PROTOCOL TO TEST CELL MIGRATION ON CULTURED BRAIN SLICES

CM = culture medium for the cell line of choice.

Soft agar plates

1) Prepare in the laminar flow hood a stack of 35mm culture dishes (~15 dishes).

2) Dissolve 0.3 g ultrapure agarose (American Bioanalytical AB00972) into 30 ml of DMEM containing 2% FBS (use a 50ml Falcon tube). There is no need to prefilter the DMEM+FBS medium. The result will be a hazy suspension.

3) Carefully microwave the suspension until it is clear: Keep the cap of the Falcon loosely applied on top of the tube during the microwaving and dissolve the agarose using several cycles of 10 or 5 seconds each. **DO NOT** allow the DMEM to spill over from the tube (it can spill if left boiling unattended).

4) Recap the Falcon tube loosely (the tube needs to vent vapor) and transfer it to the laminar flow hood. Keep swirling the tube every couple of minutes and check for the temperature of the tube exterior with a thermometer. Once the temp. is ~45 °C, filter the contents of the tube through a 0.22 µm filter (Millipore Steriflip)

5) Quickly pour ~1.5ml of 1% agarose into the 35mm culture dishes and allow the medium to gelify. Cover the dishes only halfway until the agarose is solid.

6) Wrap the dishes in saran-wrap, in packs of 4-6 dishes. Store them inverted at 4 °C. Dishes last for ~ one month.

Cell culture to prepare spheroids:

Several days before the experiment seed cells in 60mm dishes and wait until 90% confluence for transfection. Culture the cells with 5 ml CM/dish.

Optional: membrane-tracker staining (to provide fluorescence to cells that will not express fluorescent proteins). Remember: Vybrant DiI=red; DiO=green

One day before transfection (~80-85% confluence) stain the cells with Vybrant (membrane-tracker) dye, using the following protocol:

1) Prepare a 1/200 dilution of Vybrant in CM (use ~1 ml dilution / 60 mm dish).
2) Remove CM from the cells and wash 1x PBS
3) Add Vybrant and return to the incubator (37 °C) x 20 min
4) Remove Vybrant and wash 2 x CM
(Vybrant staining of the cells lasts ~ 2 weeks but gets very diluted after 1 week)

1) Transfect the cells in 60mm dishes with 4 µg of DNA (in 500 µl Optimem) and 10 µl Lipofectamine-2000 (in 500 µl Optimem), for 4 hours.

2) During the transfection, re-hydrate soft-agar plates with 1 ml CM and leave in the incubator at 37 °C for at least 2 hours before use. Discard the CM using for re-hydration and add 1ml fresh CM to the agar plates.

3) Wash transfected cells twice with DMEM or RPMI (without FBS) and trypsinize for 1-2 min at room temp.

4) Centrifuge the cells and resuspend with thorough disaggregation (important for U87MG line).

5) Seed ~ 300,000-500,000 cells / soft-agar plate if you want to use them the next day. Alternatively, seed ~75,000-100,000 cells/soft-agar plate and let the aggregates grow for 3-4 days. The first method is fast but the cells aggregate too fast and the aggregates will be big and irregular. **Optional:** to enhance aggregation and spherical shape of the aggregates, place the soft-agar plates with cells on a small, slow orbital rotator inside the incubator.

Brain slice medium: All media must be freshly prepared and filtered through 0.22 µm filter

Formula 1: (for shorter cultures, < 7 days)

• Medium Neurobasal-A without phenol-red	(Invitrogen 12349-015):	28.75 ml
• Hanks balanced buffer solution without phenol-red	(Invitrogen 14175-095):	12.50 ml
• DMEM high glucose with 25 mM HEPES and L-glutamine, (without Na pyruvate, without phenol-red)	(Invitrogen 21063-029):	6.25 ml
• 100x Sodium pyruvate	(Invitrogen 11360-070):	100 ul
• 100x L-glutamine (200 mM)	(Invitrogen 25030-081)	250 ul
• 50x Culture supplement B27	(Invitrogen 17504-044):	1.00 ml
• 100x Culture supplement G5	(Invitrogen 17503-012):	500 ul
• 100x Penicillin/Streptomycin/Amphotericin		500 ul
	Total:	50 ml

[Optional: Reduce G5 to 200ul and B27 to 400 ml and add FBS to 1% final concentration, slices will last longer but cell migration may be affected by the serum]

Note: This medium is transparent, without Phenol Red. If a pH indicator is desired you can:
a) Replace the DMEM Invitrogen 21063-029 with DMEM Invitrogen 12430-054 (contains PheRed)
b) Add only 28.5 ml of Neurobasal medium and complete with 250 ul of 0.3% w/v PheRed prepared in culture H_2O.

Formula 2: (for long-term cultures from more mature slices, from the Lab or E. A. Chiocca)

• Medium MEM without glutamine or phenol red	(Invitrogen 51200-038):	25 ml
• Hanks balanced buffer solution without phenol red	(Invitrogen 14175-095):	11.5 ml
• Fetal bovine serum, heat inactivated		12.5 ml
• 100X Glutamine (200 mM)		500 µl
• 100X Penicillin/Streptomycin/Amphotericin		500 µl
	Total:	50 ml

Note: This medium is transparent, without Phenol Red. If a pH indicator is desired you must replace the MEM and the glutamine with 26.5 ml of MEM containing phenol red and glutamine (Invitrogen 11095-080)

Preparation of slices:

Material needed in advance:

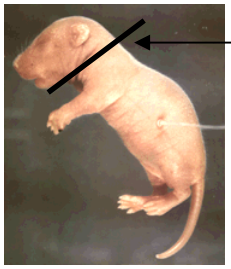
- 35 mm dishes or 6-well plates, sterile;
- MilliCel membranes, one per dish or well (~ 2-3 slices/ membrane);
- Surgical instruments, sterilized;
- Large and small spoonulas, sterilized;
- 1 box with 200 µl tips, sterilized;
- surgical absorbent spears (Sugi REF30601);
- kimwipes;
- nitrocellulose discs, 25mm x 45 µm (Millipore HAWP02500)
- clean razor blades for the tissue chopper;
- several 100-200 ml beakers with 70-95% ethanol;

Preparations in advance:

a. Several (4-5 per animal) 60mm dishes with 4ml of HBSS 1X (Invitrogen 14175-095, containing 2X Penicillin/Streptomycin and 2X Amphotericin. Place all dishes at 4 °C.
b. Turn the glass-bead sterilizer on and let it reach 250 °C.
c. Prepare a bag to collect the carcasses.
d. Prepare 2 ice buckets full of ice.
e. Under the culture hood, place several MilliCel membranes on 35mm dishes containing ~1 ml of slice medium. Allow them to re-wet and place them at 37 °C in the incubator

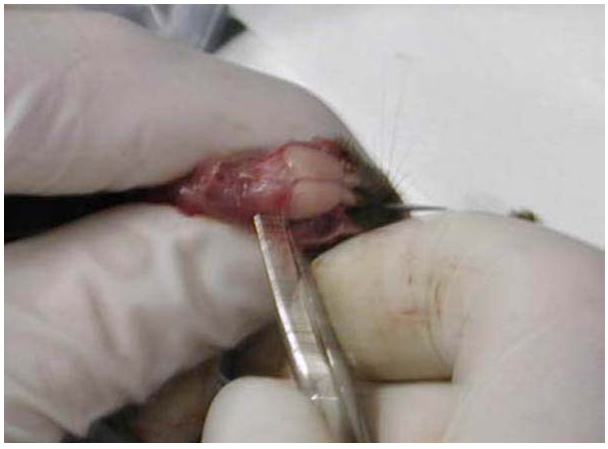
Procedure:

1) Cut the head of a pup at neck level and let if fall on cold HBSS + antibiotics on ice (discard the carcass)

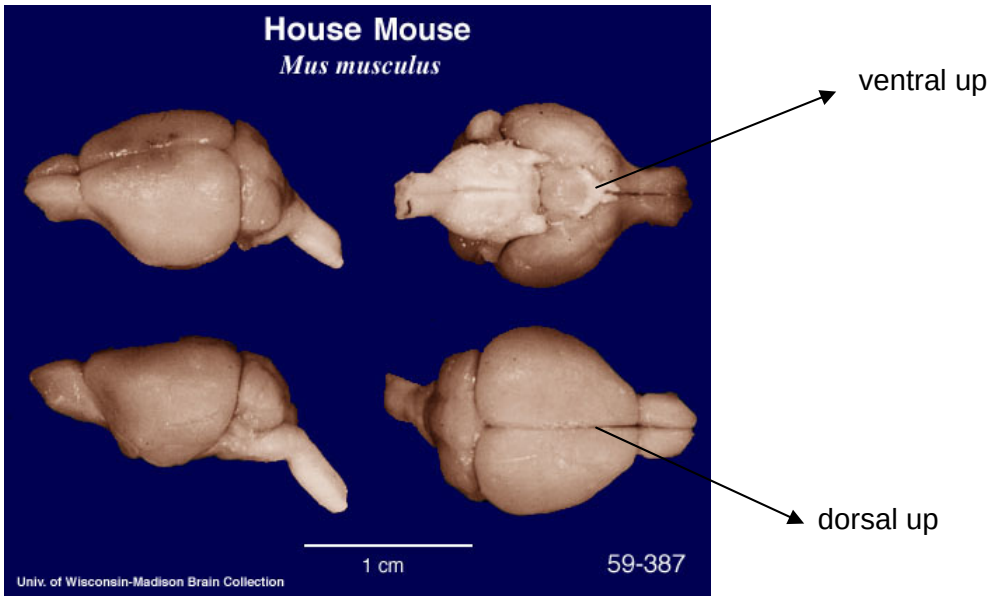


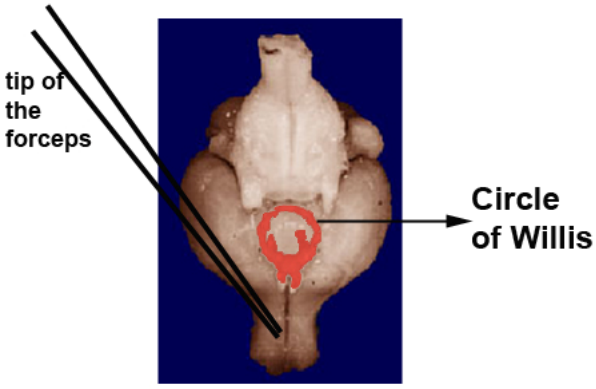
2) Peel the skin of the head carefully and cut the cartilaginous bones in the skull. Always do tangential cuts to avoid damage to the brain! Expose the brain.

3) With a small spoonula, scoop the brain/brainstem out of the skull (cut the optic nerves!) and into a new dish with clean, cold HBSS + antibiotics.



4) Under dissecting microscope, place the brain ventral up and start peeling the meninges from the **Circle of Willis** outward. Use a #5 Dumont fine forceps to peel and another to pin the brain down to the plastic dish (without using much force! The Dumonts are **EXTREMELY delicate**!!). Pin the brain down at the brainstem level to avoid damaging the hemispheres.





5) Once the lower part of the brain looks clean (no major membranes with bloody appearance), invert the brain (= dorsal up) and peel the meninges from the upper part. It is important to peel the meninges from the frontal lobes more than the caudal part of the brain. Olfactory bulbs may be removed to help with the peeling.

6) Place a 25mm nitrocellulose disk on the cutting block of the tissue chopper and wet it with cold HBSS.

7) Transfer the cleaned brain with a large spoonula onto the nitrocellulose disk rotate the cutting block until the brain is perfectly perpendicular to the blade of the chopper. With a rolled kimwipe remove as much liquid as possible from under the brain.

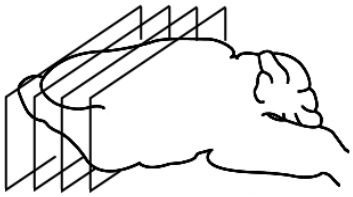
8) Set the chopper for cutting thickness = 300 (P1-P2 pups) or 350 (P0 pups) µm. Set the speed to medium level and the strength of the arm to minimum.

9) Start the chopper and let it cut through the whole brain. Stop the chopper if the brain gets squashed by the blade or if it sticks to the blade. In those cases, discard the brain.

10) Re-wet the brain with a drop or two of cold HBSS.

11) **VERY CAREFULLY** use the large spoonula to scoop the sliced brain and place it in a new dish with cold HBSS. You may use a second spoonula to help support the brain during the scooping.

12) Using two #5 Dumont forceps, separate as many slices as possible. If necessary, cut the slices at the midline to obtain two hemi-slices (easier to manipulate). The cortex will likely twist itself inward during the manipulations. If necessary, it may be removed as far as the slice has a good portion of thalamus or striatum to place cells on.



(slicing planes)

13) After separating the slices (typical yield: 5-10/animal), use a sterile Pasteur pipette with a blunt, wide tip to transfer the slices onto the MilliCell membranes. Place the slices carefully on the membranes until they are flat.

14) Remove the liquid around the slices with the surgical spears. **BE CAREFUL** not to touch the slices or they will stick to the spear!!

Placing cells on the slices:

1) Draw several marks on the lid and lower plate of each dish containing slices; this will help to reposition the dish in the same position each time you need to take pictures.

2) Using a sterile 200 µl tip and a clean razor blade, cut ~ 3-4 mm at the end of the tip to make a blunt, angled tip.

3) Using a P2 pipettor (set at 1 µl), collect a cell aggregate from the soft-agar dish under the dissecting scope. Aspirate the lowest possible amount of liquid to get the cells into the tip.

4) Carefully transfer the cells onto the slice, **without touching the slice with the tip**!! Cells should preferably be placed onto large, homogeneous areas such as the ***striatum*** or the ***thalamus*** (avoid cortex and hippocampus unless desired for a particular test). If the cells fall on a different place, do not touch them! Avoid getting close to the cells with the tip after they have been ejected.

5) If some liquid is ejected together with the cells and covers the slice, try to remove it with the surgical spears.

6) Take pictures of the cells on the slices 1-2 hours after all of them have been “seeded”. Repeat every 24 hours. Migration may be detected overnight (CNS-1 cells) or after several days (U373 cells, some glioma stem cells)