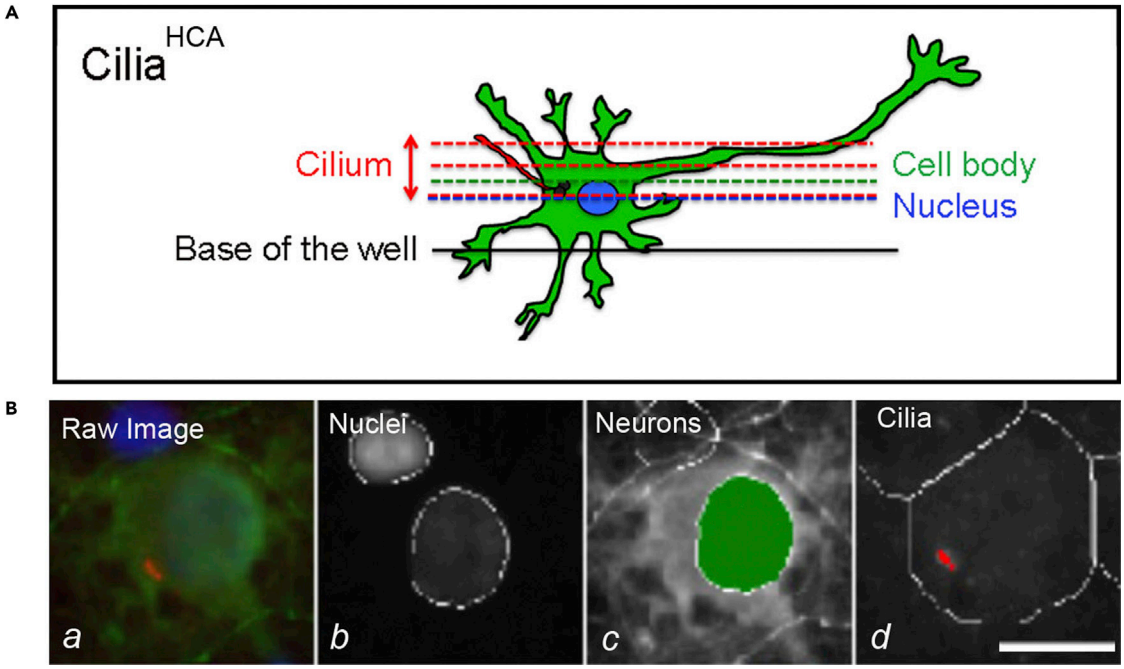

Figure 5. Algorithm for High-Content Cilia Imaging

(A) Overview of focal planes used for the cilia^{HCA}.

(B) Raw image (*a*) of hippocampal neuron transduced with GFP-tagged lentivirus (in green) stained with the adenylyl cyclase III (ACIII, cilia marker in red) and with Hoechst (nuclei in blue). Identification of nuclei (grayscale in *b*), infected neurons (green object in *c*), and cilia (red spot in *d*). White masks show the region of interest (ROI) for object identification. Image acquisition for the cilia^{HCA} was performed at 40×. Scale bar, 25 μm.

neuronal adhesion, dendritic arbor formation and maturation. Healthy hippocampal cultures at DIV13 appear 60%–70% confluent; neurites are overlapping and have formed neuronal connections (Figures 3A and 3B).

LV transduction is one of the most efficient approaches for gene manipulation of primary neuronal cultures (Karra and Dahm, 2010). In our experimental conditions, we typically obtain ~70%–80% of transduced neurons as assessed by GFP-tag expression. However, transduction efficiency may vary depending on the health status of the neuronal cultures, LV titer, targeted gene, or type of manipulation (i.e., gene overexpression versus gene silencing).

Cilia are microscopic cellular protrusion 3–5 μm long and 0.2 μm wide, thereby high-resolution cilia imaging might be a challenging task (Sarkisian and Semple-Rowland, 2019). The image projection tool of the Arrayscan XTI allows the acquisition of a stack of images particularly useful for identification and accurate reconstruction of small objects. By using this imaging setup in our cilia^{HCA}, we were able to successfully monitor ACIII^{+} cilia in primary neurons despite the lack of a confocal mode (Figure 6). Using our cilia^{HCA} we monitored ciliation in primary neurons over a time course of 20 days in culture. Our data showed that neurons start to form cilia around DIV5. By DIV13, approximately 30% of neurons become ciliated, and they remain stable until about DIV20 (Di Nardo et al., 2020).

LIMITATIONS

The dissociation protocol described here has been optimized for hippocampal neurons, and it is not intended for other cell types. Both methodology and reagents should be revised if used for other culture systems.

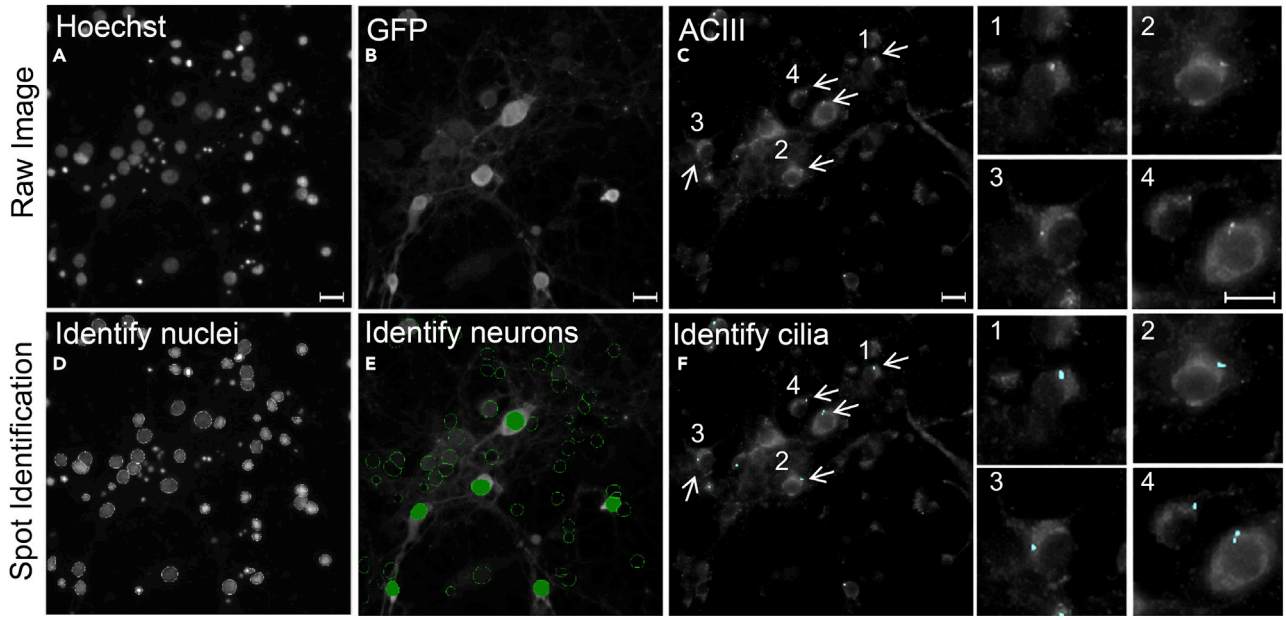

Figure 6. High-Content Cilia Imaging

Raw images (A–C) and spot detector algorithm identification (D–F) of rat hippocampal neurons stained at DIV13 with Hoechst (nuclei A and D), GFP (LV-infected neurons B and E), and ACIII (cilia, C and F). Nuclei are identified by white masks (D), neurons are identified by green spots (E), cilia are in light blue (F). Numbers 1–4 (C and F) indicate cilia that are enlarged in the zoom panels on the right. Image acquisition was performed with a 40× objective of the Arrayscan XTI. Scale bar, 25 μm.

A minor contamination of astrocytes (5%–10%) is expected when using E18 rat embryos. However, performing this protocol with newborn rats (P1-P2) will likely increase astrocytes contamination. The dissociation of embryos at earlier ages (E16-E17) will result in a lower neuronal yield.

Our cilia cell-based assay has been developed to monitor ciliation in post-mitotic hippocampal neurons. If used for other cell types, consider some adaptation (i.e., for dividing cells consider adjusting cell doubling times, confluency, and growth conditions).

TROUBLESHOOTING

Problem 1

Incomplete removal of the meninges and high contamination of astrocytes (step 2).

Potential Solution

Contaminating astrocytes might arise if meninges are not fully removed. Under these circumstances, try inhibiting astrocyte growth by adding the anti-mitotic agent cytosine arabinoside (AraC, 5 μM) to the hippocampal culture between DIV2–4. Alternatively, start over with the procedure, make sure to fully remove meninges from the brains of the embryos when preparing the culture and filter the dissociated neurons through a 40 μm pore size cell strainer before plating.

Problem 2

Sparse neuronal culture and clump formation (step 9).

Potential Solution

A potential cause of unhealthy neuronal cultures and clump formation (Figures 2C and 2D) could be low neuronal plating density, inefficient coating of the 96-well plate with PDL-borate or incomplete PDL removal from the wells. Under these circumstances, start over. Make sure that neuronal counts

are accurate, add enough PDL-borate to wells and wash it out completely with sterile Milli-Q water. It is also recommended not to freeze/thaw PDL stocks and make fresh PDL-borate right before use.

Problem 3

Low GFP fluorescence signal or poor cilia detection ((step 13); Figures 4B and 4D).

Potential Solution

Low GFP antibody staining could be due to inefficient immunofluorescent staining with either primary or secondary antibodies. Try repeating the staining using higher concentration of primary antibodies and a fresh aliquot of secondary antibody. Before repeating this step, neurons should be rinsed in PBS a couple of times to wash out the NaN_3. It is not necessary to repeat the incubation in PBT-block solution. Poor cilia detection could result from inefficient PFA fixation and/or MeOH permeabilization of the neurons. Under these circumstances, start over using freshly made PFA and precooled MeOH, and make sure that all incubations are performed as indicated in the protocol. If none of the above helps to solve the problem, try new lots of ACIII and GFP antibodies.

Problem 4

Failure in focusing and loss of image acquisition with the 40× objective (step 15).

Potential Solution

The Arrayscan XTI uses the nuclei staining at 386 nm emission for focal plane acquisition. Hoechst staining is usually very robust; however, the 40× objective is particularly sensitive to the presence of dirty objects or too sparse nuclei in the well. Therefore, out of focus imaging could be a problematic issue. It is highly recommended to use filtered tips throughout the protocol to avoid presence of small debris in the wells. In addition, perform imaging with an intra-well autofocus interval of one (refocus in each subsequent well) and adjust focal planes of each channel to the best optimal resolution. Once optimized, keep Z offsets the same throughout the scan.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Mustafa Sahin (mustafa.sahin@childrens.harvard.edu).

Materials Availability

All unique reagents generated in this study are available from the Lead Contact with a complete Material Transfer Agreement.

Data and Code Availability

All software used in this study are listed in the Key Resources Table. This study did not generate new datasets.

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AUTHOR CONTRIBUTIONS

A.D.N. designed the protocol, performed experiments, and wrote the manuscript. S.V. performed experiments. M.S. reviewed the manuscript.

DECLARATION OF INTERESTS

M.S. reports grant support from Novartis, Roche, Pfizer, Biogen, Ipsen, LAM Therapeutics, Astellas, Bridgebio, Aucta and Quadrant Biosciences unrelated to this project. He has served on Scientific Advisory Boards for Sage, Roche, Aeovian, Celgene, and Takeda. M.S. and A.D.N. have a patent pending on this work.

REFERENCES

Di Nardo, A., Lenoel, I., Winden, K.D., Ruhmkorf, A., Modi, M.E., Barrett, L., Ercan-Herbst, E., Venugopal, P., Behne, R., Lopes, C.A.M., et al. (2020). Phenotypic screen with TSC-deficient neurons reveals heat-shock machinery as a druggable pathway for mTORC1 and reduced cilia. Cell Rep. *31*, 107780.

Di Nardo, A., Wertz, M.H., Kwiatkowski, E., Tsai, P.T., Leech, J.D., Greene-Colozzi, E., Goto, J., Dilsiz, P., Talos, D.M., Clish, C.B., et al. (2014). Neuronal Tsc1/2 complex controls autophagy through AMPK-dependent regulation of ULK1. Hum. Mol. Genet. *23*, 3865–3874.

Karra, D., and Dahm, R. (2010). Transfection techniques for neuronal cells. J. Neurosci. *30*, 6171–6177.

Sarkisian, M.R., and Semple-Rowland, S.L. (2019). Emerging roles of primary cilia in glioma. Front. Cell Neurosci. *13*, 55.