Assembloids generated from human pluripotent stem cells are self-organizing, multicellular in vitro models that recapitulate aspects of cell-cell interactions and circuit assembly during neural development. Here, we present protocols to functionally monitor, in forebrain assembloids, the migration of GABAergic interneurons from the ventral to the dorsal forebrain and the activity in early cortical networks. Specifically, we describe high-resolution imaging and analysis of neuronal migration as well as calcium imaging of network activity in forebrain assembloids.

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

Imaging neuronal migration and network activity in human forebrain assembloids

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SUMMARY

Assembloids generated from human pluripotent stem cells are self-organizing, multicellular in vitro models that recapitulate aspects of cell-cell interactions and circuit assembly during neural development. Here, we present protocols to functionally monitor, in forebrain assembloids, the migration of GABAergic interneurons from the ventral to the dorsal forebrain and the activity in early cortical networks. Specifically, we describe high-resolution imaging and analysis of neuronal migration as well as calcium imaging of network activity in forebrain assembloids.

For complete details on the use and execution of this protocol, please refer to Birey et al. (2022).

BEFORE YOU BEGIN

The user should have skills in aseptic cell culture techniques, stem cell maintenance, neural organoid generation, and microscopy (laser-scanning or spinning-disk confocal microscopy is recommended). For analysis, the user should have basic coding skills in Terminal & Command Line, R, Python and Matlab. Figure 1 includes a timeline schematic of experimental flow.

Viral labeling of hCS and hSS prior to assembly

© Timing: 4–5 days

Perform viral labeling of human Subpallial Spheroids (hSS) and/or human Cortical Spheroids (hCS) using a protocol adapted from (Birey et al., 2017).

1. Transfer 2–3 hCS and/or hSS to a 24-well plate containing 500 μL neural medium (NM) supplemented with adeno-associated virus (AAV) or lentiviral virus.

   Note: Empirically determine the optimal titers using a dilution series.

2. After 24 h, add an additional 500 μL of NM. On day 3, remove all medium and replace with 1 mL NM.

   Note: The expression for AAVs and lentiviruses is usually evident at 14–21 days and 7–14 days after infection, respectively.
3. Generate lentiviruses by transfecting HEK293T cells plated on 100 mm plates (5 * 10^6/plate) with Lipofectamine-2000 (50 μL/dish).

4. After 48 h, then again after 72 h, collect viral supernatant and filter it using a centrifugal filtration device.
   a. Transfer up to 15 mL filtrate into a filtration.
   b. Put the concentrator into an aerosafety container and spin it with a swinging-bucket rotor (4,000 × g, 15 mL starting volume), or fixed-angle rotor (5,000 × g, 12 mL starting volume).
   c. Repeat this step until all the viral supernatant is concentrated.
   d. Using a P200 pipette, transfer the concentrated virus from the top of the concentrator into a cryovial, and maintain at −80°C.

**Generation of forebrain assembloids**

© Timing: 4–7 days

The protocol below describes steps for imaging migration and activity of neurons in forebrain assembloids derived from human induced pluripotent stem cells (hiPSCs) maintained feeder-free (hiPSC cultured on mouse embryonic fibroblasts or human Embryonic Stem Cells (hESCs) can also be used). Perform these assays at 15–60 days after assembly; interneuron migration into hCS is evident starting at 15 days after assembly. Interneuron integration into the hCS network is a more lengthy process; perform calcium imaging 30–60 days after assembly. For details on generating forebrain assembloids, see (Sloan et al., 2018). Successful implementation of both migration and activity assays described below depends on the generation of high-quality forebrain assembloids, and this requires one to carry out quality control measures at multiple stages, such as ensuring the maintenance of pluripotency and genomic integrity of hiPSCs by qPCR for pluripotency markers and by SNP arrays, validating efficient neural differentiation by qPCR and ICC using regional markers, and ensuring optimal viral labeling prior to assembly. For troubleshooting and tips on generating region-alized neural organoids and assembloids, see (Sloan et al., 2018; Miura et al., 2022).

**Imaging human cortical interneuron migration at high spatiotemporal resolution in forebrain assembloids**

© Timing: ~1–3 days of imaging per session

5. Use hSS labeled with an interneuron-specific viral reporter (e.g., Lentiviral Dlx1/2b-eGFP or AAV-Dlx5/6-mScarlet) to visualize hSS-derived GABAergic interneurons.

**Note:** A pre-screening of viral labeling can be performed prior to assembly with hCS. Use an inverted fluorescence microscope and 5–10× magnification to assess if hSS are optimally labeled. Refer to our studies where we describe how hSS are labeled (Birey et al., 2017, 2022; Sloan et al., 2018).
We have imaged interneuron migration in forebrain assembloids at either low (15–20 minutes per field) or at high (30–60 seconds per field) temporal resolutions. Low temporally resolved imaging protocol enables higher throughput phenotyping of mobility kinetics (i.e., distance traveled, pausing time, saltation length, saltation frequency), which we described before (Sloan et al., 2018). Here, we primarily focus on imaging cortical interneuron migration at a high spatiotemporal resolution that enables morphological investigation of cellular and subcellular dynamics.

Cells dually labeled with a structural (e.g., mScarlet) and a functional (e.g., GCaMPx) reporter can be used to record subcellular dynamics.

Imaging network activity with calcium imaging in forebrain assembloids

Timing: 7–14 days for plating on inserts, ~1 day of imaging per session

To image calcium activity in forebrain assembloid networks, place them on 0.4 μm transparent trans-well inserts for 7–14 days in order to generate a slightly more flattened morphology (Figures 2A and 2B).

This preparation is more amenable to faster imaging speeds since it allows recordings in a single imaging plane with a larger field of view (FOV; range of FOV dimensions: 0.50 × 0.50mm–1.75 × 1.75mm) and permits sampling the same assembloid over time since the assembloids are fixed in place on the inserts. A single imaging plane enables faster calcium imaging but yields a limited FOV in intact organoids (Figure 2C) therefore higher-resolution calcium imaging of intact assembloids can be performed at reduced frame rates and/or FOV area.

The choice between an intact versus a flattened preparation should be empirically determined based on the experimental questions.

Prior to assembly, virally label hSS and/or hCS with a genetically encoded calcium indicator (GCaMPx) to image calcium dynamics as a readout of neuronal activity.

We have successfully imaged activity with GCaMP expressed under promoters/enhancers that are generic (e.g., EF1α), neuronal (e.g., hSYN1) or neuron type specific (e.g., Dlx1/2, Dlx5/6, CaMKII) packaged in a lentivirus or in various AAV serotypes (we find AAV-DJ and AAV1 serotypes to be effective in infecting neurons in hCS and hSS).

We recommend using a GCaMPx vector that co-expresses a red-shifted fluorophore (e.g., mScarlet) to distinguish neurons of hSS or hCS origin in assembloids. Two distinct assembloid preparations with different viral labeling of calcium indicators and their representative calcium transient traces examples are shown in Figures 2C and 2D.
8. We typically image network calcium activity at 30–60 days after assembly, when a portion of hSS-derived interneurons has integrated in the hCS glutamatergic neuronal networks.

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and virus strains** |
| LV-Dlx1/2b-eGFP      | From J. Rubenstein (UCSF) | N/A |
| AAV-DJ-Dlx5/6-eGFP   | Addgene | 83900 |
| AAV-DJ-Dlx1/2b-mScarlet-P2A-GCaMP6s | VectorBuilder | N/A |
| AAV-DJ-Dlx5/6-GCaMP6f | Addgene | 83899 |
| AAV-DJ-hSyn1-GCaMP6s | Stanford Gene Vector and Virus Core | N/A |
| AAV1-hSyn1:-jGCaMP7s | Addgene | 104487-AAV1 |
| **Experimental models: Cell lines** |
| Human Induced Pluripotent Stem Cells | Pasca lab | N/A |
| **Chemicals, peptides, and recombinant proteins** |
| Neurobasal Medium, (minus phenol red for imaging) | Gibco | Cat# 12348017 |
| B-27 supplement without vitamin A | Life Technologies | Cat# 12587010 |
| GlutaMAX Supplement | Gibco | Cat# 35050061 |
| Penicillin-Streptomycin (10,000 U/mL) | Gibco | Cat# 15140122 |
| **Software and algorithms** |
| ImageJ (Fiji) | (Schindelin et al., 2012) | https://imagej.net/Fiji |
| MATLAB v2019b, v9.4.0 | MathWorks | https://www.mathworks.com/help/matlab/ref/rand.html |
| Python v3.6.9 | Python | https://www.python.org/ |
| DeepLabCut, v2.1.6 | (Mathis et al., 2018) | DeepLabCut/blob/master/README.md |
| R v4.1.2 | R-project | https://www.r-project.org/ |
| Prism v8.4.2-9.0.0 | GraphPad | https://www.graphpad.com/ |
| Custom-written routines in Matlab | (Birey et al., 2022) | Available upon request |
| **Other** |
| 100 mm ultralow attachment plates | Corning | Cat# 3262 |
| 24-well ultralow attachment plates | Corning | Cat# 3473 |
| 6-Well Flat-Bottom Plate | Stem Cell Technologies | Cat# 38016 |
| Cell culture inserts, 0.4 μm pore size | Corning | Cat# 353090 |
| Glass-bottom 96-well plate #1.5 cover glass | Cellvis | Cat# P96-1.5H-N |
| μ-Plate 96 Well Black, #1.5 cover glass, tc-treated | iBidi | Cat# 89626 |
| Glass-bottom 6-well plate #1.5 cover glass | Cellvis | Cat# P06-1.5H-N |
| Lipofectamine 2000 | Invitrogen | Cat# 11668030 |
| Amicon Ultra tubes 15 mL capacity, 100,000 kDa MW cutoff | Millipore | Cat# UFC910024 |
| Confocal microscope | Leica Microsystems | TCS SP8 |
| Environmental control chamber | Oko Lab | H201 T Unit-BL with 5% CO2/air perfusion |
| Analysis computer | N/A | N/A |

### MATERIALS AND EQUIPMENT

- **Microscopy:** We use a Leica TCS SP8 confocal microscope equipped with galvo and resonant scanners and an environmental control chamber (Oko Lab, H201 T Unit-BL with 5% CO2/air perfusion). As an alternative, a spinning disk confocal microscope can be used. We recommend the use of green (excitation: 488 nm laser) or red-shifted (excitation: 555–594 nm laser) fluorophores.
Computer Hardware: As per Deeplabcut developers’ suggestions, at least 8GB memory (e.g., NVIDIA GeForce 1080 Ti or 2080 Ti) should be used to train a GPU-based network. A CPU-based training and evaluation is also possible even though it is considerably slower for ResNets. If a local GPU is not available, a network can be trained remotely using the Deeplabcut routine found at Mathis et al. (2018).

Computer Software: Deeplabcut requires Python/Anaconda. We perform power analysis with R, and calcium imaging analysis with custom-written routines in MATLAB. For additional statistical analysis and data visualization, we use GraphPad Prism and R.

### Neural media table

| Neural media (NM) components | Storage                  | Final concentration | Amount  |
|------------------------------|--------------------------|---------------------|---------|
| Neurobasal™ Medium, (minus phenol red for imaging NM) | 4°C, stable for 1 year | 485 mL              |
| B-27 supplement without vitamin A | –2°C, stable for 1 year | 1:50 into Neurobasal™ Medium | 10 mL   |
| GlutaMAX™ Supplement         | 4°C, stable for 2 years  | 1:100 into Neurobasal™ Medium | 5 mL    |

Once supplemented, NM is stable for up to one week when stored in the dark at 2°C–8°C. Prepare the imaging medium by pre-warming the desired volume in a water bath for 5 minutes. For migration imaging in 96-well plates, prepare ~400 μL of imaging NM per well (one assembloid per well). For calcium imaging on inserts, prepare ~2.5mL per well.

### STEP-BY-STEP METHOD DETAILS

**Imaging human cortical interneuron migration at high spatiotemporal resolution in forebrain assembloids**

**Steps:**

1. Transfer the forebrain assembloids labeled with LV-Dlx1/2b-eGFP to a glass-bottom 96-well microplate in 300–350 μL imaging NM. Incubate assembloids in an environmentally controlled chamber for 30–60 minutes before imaging.

   **Note:** Imaging NM contains neurobasal-A without phenol red, B-27 supplement without vitamin A, GlutaMax (1:100) and penicillin and streptomycin (1:100). See neural media table for details.

   **Note:** It is important to make sure that assembloids are settled at the bottom of the well before imaging. Perform a short (10–15min) pre-imaging recording to exclude drift in the position of the assembloid.

   **Note:** The user can use a square-well 96-well imaging plate instead of a round welled 96-well imaging plate. Placing assembloids on a corner of the well can reduce drift.

   **CRITICAL:** It is important to use the central wells on the plate to ensure that the microscope objective can safely move and image cells.

2. Select the FOV and start the imaging session.
Note: We have used a 20 × (0.75 NA) objective with 2–2.5 × zoom at 1024 × 1024 resolution to achieve the necessary magnification and resolution.

Note: The specific imaging parameters (e.g. laser power, signal averaging, zoom) should be empirically determined based on the experimental needs, microscope specifications and viral reporter used.

△ CRITICAL: The use of sparsely labeled FOVs (Figure 3A) can minimize the likelihood of capturing too many interneurons with overlapping trajectories, which makes the downstream analysis more challenging.

Note: We have imaged 15–45 seconds per volume (i.e., z-stack; 9–15 μm per stack) to temporally resolve the cellular dynamics involved in the nucleokinesis events. Hours-long imaging at faster imaging rates could induce phototoxicity, although this may be possible with a spinning disk confocal microscope.
**Optional:** If an experiment requires pharmacological modulation, perform a half-volume medium change with fresh medium containing the drug at twice the working concentration.

**Optional:** Adjust the fields for minor shifts prior to post-drug imaging.

**Subcellular tracking of migrating human cortical interneurons using Deeplabcut**

**Timing:** ~1–2 days per dataset

This section will describe a step-by-step protocol for setting up the analysis pipeline for markerless tracking of cellular and subcellular ROIs using Deeplabcut.

3. Prepare the imaging files for Deeplabcut.
   a. Correct for drift using Fiji with the Linear Stack Alignment plugin.
   b. Smoothen using Fiji with the Gaussian Blur 3D plugin.
   c. Crop the image to only include an individual interneuron undergoing at least one saltation.
   d. Record the pixel / micron ratio of the image in Fiji under “Image > Show info…”: It is listed as “Resolution”.
   e. Save as .avi file.
4. Install Deeplabcut following developers’ instructions.
5. Launch Deeplabcut, initiate a new project and fill in the relevant details.
6. In the Config file, rename the ROIs accordingly. ROI names are located under “Annotation data set configuration / body parts” and “Plotting configuration / skeleton”.
7. Automatically extract 19 training frames using the k-means algorithm.

   **Note:** We successfully used the default parameters (cluster step = 1, network = resnet_50, augmentation = imgaug).

Manually annotate ROIs in each training frame for each video and train the network for up to 100,000 iterations or until the loss is plateaued.

   **Note:** This step can take several hours.

8. Evaluate network and analyze videos with filtered predictions. **Figure 3A** shows tracking of different ROIs of a representative migrating interneuron.

   **Note:** Videos used for analysis are the same videos used for training.

9. Export results as a .csv file. The output is X and Y coordinates in pixels per ROI.
10. Convert X and Y pixel coordinates to Euclidean distances in micron using the pixel/micron ratio. These values can be used to estimate the relative positional information of each ROI across the time series. We provide a conversion template under “quantification and statistical analysis”.

   **Note:** Outlier frames can be extracted, and the network can then be retrained for more accurate ROI identification.

   **△ CRITICAL:** Detect occasional ROI mismatch by outlier likelihood measure displayed in the .csv readout and use a smoothing function (e.g. moving median function in MATLAB (k=100; **Figure 3B**)) to correct for such artifacts.

**Optional:** Visually validate the tracking fidelity by converting X and Y pixel coordinates to time series ROIs for each movie in FiJi and confirm that each ROI is localized to appropriate subcellular locus per frame.
Imaging network-level calcium activity in forebrain assembloids

© Timing: 7–14 days for plating on inserts, ~1 day of imaging per session

This section will describe a step-by-step protocol for setting up a calcium imaging experiment to assess network activity in forebrain assembloids.

11. Seed GCaMPx-labeled forebrain assembloids 7–14 days before imaging on 0.4 μm-transparent trans-well inserts to achieve a flattened geometry (Figures 2A and 2B).

12. Transfer the inserts with assembloids to glass-bottom 6-well imaging plates containing 1.5–2 mL of imaging NM medium, positioned in the environmentally controlled imaging chamber of the confocal microscope, and incubate for 30–60 minutes before imaging.

13. Confirm the orientation of the assembloids (hCS side versus hSS side) by the expression of the red-shifted marker and set the imaging parameters.

Note: It is important to eliminate any bleed-through between channels before imaging; a quick test can be performed where only a single laser line is turned on while both GCaMPx and red-shifted detectors are on.

Note: Using a Leica TCS SP8 equipped with a resonant scanner and a 10× (0.4 NA) objective, we have imaged calcium activity at cellular resolution at the imaging speeds ranging from 4–14 Hz, (FOV 0.75 × 0.75mm to 1.8×1.8mm, single imaging planes were used without a z-stack).

Note: A 10–15-minutes imaging session is usually sufficient to sample a representative activity profile of the network.

14. For analysis, identify the cell somas (using the red-shifted marker or using the standard deviation projection of the GCaMP signal over time).
   a. Collect mean gray values from the soma using Fiji.
   b. Transform mean gray values to relative changes in fluorescence: \( \frac{dF}{F_0} = \frac{F(t) - F_0}{F_0} \), where \( F_0 \) represents average gray values of the time series of each ROI.

Optional: For longitudinal, within-assembloid experiments, mark the orientation of the insert with a sterile marker after the first imaging session and transfer the insert back to the 6-well culture plate with 1.5 mL of NM. The landmark can then be used to position the insert accordingly and find the same FOV during subsequent imaging sessions.

EXPECTED OUTCOMES

Imaging and analysis of human cortical interneuron migration at high spatiotemporal resolution in forebrain assembloids

Extract positional information on subcellular ROIs. See examples of expected results in (Birey et al., 2022).

Imaging network-level activity with calcium imaging in forebrain assembloids

Record network-level calcium activity in forebrain assembloids, which can include both uncorrelated calcium transient and synchronous activity events. See examples of expected results in (Birey et al., 2022).

QUANTIFICATION AND STATISTICAL ANALYSIS

Sample size & power analysis

Perform pilot studies to estimate variability of phenotypes; then use R packages effectsize and pwr to calculate effect size and sample size for a well-powered study.
CRITICAL: When comparing cells from assembloids derived from patients vs controls, include cells from several hiPSC lines that are sex and ancestry matched and perform multiple differentiations and imaging sessions.

Deeplabcut analysis
Table 1 shows how to convert Deeplabcut output (X and Y coordinates in pixels) to XY plots in microns. The scale is the pixels/micron ratio from each TIFF file corresponding to each cell. Extract this number from Fiji (Analyze>Set Scale…) and input it into the table.

Note: Subtract a given xy (micron) value from the previous value to obtain “relative” xy (micron), where xy (micron) = 0 at time = 0.

LIMITATIONS
Imaging and analysis of cell migration at high spatiotemporal resolution in forebrain assembloids
Imaging in assembloids is a relatively low-throughput experimental approach partly due to the long-term nature of these experiments (neural differentiation, viral labeling, assembly, high resolution imaging of smaller FOVs, etc). Obtaining a well-powered dataset from multiple hiPSC lines and differentiations is time consuming and requires extensive imaging time (See notes on power analysis under “quantification and statistical analysis”). Training the Deeplabcut network should ideally be extended to include large number of migrating interneurons. This could be a limitation as the datasets could get prohibitively large to train.

Imaging network-level activity with calcium imaging in forebrain assembloids
Although the use of trans-well inserts to achieve a flattened geometry helps with imaging large FOVs with relatively high frame rates, this process can reduce the quality of imaging.

TROUBLESHOOTING
Imaging and analysis of human cortical interneuron migration at high spatiotemporal resolution in forebrain assembloids

Problem 1
There is excessive drift during imaging (Step 3a).

Potential solution
This can happen if the samples do not settle enough during the acclimation period. Extend the pre-imaging incubation period. Minimize entry to the confocal room during imaging.

Problem 2
There is bleaching observed during imaging (Step 3a,14).

Potential solution
Correct for signal decay using Fiji plugin Bleach Correction (Simple Ratio).

Problem 3
I am having difficulty catching migrating interneurons (Step 3a).

Table 1. Deeplabcut conversion template
| x (pixels) | y (pixels) | Scale (pixels/micron) | x (micron) | y (micron) | xy (micron) |
|-----------|-----------|-----------------------|-----------|-----------|------------|
| A         | B         | C                     | D=A/C     | E=B/C     | =SQRT(POWER(D,2)+POWER(E,2)) |

△ CRITICAL: When comparing cells from assembloids derived from patients vs controls, include cells from several hiPSC lines that are sex and ancestry matched and perform multiple differentiations and imaging sessions.
Potential solution 1
Given that it is challenging to identify actively migrating cells due to the slow kinetics of the jumps (every 3–4 hours), we recommend imaging sessions that are at least 8–10 hour-long. We detected no noticeable phototoxicity in imaging session up to 24 hours when the parameters above are used. Shorter imaging periods can be opted for if a multi-point paradigm is used to capture multiple cells in each session (we use the “live data” module of the SP8 software).

Potential solution 2
The lack of migration could indicate issues with differentiation or assembly of organoids. Perform a lower resolution/magnification migration imaging for longer imaging periods (as described in (Sloan et al., 2018)) to verify if there are migrating cells in assembloids.

Problem 4
Deeplabcut tracking frequently mismatches the position of a particular ROI (Step 10).

Potential solution
Deeplabcut’s performance in tracking highly dynamic subcellular ROIs might be variable. Increase the number of training frames extracted from each video using the numframes2extract in the config file. Include more samples in the training dataset.

Problem 5
Deeplabcut tracking occasionally mismatches the position of a particular ROI (Step 10).

Potential solution
Use the MATLAB function moving median to correct for occasional artifacts in ROI matching (Figure 3B).

IMAGING NETWORK-LEVEL ACTIVITY WITH CALCIUM IMAGING IN FOREBRAIN ASSEMBLOIDS

Problem 6
The assembloids are detaching from inserts (Step 11).

Potential solution
During medium changes, remove excess media from the top of the assembloids to maintain an air/liquid interface.

Problem 7
There is no activity (Step 14).

Potential solution
This indicates an issue with the quality of the forebrain assembloids or the imaging setup. Perform additional tests to assess assembloid health (cleaved caspase ICC, RT-qPCR for region-specific markers) and verify imaging parameters.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Sergiu Pasca (spasca@stanford.edu).

Materials availability
This study did not generate new unique reagents.
Data and code availability
Any information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS
F.B. generated forebrain assembloids, performed migration and calcium imaging experiments and analyses. F.B. implemented the Deeplabcut pipeline and downstream analysis. F.B. wrote the manuscript with input from S.P.P. S.P.P. supervised all aspects of the work.

DECLARATION OF INTERESTS
Stanford University holds patents and has provisional patent applications covering the generation of brain region-specific organoids and assembloids (F.B. and S.P.P.).

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