Protocol

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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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Protocol
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% CO₂ 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 CO₂ 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 H₂O, filter sterilized with 0.2 μm membrane filter, aliquoted and stored in small aliquots (1 mL) at −20°C.
Note: Dissolve ingredients in sterile Milli-Q H$_2$O. 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 H₂O (100 μL/well). Replace last wash with 80 μL/well of plating medium (PM), keep plate in a 37°C, 5% CO₂ 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# A801915 |
| Boric acid | American Bioanalytical | Cat# A800250 |
| TWEEN 20 | Sigma | Cat# P1379 |
| Triton X-100 | Sigma | Cat# X100-500ML |
| BSA, Fraction V | American Bioanalytical | Cat# A800440 |
| Glycine | Sigma | Cat# G8790 |
| Sodium tetraborate | Sigma | Cat# 221732 |
| Hoechst | Invitrogen | Cat# H3570 |
| Hank’s balanced salt solution (w/o Ca²⁺) | Thermo Fisher Scientific | Cat# 14170-112 |
| Neurobasal medium | Thermo Fisher Scientific | Cat# 21103-49 |
| B27 Supplement (50x) | 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# L5003126 |
| L-Cysteine | Sigma | Cat# C7352 |

(Continued on next page)
**MATERIALS AND EQUIPMENT**

Here is a list of reagents needed.

| 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 (NaN₃) | Sigma | Cat# S2002 |
| MgCl₂ | American Bioanalytical | Cat# A801310 |
| Kynurenic acid | Sigma | Cat# K3375 |
| HEPES | Calbiochem | Cat# 391338 |
| KCl | Sigma | Cat# P1767 |
| Na₂HPO₄ | Sigma | Cat# S5136 |
| KH₂PO₄ |American Bioanalytical | Cat# A801660 |

**Critical Commercial Assays**

| Critical Commercial Assays | SOURCE | IDENTIFIER |
|----------------------------|--------|------------|
| Pure Link Plasmid Kit | Thermo Fisher Scientific | Cat# K21007 |

**Recombinant DNA**

| Recombinant DNA | SOURCE | IDENTIFIER |
|-----------------|--------|------------|
| Luciferase-ctrl-sh-GFP | Previously reported (see text for details) | N/A |

**Other**

| Other | SOURCE | IDENTIFIER |
|-------|--------|------------|
| 96-Well plate | Greiner Bio-One | Cat# 655090 |
| Cell strainer 40 μm | CellTreat | Cat# 229481 |
| Fisherbrand Isotemp Water Bath | Fisher Scientific | N/A |
| Hemacytometer | Hauser 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) CO₂ Incubator | N/A |
| 0.2 μm membrane filter | EMD Millipore | Cat# SLGPR33RS |
| 0.45 μm membrane filter | EMD Millipore | Cat# SLHVR33RS |
| 6 cm dish | Coming | Cat# 354401 |

**Software and Algorithms**

| Software and Algorithms | SOURCE | IDENTIFIER |
|--------------------------|--------|------------|
| 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 v6.0 | Thermo Fisher Scientific | N/A |

**Plating Medium (PM)**

| Reagent | Final Concentration | Amount |
|---------|---------------------|--------|
| B27 supplement (50X) | 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 |
**10× Dissociation Medium (10× DM)**

| Reagent                                           | Final Concentration | Amount |
|---------------------------------------------------|---------------------|--------|
| MgCl₂ (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 Ca²⁺)          | 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).

**1× Dissociation Medium (1× DM)**

| Reagent                                           | Final Concentration | Amount |
|---------------------------------------------------|---------------------|--------|
| 10× DM                                            | 1×                  | 50 mL  |
| Penicillin-streptomycin (10,000 U/mL)              | 100 U/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    |
| Na₂HPO₄ (MW 142)                                  | 101 mM              | 14.4 g |
| KH₂PO₄ (MW 136)                                   | 18 mM               | 2.4 g  |
| Milli-Q H₂O                                       | n/a                 | Up to 1 L |

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).
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 H₂O | 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 H₂O | 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 H₂O | n/a                 | Up to 100 mL|

Note: Dissolve PFA in 90 mL of sterile Milli-Q H₂O. 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 1 min.

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: \[ \frac{4 \times \text{clear squares} \times 2 \times 5}{400} = n \times 10^6 \text{neurons/mL}. \] 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.
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 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).
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<sub>GFP</sub>) (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<sub>2</sub> 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 a 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\textsuperscript{ACIII}) in neuronal cultures transduced with LV\textsuperscript{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 (NaN₃). 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 (NaN₃).

Pause Point: Plates can be imaged right away or within 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.
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
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 ciliaHCA, we were able to successfully monitor ACIII+ cilia in primary neurons despite the lack of a confocal mode (Figure 6). Using our ciliaHCA 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.
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 NaN3. 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 40x 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 40x 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.

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