Journal of Visualized Experiments

Migration, chemo-attraction and co-culture assays for human stem cell derived endothelial cells and GABAergic neurons

--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60295R1
Full Title:	Migration, chemo-attraction and co-culture assays for human stem cell derived endothelial cells and GABAergic neurons
Keywords:	Endothelial Cells; interneurons; cell migration; neuro-vascular interactions; co-culture; chemo-attraction
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Belmont, Massachusetts, United States of America

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Editor
Journal of Visualized Experiments (JoVE)
1 Alewife Center, Suite 200
Cambridge, MA 02140

July 2nd, 2019

Dear Dr. Steindel,

Re: JoVE60295 "Migration, chemo-attraction and co-culture assays for human stem cell derived endothelial cells and GABAergic neurons

Thank you for reviewing our manuscript and giving us the opportunity to address the reviewers' comments. The revision fully addresses the editorial comments as well as the specific concerns expressed by the reviewers, as described in our letter of response.

Our revision includes a copy of the manuscript with all changes highlighted in red and filmable content shaded in yellow. The manuscript has been formatted according to JoVE guidelines and checked for spelling and grammar issues.

We hope that you will find our response and revision satisfactory.

Thanking you,

Sincerely yours,

Anju V

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TITLE

Migration, Chemo-Attraction, and Co-Culture Assays for Human Stem Cell Derived Endothelial Cells and GABAergic Neurons

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KEYWORDS:

endothelial cells; interneurons; cell migration; neuro-vascular interactions; co-culture; chemo-attraction

SUMMARY:

We present three simple in vitro assays–the long-distance migration assay, the co-culture migration assay, and chemo-attraction assay–that collectively evaluate the functions of human stem cell derived periventricular endothelial cells and their interaction with GABAergic interneurons.

ABSTRACT:

Role of brain vasculature in nervous system development and etiology of brain disorders is increasingly gaining attention. Our recent studies have identified a special population of vascular cells, the periventricular endothelial cells, that play a critical role in migration and distribution of forebrain GABAergic interneurons during embryonic development. This, coupled with their cell-autonomous functions, alludes to novel roles of periventricular endothelial cells in the pathology of psychiatric disorders like schizophrenia, epilepsy, and autism. Here, we have described three different in vitro assays that collectively evaluate the functions of periventricular endothelial cells and their interaction with GABAergic interneurons. Use of these assays, particularly in a human context, will allow us to identify the link between periventricular endothelial cells and brain disorders. These assays are simple, low cost, and reproducible, and can be easily adapted to any adherent cell type.

INTRODUCTION:

Endothelial cells form the lining of blood vessels and mediate important functions that include maintenance of vessel wall permeability, regulation of blood flow, platelet aggregation, and formation of new blood vessels. In the brain, endothelial cells form part of a critical blood-brain-barrier that tightly controls exchange of materials between brain and bloodstream[1]. Our studies in the past decade have identified novel neurogenic roles of brain endothelial cells that have significant implications for brain development and behavior[2-5]. We have shown that mouse embryonic forebrain is vascularized by two distinct subtypes of vessels, the pial vessels and the periventricular vessels, that differ in anatomy, origin, and developmental profile[2]. Endothelial cells lining these two vessel subtypes show distinct differences in their gene expression profiles. While pial endothelial cells mostly express genes related to inflammation and immune response, periventricular endothelial cells are uniquely enriched in expression of genes associated with neurogenesis, neuronal migration, chemotaxis, and axon guidance[3]. Periventricular endothelial cells also house a novel GABA signaling pathway that is distinct from the traditional neuronal GABA signaling pathway[5]. Concomitant with its gene expression, periventricular endothelial cells were found to regulate migration and distribution of GABAergic interneurons in the cortex. During embryonic development, periventricular endothelial cells undergo long distance migration along a ventral-dorsal gradient to establish the periventricular vascular network[2,3]. This migratory route is mirrored a day later by interneurons. Migrating interneurons physically interact with the pre-formed periventricular vascular network and use it as a guiderail to reach their final destination in the neocortex. In addition to acting as a physical substrate, periventricular endothelial cells serve as the source of navigational cues for migrating neurons. Periventricular endothelial cell secreted GABA guides interneuron migration and regulates their final distribution patterns[4]. Defects in interneuron migration and distribution are associated with psychiatric disorders such as autism, epilepsy, schizophrenia and depression[6-10]. Therefore, study of periventricular endothelial cell functions and their influence on interneurons migration in human context becomes critical for addressing the pathogenesis of these disorders.

We have generated human periventricular-like endothelial cells from human embryonic stem cells in our laboratory[11], using induced pluripotent stem cell (iPSC) technology[12,13]. To validate whether human periventricular endothelial cells faithfully mimic mouse periventricular endothelial cells, and to quantitatively assess their influence on interneuron migration, we developed three in vitro assays: a long-distance migration assay, a co-culture migration assay, and a chemo-attraction assay. Here we describe protocols for these assays in detail. All three assays are based on the usage of silicone culture inserts to create small rectangular patch of cells (of fixed dimensions) surrounded by cell-free space. Migration distance is evaluated by measuring the distance between the final positions of cells from the border of the rectangular patch that has been outlined on day 0. In the long-distance migration assay, human periventricular endothelial cells are seeded as a patch in the center of a 35 mm dish, and the distances traveled by the cells over a long range of time are calculated. In the co-culture migration assay, human periventricular endothelial cells are co-seeded with human interneurons as one patch in a 35 mm dish. This setup allows examination of the effect of direct physical interactions of these two cell types on the rate of migration of interneurons. The chemo-attraction assay measures the migration of interneurons in response to GABA release by human periventricular endothelial cells. Interneurons are seeded as a rectangular patch, with human periventricular

endothelial cells and control non-periventricular endothelial cells seeded as similar sized patches on either side. Each of the cell patches are separated by a cell-free gap of 500 µm. Response of interneurons is assessed by quantifying the number of cells that have migrated towards periventricular endothelial cells compared to control non-periventricular endothelial cells.

These assays provide robust assessment of human periventricular endothelial cell functions and their influence on interneuron migration. The novel setup of long-distance assay and co-culture migration assay provides cell-free space in the range of centimeters (~1-1.5 cm) to allow detection of long-distance migration. A summary of the features of our assays compared to other popular assays is presented in **Table 1**. Collectively, the assays described here will serve as a platform for assessing "diseased" periventricular endothelial cells and interneurons generated from iPSCs of brain disorders like schizophrenia, autism or epilepsy. These assays can also be used to determine how different conditions (e.g. inhibitors, ligands, RNAi) affect cell migration. Finally, these assays can be optimized for other cell types to measure long-distance migration, chemo-attraction or cell-cell mediated migration.

PROTOCOL:

1. Culture and storage of human periventricular endothelial cells

1.1. Maintain human periventricular endothelial cells on basement-membrane-matrix-coated (see **Table of Materials**) 6-well plates in periventricular endothelial cell medium (E6 medium containing 50 ng/mL VEGF-A, 100 ng/mL FGF2 and 5 µM GABA) at 37 °C and 5% CO_2. Change medium every alternate day.

1.2. Thaw basement membrane matrix in 4 °C, and make a 1:100 solution by diluting it in cold DMEMF/12 medium. Coat each well of a 6-well plate with 1 mL of matrix solution. Incubate plates at 37 °C for at least one h before use.

1.3. Allow human periventricular endothelial cells to reach a confluency of 80%–90%. Aspirate medium from the well. Wash the wells once with 1 mL of sterile 1X PBS per well.

1.4. Detach cells by adding 1 mL of cell dissociation solution (see **Table of Materials**) per well. Incubate at 37 °C for 5 min. After 5 min, add 1 mL of periventricular endothelial cell medium. Transfer the cell solution into a 15 mL conical tube.

NOTE: We use Accutase for cell dissociation here, as opposed to TrypLE in sections 3 and 4.

1.5. Centrifuge cells at 500 x *g* for 5 min at room temperature, aspirate the supernatant and resuspend the cell pellet in 1 mL of periventricular endothelial cell medium.

1.6. Count live cells using trypan blue exclusion method. Seed cells in fresh matrix-coated plates at a density of 1.2 x 10^5 cells/cm^2. Incubate at 37 °C and 5% CO_2.

1.7. Store human periventricular endothelial cells by cryopreserving in freezing medium (90% periventricular endothelial cell medium and 10% DMSO).

1.7.1. Dissociate and collect cells following steps 1.3 and 1.4 above. Count cells in the solution by the trypan blue exclusion method.

1.7.2. Centrifuge cells at 500 x *g* for 5 min at room temperature. Aspirate the supernatant and resuspend the cell pellet at 5 x 10^6 cells/ mL of freezing medium.

1.7.3. Dispense 1 mL of freezing medium plus cells per cryovial. Place the vials in isopropanol-filled chamber and cool overnight in -80 °C at 1 °C/min. Transfer vials to a liquid nitrogen tank on the next day for long term storage.

2. Preparation of human periventricular endothelial cells for assay

2.1. Allow human periventricular endothelial cells to reach 70%–80% confluency.

2.2. Dissociate cells following steps 1.3 to 1.5 as described above. Count cells using the trypan blue exclusion method.

3. Preparation of human GABAergic interneurons for assay

NOTE: Human induced pluripotent stem cell (iPSC)-derived GABAergic interneurons and the neuronal medium were commercially purchased (see **Table of Materials**). The neurons are generated by differentiating a human fibroblast-derived iPSC line following a protocol developed by the manufacturer. The cells were thawed and cultured according to manufacturer's protocol.

3.1. Thaw human GABAergic interneurons and culture them in 12-well plate for two weeks to a confluency of 70%–80%.

3.2. On the day of assay, warm cell dissociation solution (see **Table of Materials**) and an aliquot of neuronal medium at 37 °C for 10 min before use.

3.3. Aspirate medium from each well containing the cells. Wash cells with 1 mL of sterile 1x PBS per well.

3.4. Detach cells by adding 0.5 mL of pre-warmed dissociation solution per well and incubating at 37 °C for 5 min. Add 1 mL of neuronal medium per well. Transfer cell solution into a 15 mL conical tube. Gently triturate to dissociate cell clumps.

3.5. Centrifuge cells at 380 x *g* for 5 min at room temperature, aspirate the supernatant and resuspend the cell pellet in 1 mL of neuronal medium. Count live cells using the trypan blue exclusion method.

4. Preparation of control human endothelial cells for assay

NOTE: Control human iPSC-derived endothelial cells and endothelial cell medium were commercially purchased (**Table of Materials**). These endothelial cells are generated by differentiating a human fibroblast-derived iPSC line to endothelial fate following a protocol developed by the manufacturer. The cells were thawed and cultured on Fibronectin substrate according to manufacturer's protocol. Fibronectin-coated plates were prepared following manufacturer's protocol.

4.1. Thaw control human endothelial cells and culture them in 6-well plate to a confluency of 80%–90%.

4.2. On the day of the assay, warm cell dissociation solution (see **Table of Materials**) and an aliquot of endothelial medium at 37 °C for 10 min before use.

4.3. Aspirate the medium from each well containing the cells. Wash cells with 1 mL of sterile 1x PBS per well.

4.4. Detach cells by adding 0.5 mL of pre-warmed dissociation solution per well. Incubate at room temperature for 5 min. Add 1 mL of endothelial cell medium per well to neutralize the dissociation solution. Transfer cell solution into a 15 mL conical tube.

4.5. Centrifuge cells at 200 x *g* for 5 min at room temperature. Aspirate supernatant and resuspend the cell pellet in 1 mL of endothelial cell medium. Count live cells using the trypan blue exclusion method.

5. Preparation of one-well culture inserts

5.1. Thaw 1 mg/mL laminin solution at room temperature or overnight at 4 °C.

5.2. Coat an appropriate number of 35 mm dishes with 0.01% poly-L-ornithine solution (1 mL per dish). Incubate the dishes in room temperature for at least 1 h.

5.3. Dilute 1 mg/mL laminin solution 1:300 in sterile water to a final concentration of 3.3 µg/mL immediately before use.

5.4. Completely aspirate poly-L-ornithine from each dish. Rinse each dish thoroughly 3x with sterile water and aspirate completely to avoid poly-L-ornithine-induced cell toxicity.

5.5. Add 1 mL of 3.3 µg/mL laminin solution to each dish and incubate at 37 °C overnight or least 1 h. Remove laminin solution from the dish immediately before use.

NOTE: Alternatively, store the laminin-containing dishes in 4 °C. Equilibrate the dishes in a 37 °C cell culture incubator before use.

5.6. Cut three sides of one well of a two-well silicone culture insert (**Figure 1A**) using a sterile blade to generate a one-well insert **(Figure 1B).**

NOTE: Keep the two-well insert firmly attached to the surface of the original packaging while cutting to ensure a smooth cut and protect the adhesiveness of the insert.

5.7. Aspirate laminin solution from the dishes.

NOTE: Do not wash the dishes with sterile PBS or water after laminin incubation. Wet surfaces will prevent tight adhesion of the culture insert.

5.8. Remove one-well insert with sterile tweezers and place it in the center of the poly-L ornithine/Laminin coated dish. Use tweezers to press along the edges of the insert to fix it to the surface of the dish.

5.9. Carefully turn the dish upside down to verify that the insert is firmly adhered.

5.10. Keep the dish upside down and mark the boundary of the insert compartment using a permanent black marker with an ultra-fine tip (**Figure 1C**).

6. Long distance migration assay

6.1. Suspend human GABAergic interneurons at a concentration of 3 x 10^4 cells/70 µL of neuronal medium. Seed 70 µL of cell solution inside each one-well culture insert.

NOTE: The seeding density of interneurons is as per manufacturer's recommendation.

6.2. Suspend human periventricular endothelial cells at a concentration of 3 x 10^4 cells/70 µL of periventricular endothelial cell medium. Seed 70 µL of cell solution inside each one-well culture insert.

NOTE: The number of human periventricular endothelial cells seeded maintains a 1:1 ratio with the number of seeded neurons.

6.3. Add 1 mL of neuronal medium in neuron dish to fill the area around the insert and prevent the coating from drying. Similarly, add 1 mL of periventricular endothelial cell medium in periventricular endothelial cell dish.

NOTE: Add medium slowly along the edge of the dish so that the insert is not disturbed.

6.4. Check under a microscope to verify that cells are not leaking from the insert compartment.

6.5. Incubate cells for 24 h at 37 °C and 5% CO_2. After 24 h incubation, check under microscope

to verify that cells have attached properly and there is no overnight leak.

6.6. After 48 h post-seeding, gently remove the insert using a sterile tweezer. Check under the microscope to verify that the cell layer remains undisturbed (day 0).

6.7. Remove medium from the neuron dish and add 1 mL of fresh neuronal medium. Similarly, remove medium from the periventricular endothelial cell dish and add 1 mL of fresh periventricular endothelial cell medium.

NOTE: Set aside the required number of dishes and fix with 4% PFA for day 0 images.

6.8. Incubate cells for 5 days at 37 °C and 5% CO_2. After 5 days, remove medium, fix cells with 4% PFA for 10 min, and wash 3x with 1x PBS.

6.9. Stain neurons with anti-human β-Tubulin or anti-human MAP2 antibody, and endothelial cells with anti-human CD31 antibody. At the end of immunostaining, add 1 mL of antifade mounting medium to each dish.

7. Co-culture migration assay

7.1. Co-suspend 3 x 10^4 GABAergic interneurons and 3 x 10^4 human periventricular endothelial cells in 70 µL of co-culture medium (50% periventricular maintenance medium without GABA and 50% neuronal medium). Seed this cell solution inside the one-well insert compartment. Prepare an appropriate number of such assay dishes.

NOTE: GABA was not added in the co-culture medium to exclude the effect of exogenous GABA on migration.

7.2. Check under a microscope to verify that cells are not leaking from the insert compartment.

7.3. Slowly add 1 mL of co-culture medium along the side of the dish to prevent the coating from drying.

NOTE: Add medium slowly along the edge of the dish so that the insert is not disturbed.

7.4. As a first control, seed 3 x 10^4 human GABAergic interneurons only in 70 µL of co-culture medium per one-well insert. Prepare an appropriate number of such dishes.

7.5. As a second control, co-seed 3 x 10^4 GABAergic human interneurons with 3 x 10^4 control human endothelial cells in 70 µL of co-culture medium per one-well insert. Prepare an appropriate number of dishes.

7.6. Incubate the dishes for 24 h at 37 °C and 5% CO_2. After 24 h incubation, check under a microscope to verify that cells have attached properly and there is no leak.

7.7. After 48 h of seeding, gently remove the insert using a sterile tweezer. Check under the microscope to verify that the cell layer is not disturbed (day 0).

7.8. Remove medium and add 1 mL of fresh co-culture medium.

NOTE: Set aside an appropriate number of dishes for acquiring day 0 images.

7.9. Incubate cells for 5 days at 37 °C and 5% CO_2.

7.10. After 5 days, remove medium, fix cells with 4% PFA for 10 min, and wash 3x with 1x PBS.

7.11. Stain with anti-human β-Tubulin or anti-human MAP2 antibody to label the neurons. At the end of immunostaining, add 1 mL of antifade mounting medium to each dish.

8. Chemo-attraction assay

8.1. Place a three-well culture insert in the center of a poly-ornithine/Laminin coated 35 mm dish using sterile tweezers.

8.2. Turn the dish upside down. Mark the boundary of the insert compartment using a permanent black marker with ultra-fine tip.

8.3. Seed 3 x 10^4 human GABAergic interneurons in the middle compartment in 70 µL of neuronal medium (**Figure 3A**).

8.4. Seed 10^4 human periventricular endothelial cells in 70 µL of periventricular endothelial cell medium and 10^4 control endothelial cells in 70 µL of control endothelial cell medium in the two outer compartments respectively (**Figure 3A**).

8.5. Add 1 mL of co-culture medium (50% periventricular maintenance medium without GABA and 50% neuronal medium) along the side of the dish to prevent the coating on the dish from drying.

8.6. Check under microscope to verify that cells are not leaking from the insert compartment.

8.7. Incubate cells for 24 h at 37 ^{o}C and 5% CO_2. After 24 h incubation, check under a microscope to verify that cells have attached properly and there is no leak.

8.8. After 48 h of seeding, gently remove the insert using a sterile tweezer. Check under the microscope to verify that the cell layer is not disturbed (day 0).

8.9. Remove medium and add 1 mL of fresh co-culture medium.

NOTE: Set aside required number of dishes for day 0 images.

8.10. Incubate cells for 36 h at 37 °C and 5% CO_2. After 36 h, aspirate medium, fix cells with 4% PFA for 10 min, and wash 3x with 1x PBS.

8.11. Stain human GABAergic interneurons with anti-human β-Tubulin or anti-human MAP2 antibody. At the end of the staining procedure, add 1 mL of antifade mounting medium in each dish.

9. Imaging and data analysis

9.1. Place the immuno-stained assay dish under a microscope at 4X magnification.

9.2. Keep one long-edge of the rectangular boundary (made in step 5.10 above) in the field of view. Take images of cells in the cell free-space adjacent to that boundary. Acquire images along the right long-edge and the left long-edge of the rectangular boundary (**Figure 2B**)**.**

NOTE: Cells positioned diagonally with respect to the rectangle are not considered due to ambiguity in selecting the short- or long-edge as starting mark. Also, numbers of cells migrating across the short-edge are often significantly fewer (possibly due to lesser number of starting cells along the short-edge) and are not considered.

9.3. Open the images in ImageJ. Calculate distance between each cell and the boundary mark (**Figure 2D**) using ImageJ.

9.4. To assess migration in terms of cell numbers, set a specific distance from the boundary in the acquired image in ImageJ. Count the number of cells that are present within this distance. Calculate average number, standard deviation, and statistical significance using appropriate software.

REPRESENTATIVE RESULTS:

The steps to set up one-well culture insert inside a 35 mm dish are shown in **Figure 1**. Long distance migration assays and co-culture migration assays used a one-well insert to seed the desired number of cells in the center of a poly-ornithine/Laminin coated 35 mm dish. On day 0, cells were present as a rectangular patch (**Figure 2A,C**). In day 0 images, the day 0 line could be easily identified by the sharp edge of the cell layer (white dotted line in **Figure 2C**). By 48 h, cells had migrated out into the cell free space (**Figure 2B,D**). In post-day 0 images, the black border drawn around the insert (at the back of the dish) could be clearly observed as a black gap. The edge of the gap was assigned as the day 0 line (white dotted line in **Figure 2D**). As mentioned in Step 9.2, only those cells which fell in the area adjacent to the right and left long-edges of the cell layer (yellow area in **Figure 2B**) were considered for data analysis. The distance travelled by a cell was measured by calculating the distance between the cell (white arrow in **Figure 2D**) and the day 0 line. Immunocytochemical staining with anti-active Caspase 3 antibody, a marker of apoptosis, showed no apoptotic signal in the seeded cells (**Figure 2E**). In co-culture migration

assay, when interneurons were co-seeded with human periventricular endothelial cells, neurons travelled farther distances compared to when interneurons were seeded alone or when co-seeded with control endothelial cells (**Figure 2F**). Also, for the same distance range, a higher number of interneurons migrated out when co-seeded with periventricular endothelial cells in comparison to interneurons in the other two groups. This shows that, like mouse periventricular endothelial cells, human periventricular endothelial cells promote human interneuron migration.

In the chemo-attraction assay, using three-well culture inserts, human interneurons were seeded as a small rectangular patch in a 35 mm poly-ornithine/Laminin coated culture dish. Periventricular endothelial cells and control non-periventricular endothelial cells were seeded as patches on either side of the neuronal patch, with the gap between each patch being 500 µm (**Figure 3A**). The number of interneurons that migrated towards periventricular endothelial cells versus control endothelial cells was quantified after 36 h. A significantly higher number of interneurons migrated towards periventricular endothelial cells compared to control endothelial cells (**Figure 3B,C**), confirming that GABAergic interneurons respond selectively to chemo-attractive cues secreted by human periventricular endothelial cells.

FIGURE AND TABLE LEGENDS:

Figure 1. Preparation of the culture insert. (**A**) A two-well culture insert. (**B**) A one-well insert fixed in the center of a 35 mm dish. (**C**) The outline of the rectangular patch as observed after removing the insert.

Figure 2. Schema and representative result of migration assay. (**A**) Schema of cell layer (red rectangle) on day 0. (**B**) Schema of cells migrating out into the cell-free space. Red dots indicate migrating cells. The yellow region marks the area that is imaged for data acquisition. The dotted box in **A** and **B** corresponds to the area shown in panels **C** and **D**. (**C**, **D**) Representative fluorescent images of anti-β-tubulin antibody labelled interneurons on day 0 (**C**) and day 2 (**D**) of the migration assay. The white dotted line marks day 0. The yellow line in **D** indicates the distance travelled by a cell (marked by white arrow) in 48 h. (**E**) Neurons (on day 0) are co-labeled with anti β-tubulin antibodies (red) and anti-active Caspase 3 antibodies (green), which mark apoptotic cells. Nuclei are stained with DAPI (blue). Apoptotic cells were not detected in seeded cells. (**F**) Graph from day 5 of the co-culture assay, where the number of interneurons that have migrated is plotted against distance travelled. In comparison to interneurons that were seeded alone or co-seeded with control endothelial cells, interneurons co-seeded with periventricular endothelial cells migrated out in higher numbers, and also travelled farther distance**.** Data represents mean ± S.D (n = 5; $^{**}p<0.01$, $^{***}p < 0.001$, Student's t test). Scale bars = 100 µm. IN= interneurons; PV EC= periventricular endothelial cells.

Figure 3. Chemo-attraction assay. (**A**) Schema of the chemo-attraction assay. Using a three-well culture insert, interneurons (IN) were seeded in the middle (green dotted rectangle), while periventricular endothelial cells (PV ECs; orange dotted rectangle) and control endothelial cells (ECs; yellow dotted rectangle) were seeded on either side. (**B**) Images of β-Tubulin labeled interneurons showing robust migration towards periventricular endothelial cells but not towards

control endothelial cells. (**C**) Quantification of the chemo-attractive response of interneurons. A significantly higher number of neurons migrated towards periventricular endothelial cells than towards control endothelial cells. Data represents mean ± S.D (n = 5; *$p < 0.05$, Student's t test). Scale bars = 100 μm.

Table 1: Comparison of assay methods. (**A**) Comparison of common in vitro migration assays with the long-distance migration assay and co-culture assay. (**B**) Comparison of common chemotaxis assays with the chemo-attraction assay.

DISCUSSION:

Here, we described three in vitro assays that together provide quantitative assessment of human periventricular endothelial cell-specific properties. These assays will be valuable in gaining mechanistic insights into the interaction of human periventricular endothelial cells with human interneurons. Experiments using ligands, inhibitors, or cells with gene-specific knockdown or overexpression will identify or validate molecular players that mediate endothelial cell-guided interneuron migration or long-distance migratory properties of periventricular endothelial cells. These assays can also be modified to perform live-cell time-lapse migration studies. Furthermore, there is evidence for interaction of endothelial cells with cells other than interneurons. Studies from our group and others have alluded to the influence of periventricular endothelial cells on patterning of projection neurons and proliferation of neural precursor cells[5,14,15]. It would be of interest to test these possible interactions using our assay settings. Finally, these assays will serve as a platform for assessment of diseased periventricular endothelial cells. Our work has established novel autonomous links between the periventricular vascular network and the origin of psychiatric disorders like schizophrenia, epilepsy, autism, and major depression[3,5]. These assays will be invaluable in identifying potential defects in long distance migration, chemo-attraction, or juxtracrine signaling of diseased-periventricular endothelial cells in these psychiatric disorder conditions.

These assays are simple, reproducible, and low cost, and they can be modified to measure cell migration and effects of co-culture or chemo-attractive cues on migration in various cell types, except for non-adherent cells. There are some critical steps that need to be followed to obtain accurate and reproducible results. First, it is critical to optimize seeding cell number for each assay. The number of cells to be seeded in a single compartment should depend on cell type, desired level of confluency, and assay-specific factors like co-culture ratio. Second, it is necessary to optimize the cell culture medium for each assay. In the co-culture migration assay and the chemo-attraction assay, where more than one cell type is seeded in a single dish, the assay medium should be conducive to all cell types. In pilot experiments, we examined the effect of co-culture medium on viability (using trypan blue exclusion method) and morphology (using immunocytochemistry) of each cell type. We cultured human GABAergic neurons with co-culture medium for one week and observed no significant difference in viability and morphology of neurons in co-culture medium compared to neurons cultured in neuronal medium. In a similar fashion, periventricular endothelial cells and control endothelial cells, cultured in co-culture medium for two passages, did not show any significant variation in cell survival and morphology.

Third, since rate of migration varies among different cell types, it is important to determine the time frame for each assay for the cell type(s) being studied. Fourth, it is critical to handle the culture inserts carefully. Inserts should be fixed firmly on the dish by gently pressing with a fingertip. The dish should be turned upside down to verify that the insert is not moving. Care should also be taken while removing the insert so as not to disturb the cell layer. Finally, it is recommended to increase sample size to reduce experimental variability.

In conclusion, these assays will significantly expand our understanding of human periventricular endothelial cell biology and its role on brain development in normal and diseased conditions.

ACKNOWLEDGMENTS:

This work was supported by awards from the National Institute of Mental Health (R01MH110438) and National Institute of Neurological Disorders and Stroke (R01NS100808) to AV.

DISCLOSURES:

Authors declare no conflict of interest.

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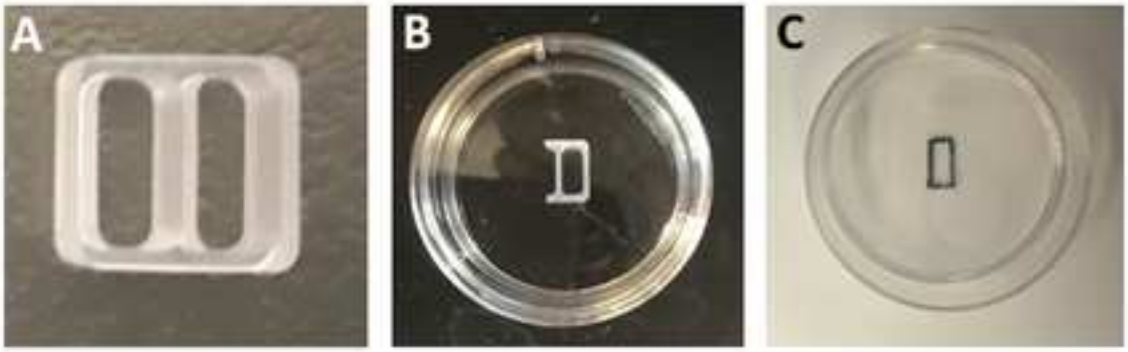

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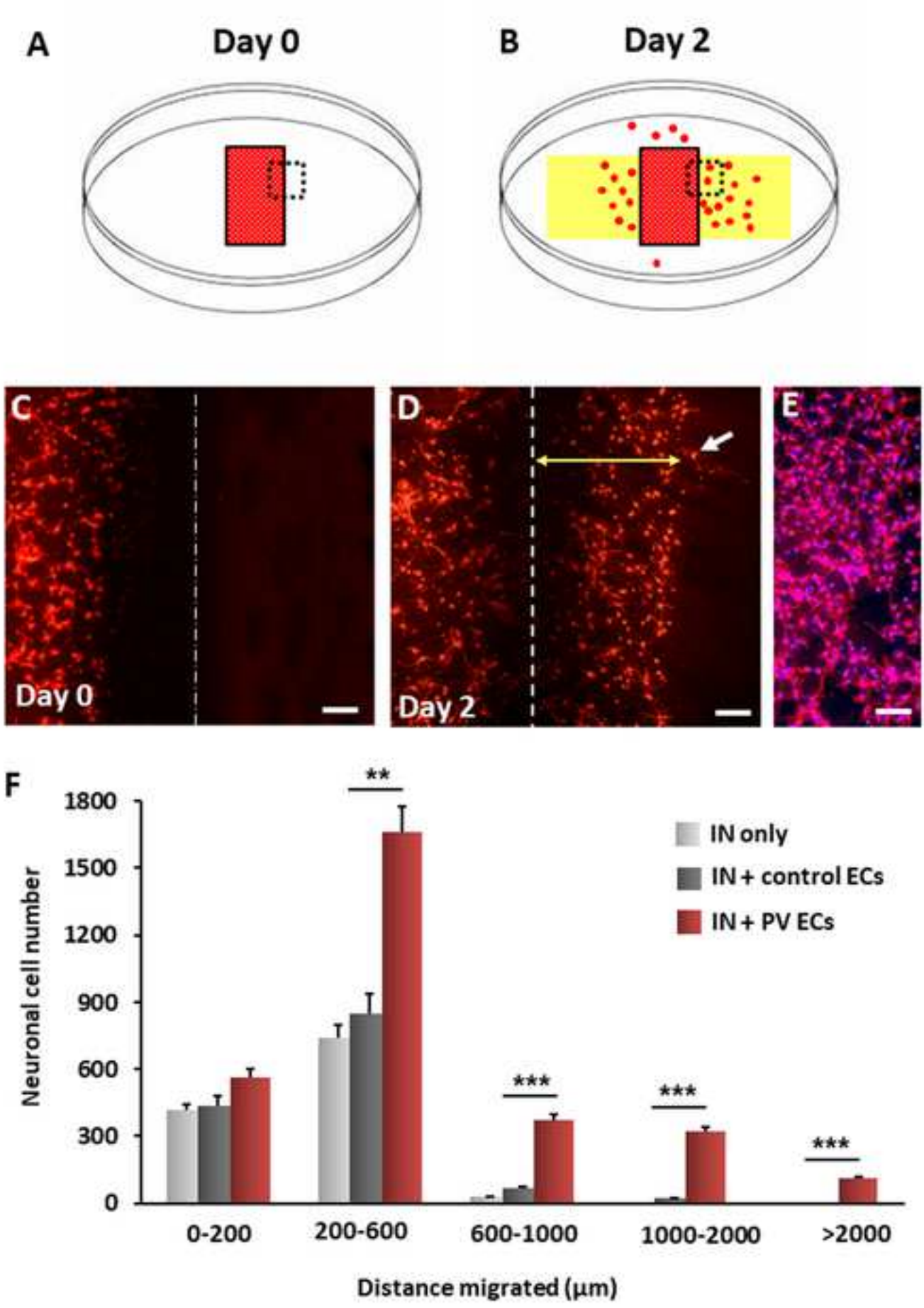

Figure 3

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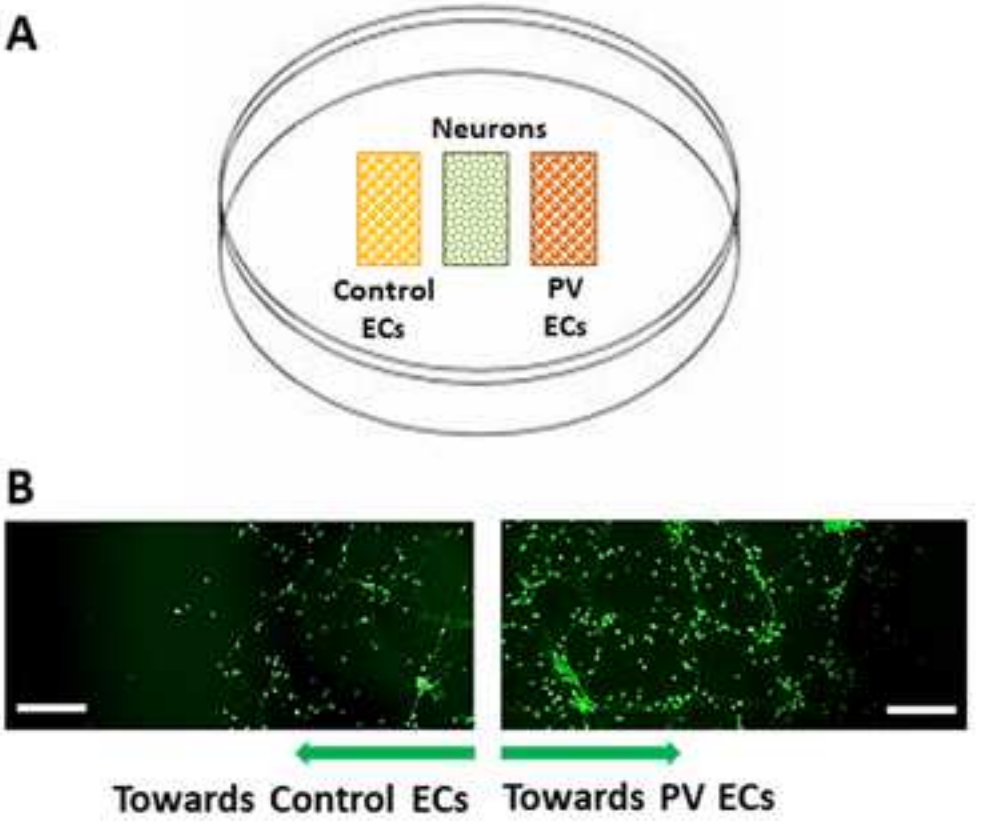

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A	Advantages
Boyden chamber assay[16,17]	· Technically non demanding · Suitable for adherent and non-adherent cells · Can be modified to study effect of paracrine signaling or chemo-attractants on cell migration
Scratch assay[18]	· Endpoint or kinetic · Technically non demanding
Long distance migration assay	· End point or Kinetic · Allows study of long-distance migration between 1.5 to 2 cm · Technically non demanding
Co-culture migration assay	· End point or kinetic · Allows study of the effect of direct cell-cell contact on migration · Allows migration length of up to 1.5 to 2 cm · Technically non-demanding
B	**Advantages**
Boyden chamber assay	· Technically non-demanding · Suitable for adherent and non-adherent cells
Under-agarose assay[19]	· Technically non-demanding · Two or more chemo attractive signals can be assayed in one set up
Capillary chamber migrati	· Endpoint or kinetic · Suitable for adherent or suspension cells
Microfluidic device[22]	· Generates controllable and stable concentration gradient · Allows single cell -level resolution
Chemo-attraction assay	· End point or kinetic · Gradual concentration gradient · Suitable for real time or fluorescent imaging · Technically non-demanding

Limitations
· Endpoint assay. Not suitable for real time imaging · Not suitable for study of effect of direct cell-cell interaction on migration
· Assays migration length of a few hundred micrometers. Not suitable for distance migration in the range of 1-2 cm. · Not suitable for suspension cells · Variations in scratch area
· Not suitable for suspension cells
· Not suitable for suspension cells

Limitations
· Endpoint assay. Not suitable for live imaging · Steep concentration gradient
· Not suitable for adherent cells. Restricted mostly to blood cells · Difficult visualization of cells in agarose
· Needs special chambers
· Needs sophisticated devices and tools · Technically demanding and steep learning curve · Complex imaging and data analysis
· Not suitable for suspension cells

Table of Materials

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Name of Material/Equipment	Company	Catalog Number
Accutase dissociation solution	Millipore Sigma	SCR005
Anti-human β-Tubulin antibody	Biolegend	802001
Anti-human CD31 antibody	Millipore Sigma	CBL468
Anti- MAP2 antibody	Neuromics	CH22103
Anti-active Caspase 3 antibody	Millipore Sigma	AB3623
Control human endothelial cells	Cellular Dynamics	R1022
Control endothelial Cells Medium Supplement	Cellular Dynamics	M1019
Cryogenic vials	Fisher Scientific	03-337-7Y
DMEMF/12 medium	Thermofisher Scientific	11320033
DMSO	Sigma-Aldrich	D2650
E6 medium	Thermofisher scientific	A1516401
FGF2	Thermofisher Scientific	PHG0261
Fibronectin	Thermofisher Scientific	33016-015
Freezing Container	Thermofisher Scientific	5100
GABA	Sigma-Aldrich	A2129
Hemacytometer	Sigma-Aldrich	Z359629
Human GABAergic neurons	Cellular Dynamics	R1013
Human GABAergic neurons base medium	Cellular Dynamics	M1010
Human GABAergic neuron Neural supplement	Cellular Dynamics	M1032
Laminin	Sigma	L2020
Matrigel	Corning	356230
Mounting Medium	Vector laboratories	H-1200
poly-Ornithin	Sigma	p4957
PBS	Thermofisher scientific	14190
Trypan blue	Thermofisher scientific	15250061
TrypLE	Thermofisher Scientific	12563011
VEGF-A	Peprotech	100-20
VascuLife VEGF Medium Complete Kit	Lifeline Cell Technologies	LL-0003
2-well silicone culture-Insert	ibidi	80209
3-well silicone culture-Insert	ibidi	80369
35 mm dish	Corning	430165
15-ml conical tube	Fisher scientific	07-200-886

4% PFA solution	Fisher Scientific	AAJ19943K2
6-well tissue culture plate	Fisher Scientific	14-832-11
Inverted phase contrast microscope	Zeiss	Zeiss Axiovert 40C
Fluorescent microscope	Olympus	FSX-100

Comments/Description

Cell dissociation solution (for periventricular endothelial cells, step 1.4)

Basement membrane matrix

Cell dissociation solution (for GABAergic interneurons and endothelial cells, sections 3 and 4)

Component of control human endothelial cell medium

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Author(s):	Debkanya Datta and Anju Vasudevan

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Response to reviewers' comments (Manuscript # JoVE60295)

1. Response to editorial comments

General:

1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*

Response: The revised manuscript has been properly proofread.

2. *Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.*

Response: We have formatted the manuscript as per the above guidelines.

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For example: Accutase, TrypLE, Milli-Q, Sharpie.

Response: We have removed reference to Milli-Q, Sharpie. We have replaced Accutase with 'acutase cell dissociation solution" (page 3, step 1.4). We have retained the use of "TrypLE" as it is the dissociation reagent recommended for optimal use of GABAergic interneurons and control endothelial cells.

Protocol:

1. *There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.*

Response: Filmable protocol has been highlighted in yellow. Some of the steps post-cell seeding are common in all three assays. We have highlighted these steps when mentioned first (in the long-distance migration assay) to adhere to the 2.75 page limit.

2. *For each protocol step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to*

perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Response: We have adhered to these guidelines in the revised manuscript.

Specific Protocol steps:
1. *1.2.1/1.3.1: What should the cell density/confluence be here?*

Response: We have mentioned the percentage cell confluency required at these two steps. (page 4, step 3.1; page 5, step 4.1).

2. *3.5/4.5: Please explain what proper seeding looks like.*

Response : By "proper seeding' we meant cells that adhered properly and there was no leakage from the insert compartment during overnight seeding. We have included this in the revised manuscript (page 6, step 6.5; page 7, step 7.6; page 8, step 8.7).

Figures:
1. *Please remove 'Figure 1' etc. from the Figures themselves*.

Response: We have removed the texts "Figure 1", "Figure 2", "Figure 3" etc. from the actual figures.

2. *Define the scale in the appropriate Figure Legend.*

Response: We have added scale bar for all microscopic images and defined the scale in the figure legend.

3. *Figure 2E, 2F, 3C: The legend mentions mean ± S.D., but there are no error bars.*

Response: We have added error bars in the revised graphs.

References:
1. *Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.*
2. *Please do not abbreviate journal titles.*
3. *Please do not include references that have not been accepted yet (the current reference 10).*

Response: We have formatted the references and excluded reference #10.

Table of Materials:
1. *Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.*

Response: We have updated the Table of Materials and provided information on all materials and equipment used.

Response to comments from Reviewer #1:

In this manuscript, the authors proposed protocols for three in vitro assays - the long-distance migration assay, the co-culture assay and the chemo-attraction assay - using co-cultures of paraventricular endothelial cells with or without human induced GABAergic interneurons.

These assays may allow for live cell time-lapse imaging which offers an advantage over conventional cell migration assays such as Transwell systems that only display the end result of the migration process. However, there are some concerns need to be addressed:

Response: We thank the reviewer for appreciating the advantage of our assays.

Major points:

1. *It will be nice to include a chart to compare and contrast assays described in this manuscript with other existing relevant assays.*

Response: We have included a chart (Table 1) that compares the features of our assays with those of other relevant assays that are commonly used.

2. *The authors need to provide more information on 1) the origin, stage, and subtypes (eg. SST or PV) of iGABA neurons, 2) contents of culturing media, 3) any adverse effect of mixed medium on the growth and morphology of endothelial cells and iGABA neurons, 4) the origin of control endothelial cells, and 5) how they optimized the seeding density of iGABA neurons and endothelial cells.*

Response:

1) We have purchased the human GABAergic neurons from a commercial vendor (mentioned in NOTE, step 3, page 4). These are derived by differentiating a human fibroblast-derived iPSC line. These neurons have been subtyped for regional and neural markers by the company and are available from them upon request. We have not characterized these cells further for SST, PV subtype specificity.
2) We have provided complete information on the content of culturing medium of human periventricular endothelial cells (page 3, step 1.1).
3) For GABAergic interneurons and control human endothelial cells, culture media are purchased from the vendor (mentioned respectively in NOTE, step 3, page 4;

NOTE, step 4, pages 4 and 5). The information is also listed in Table of Materials.

4) Adverse effects of co-culture medium on neurons and endothelial cells have been tested by cell viability assay and morphology study using immunocytochemistry. This has been mentioned in the discussion of our revised manuscript (page 11).
5) Control endothelial cells are obtained from the differentiation of a human fibroblast-derived iPSC line. Further information on the source of fibroblasts or differentiation method is available from the vendor.
6) Seeding density of GABAergic neurons were as per manufacturer's recommendation (information provided in NOTE, step 6.1, page 6). The seeding density of control endothelial cells and human periventricular endothelial cells were followed to maintain 1:1 ratio with seeded neurons (mentioned in NOTE in step 6.2, page 6).

3. *The authors may use simple plots instead of 3-D plots in Figure 2, 3, and 4.*

Response: We thank the reviewer for this suggestion. We have changed our graphs and used simple bar graph plots in the revised version.

4. *The authors need to revise the sentence on page 4 "paraventricular endothelial cells were co-seeded with the GABA-interneurons to mimic the conditions in vivo", which seems to be an overstatement.*

Response: We have rephrased this sentence (page 2).

5. *It is not clear why Figure 1C was included.*

Response: Original Figure 1C was included to show the outlined rectangular patch inside which cells are seeded. We have included a better image to represent this in the revised Figure 1. This is mentioned in the text (page 6, step 5.10) and in revised figure legend for Figure 1 (page 10).

6. *The authors may want to check apoptotic cells during the migration assay.*

Response: We have confirmed absence of apoptosis in the cells by staining with anti-active Caspase 3 antibody. Data has been included in revised Figure 2E and mentioned in the Representative Results section (page 9) and in Figure legends for Figure 2 (page 10).

7. *Size bars are missing in Figures 1,2C and D, and 3B.*

Response: We are sorry to have missed the scale bars. We have added scale bars for all microscopic images and defined the scale in the figure legends.

8. *How the authors normalized percentages of cells in Figure 3C? Also, it looks like less than 3% of cells migrating in Figure 2F. What was the criteria for cell migration?*

Response: We have changed the original plot in Figure 3C. In the new graph, we have removed the normalization and plotted the graph as relative number of neuronal cells that have migrated towards periventricular endothelial cells or control endothelial cells.

In Figure 2, we have re-represented cell number versus distance migrated as a single graph (in Figure 2F). For clarity and consistency, in this graph too we have plotted migrating neuronal cell number versus distance travelled.

Minor Concerns:
Not applicable.

Response to comments from Reviewer #2:

Manuscript Summary:
This protocol provides a good resource as to assay the interneuron migration affected by periventricular endothelial cells, and can be easily applied to monitor migration of many other cell types.

Response: We thank the reviewer for the positive remarks.

Major Concerns:
None.

Minor Concerns:
1. *It is better to clarify (and state the detailed steps) as to whether and how the differentiated periventricular endothelial cells can be regularly passaged and stored before performing the assays.*

Response: We have added an extra section in protocols detailing the steps for routine culture and freezing of human periventricular endothelial cells. This is the new step 1 (pages 3, 4).

2. *Line 116: before detach cells by adding Accutase, should the medium in the wells be removed first?*

Response: The medium needs to be removed first before adding Accutase. We missed this step, and thank the reviewer for pointing it out. We have added this step in the revised manuscript (page 3, step 1.3).

3. *Line 125, Line 134: before detach cells by adding TrypLE, should the medium in the wells be removed first?*

Response: We have added this step in the revised manuscript (page 4, step 3.3; page 5, step 4.3).

4. *For Steps 1 to 3 (page 5), it is better to put more detailed steps about how the authors prepare Matrigel, poly-L-ornithine/laminin, fibronectin coated plate, respectively.*

Response: We have provided more detailed steps on preparation of Matrigel (page 3, step 1.2) and poly-L-ornithine/Laminin (page 5, steps 5.1 and 5.3).

We purchased control human endothelial cells from a company (mentioned in Table of Materials). We followed manufacturer's recommended protocol for culturing these cells on Fibronectin substrate. Fibronectin coated plates were prepared according to manufacturer's protocol. In the revised manuscript, we have omitted the mention of fibronectin-coated plates. Instead, we have referred to follow the manufacturer's protocol for preparation of fibronectin coated plates 9 (mentioned in NOTE following step 4, page 5).

5. *The numerical order of the steps could be confusing:*
Page 4: 1. Cell Preparation
Page 5: 1. Preparation of human periventricular endothelial cells
Page 5: 3. Preparation of control human endothelial cells
Page 6: 2. Preparation of One-well Culture Inserts
The author should put the numerical orders in a better way.

Response: We have changed the numbering order. The new format consists of major steps 1, 2, 3 and sub-steps 1.1, 2.1, etc.

6. *Line 176, 178: what kind of medium is used?*

Response: For neuron dish, neuronal medium is used, and for endothelial cell dish, endothelial cell medium was used. We have revised the line and added information on the kind of medium in the revised manuscript (page 7, step 6.7).

7. *Line 213, 214: it is better to link the text to Figure 3A here as well.*

Response: We have linked this step to Figure 3A (page 8, step 8.4).

Response to comments from Reviewer #3:

Manuscript Summary:
The manuscript by Dutta and Vasudevan provides new assays to assess the interaction between specialized human iPSC-derived endothelial cells and interneurons.

Response: We thank the reviewer for commenting on the novelty of the assays.

Minor Concerns:
***1.** While the assays are described well and in details that can be reproduced, the manuscript can benefit from a better introduction of the endothelial subtypes in brain and their function both during development and in adult brain.*

Response: We have provided information about the different endothelial subtypes in the embryonic forebrain and their distinctive features (page 2).

2.*Similarly, the discussion could include potential effects of these endothelial cells on brain cells other than interneurons*,

Response: We have discussed the potential effects of these endothelial cells on neural progenitors and projection neurons (page 11).

3. Typos and grammatical mistakes should be checked.
Line 30….vasculature in nervous system
Line 43… alludes to
Line 69… patterns

Response: We have corrected these mistakes in the revised version.

4. lines 74/75…. Using the stem cell technology….should this be iPSC technology….

Response: We have replaced “stem cell technology” with “iPSC technology” (page 2).

5. please add a brief 1-2 lines description of the technology used in the lab that was previously published.

Response: In-depth protocol for generation of human periventricular endothelial cells has been developed in the lab, and is part of a manuscript that is currently undergoing peer review.