Part II: Cellular Neuroscience Practical

1. Safety information
2. Practical protocol/methodology

1. Safety Note for Cellular Neuroscience

All students must follow the instructions accurately and adhere to the principles of Good Laboratory Practice (GLP) at all times as set out below:

- **Do not eat, drink, smoke or apply make-up.**
- **Wear a lab coat, and fasten it correctly.**
- **Do not pipette anything by mouth.**
- **For the parts of the practical where 4% formaldehyde or MTS solution are being used wear vinyl gloves. Do not wear gloves in corridors.**
- **Always use the correct route for waste disposal. All tissue culture plates, pipettes, etc must be placed in the Sterilin bags for autoclaving.**
- **Use eye protection when using MTS solution or 4% formaldehyde.**
- **Keep benches tidy and floor areas and corridors unobstructed.**
- **Know the drill for emergency evacuation.**
- **Know who your nearest first-aiders are.**
- **All** Accidents **and** Dangerous Occurrences **must be reported.**

For the part of the practical where you are fixing your cells with 4% formaldehyde, use a fume hood as directed

2. Practical Protocols/methodology

You will come to the practical each day already having planned your experiment. These notes are for guidance, but each procedure will be demonstrated.

Monday 3rd December (DAY 1)

Setting up 3T3 cell proliferation assay

*Remember to use the sterile technique as demonstrated. Label the Pasteur pipette box, the boxes of blue and yellow Gilson tips and the bottle of DMEM provided as you will retain them **for your own use** throughout the practical. Keep them sterile. **NB: NEVER use tips belonging to another group; ask if you need another box.***

1. You will be provided with a plate of 3T3 cells. Look at them under an inverted microscope and draw a quick sketch of their appearance.

2. In a sterile tissue culture hood aspirate the growth medium from the plate using a sterile Pasteur pipette.

3. With a sterile 5 ml pipette, add 5 mls of warmed trypsin solution to the plate then place it in the 37°C tissue culture incubator (8% CO_2) for 10 minutes to allow the cells to round up and detach from the bottom of the plate.

4. [During trypsinisation, make up 5 ml of DMEM containing 10% FCS ready for neutralizing the trypsin (see step 5). Also make up enough DMEM containing the % of FCS (probably DMEM/0.5% FCS) that you have decided to use for plating the cells into 96-well plates (steps 7 & 9).]

5. Remove the plate of trypsinising cells (step 3) from the incubator & check under the inverted microscope that the cells have indeed rounded up and are detaching. If so, add 5 mls of DMEM containing 10% FCS to neutralise the trypsin and gently triturate about five times to make sure all the cells are washed off.

6. Transfer the 10 mls of cell suspension to a sterile Universal tube (in the tissue culture hood) and place in a bench-top centrifuge (remember to use a balance tube). Centrifuge the suspension at 1000 RPM for 5 min to pellet the cells.

7. Carefully aspirate the trypsin/medium from the cell pellet using a Pasteur pipette. Add 5 mls of DMEM containing FCS (at the percentage you have decided to use in your plan, made up in step 4, probably DMEM/0.5% FCS) and re-suspend the pellet by triturating up and down firmly approximately twenty times with a sterile 10 ml pipette. The trituration should be vigorous, but minimize bubble formation. Remove a small volume (~0.25 mls) of the cell suspension and transfer to a sterile Bijou tube.

8. Using a sterile tip and Gilson pipette, transfer some (~ 20 µl) cell suspension from the Bijou tube to fill the haemocytometer chamber by capillary action. Determine the number of cells per ml in your cell suspension using the haemocytometer and either the upright or inverted microscopes. Discard the cells remaining in the tip and Bijou tube into a Sterilin bag.

9. Once you have determined the number of cells per ml in your cell suspension, dilute it down using the %FCS DMEM (made up in step 4, probably DMEM/0.5% FCS) that you have chosen to use, to give the final cell density required.

10. Add the correct volume of cell suspension to each well in the 96-well plate(s). Remember that the maximum volume is 200 µl and the minimum is 50 µl. Bear in mind that, to assay for cell number at the end of the experiment, 20µl MTS solution must be added to **100 µl** of growth medium per well.

11. Remember to include a BLANK on EVERY PLATE, of 100 µl of basal medium or DMEM, usually in well A1.

12. Label and date plates and place them in the incubator until the next day.

Tuesday 4th December (DAY 2)

(a) Assaying starting cell number in 3T3 proliferation assay ('Day 0 Control')

If cells are healthy (check with demonstrator), carefully add 20 µl of MTS solution to the 100 µl medium in the well. **COSHH note: Vinyl gloves must be worn when handling MTS solution, and safety glasses worn.** (Ask a demonstrator for MTS solution). Replace plate in incubator, clearly labelling it with your name and the time of MTS addition. A demonstrator will read your plate in an ELISA plate reader at 490 nm after exactly 3 hours.

(b) Adding growth factors/inhibitors to 3T3 proliferation assay

1. Before proceeding, check with a demonstrator that your experiment is worth continuing. There is no point adding growth factors/inhibitors to dead or infected cells.

2. If cells are OK, carefully make up all the growth factor and inhibitor dilutions in DMEM/%FCS medium according to your plan. Obtain growth factor & inhibitor aliquots from a demonstrator.

3. Carefully add medium containing growth factors and inhibitor solutions to appropriate wells of your 96-well plate(s) according to your plan. Place plate(s) in the incubator and leave until the day the MTS assay is required.

4. Add 100 µl to two spare wells to act as additional 'blanks.' Clearly mark which one well you want the demonstrators to use to blank the plate reading machine.

Wednesday 5th December (DAY 3)

Assaying cell number in 3T3 proliferation assay (if you set up a plate to measure relative cell number after 1 Day in culture)

1. Before proceeding, take your 96-well plate(s) from the incubator and check with a demonstrator that your experiment is worth continuing. There is no point adding MTS

reagent to infected cells, as you will be assessing the number of bacteria or fungi rather than 3T3 cells.

2. If OK, add 20 µl of MTS reagent very carefully to the 100 µl medium in each well. **COSHH note: Vinyl gloves must be worn when handling MTS solution, and safety glasses worn.** (Ask a demonstrator for MTS). Replace plate in incubator, clearly labelling with your name and the time of MTS addition. A demonstrator will read the plate in an ELISA plate reader at 490 nm after exactly 3 hours.

Thursday 6th December (DAY 4)

Assaying cell number in 3T3 proliferation assay (if you set up a plate to measure relative cell number after 2 Days in culture)

Check with a demonstrator that your experiment is worth continuing. If OK, add 20 µl of MTS reagent very carefully to the 100 µl medium in each well. **COSHH note: Vinyl gloves must be worn when handling MTS solution, and safety glasses worn.** (Ask a demonstrator for MTS). Replace plate in incubator, clearly labelling with your name and the time of MTS addition. A demonstrator will read the plate in an ELISA plate reader at 490 nm after exactly 3 hours.

Friday 7th December (DAY 5)

Assaying cell number in 3T3 proliferation assay

Check with a demonstrator that your experiment is worth continuing. If OK, add 20 µl of MTS reagent very carefully to the 100 µl medium in each well. **COSHH note: Vinyl gloves must be worn when handling MTS solution, and safety glasses worn.** (Ask a demonstrator for MTS). Replace plate in incubator, clearly labelling with your name and the time of MTS addition. A demonstrator will read the plate in an ELISA plate reader at 490 nm after exactly 3 hours.

Setting up dissociated cerebellar cultures

Remember to use sterile technique as demonstrated on day 1!

1. Three LabTek slides are provided. Take those to be used and coat with poly-L-lysine (PL) by adding 200 µl of the sterile X1 PL solution to each well. Replace lids and leave at the back of the tissue culture hood for at least 30 min. Label the cloudy part of the slides with your name, and information for you to distinguish your slides from one another.
   (* If you are planning to stain for oligodendrocytes in your experiment, you need a separate slide(s) for this as the anti-oligodendrocyte antibody needs a different staining protocol. This will be easier to manage if you keep all wells in which you wish to stain for oligodendrocytes together on this slide(s).)

2. While the slides are coating, transfer 5 mls of warmed trypsin solution to a sterile Universal tube, using the tissue culture hood. Label it with your initials & give it to me or the demonstrator, who will add to it (in a tissue culture hood) pieces of rat post-natal day four (PND4) cerebellum. Place the culture in the incubator for 15 min, with the top only loosely screwed on.

3. After 15 min, remove from incubator and, in the tissue culture hood, triturate twenty times using a sterile 10 ml pipette to dissociate the cerebellum. Put your tube back into the incubator for a further 5 min, again with the top only loosely screwed on.

4. After 5 min, remove the tube from the incubator and triturate the trypsin-cerebellum solution again ten (or more) times until you have cloudy suspension of cells (will still be some large clumps, this is OK).

5. Neutralise the trypsin by adding 5 mls DMEM/10% FCS medium with a sterile 5ml pipette, triturate to mix, then centrifuge at 2800 RPM for 5 min, using a balance tube. Whilst your tube is centrifuging, aspirate the PL solution from the LabTek Slides with a Pasteur pipette and wash by adding several drops of sterile distilled water per well with a sterile 10 ml pipette. Aspirate off the water and leave the slides at the back of the tissue culture hood with the lids on.

6. After centrifugation, remove your tube from the centrifuge carefully and take it to the tissue culture hood. Carefully remove supernatant from your tube, taking care not to disturb the pellet, **with a 10 ml or 5 ml sterile pipette, or sterile blue tip, not suction with a Pasteur pipette**. It is better to leave some supernatant, than to remove it all but risk removing/disturbing the pellet.

7. Re-suspend the pellet in 3 mls DMEM/5% FCS medium by triturating about 30 times with a sterile 5 ml pipette to achieve a cloudy cell suspension (may still be some visible large clumps, that's OK).

8. Leave the tube to stand for 2-3 minutes to allow the larger clumps to settle to the bottom. Carefully remove ~2-2.5 ml (or more) of the cloudy cell suspension to a fresh sterile bijou tube, leaving behind suspension containing any larger clumps.

9. Carefully remove ~20 µl from the cloudy cell suspension using a sterile tip and Gilson pipette and transfer to a haemocytometer to count the number of cells per ml. Dilute the cell suspension in DMEM/5% FCS medium to give the required cell density according to your plan (Recommended: 100,000 – 200,000 cells per well).

10. Add the required volume of cell suspension per LabTek well. Remember that the maximum volume possible is 500 µl and the minimum is 200 µl, and you need to add FGF2 (see point 11 next).

11. Obtain FGF2 from a demonstrator, make up FGF2 dilutions and carefully add to the cerebellar cell suspension previously plated in your 8-chamber slide, according to your plan. Place plate(s) in the incubator and leave until fixing and staining on Day 6 (Monday).

12. Place slides (labelled and dated) in the incubator for culture over the week-end. They will be fixed and stained on Day 6 (Monday 10th Dec).

Monday 10th December (Day 6)

Fix and immunostain cerebellar cell cultures

Sterility is not required for this technique.   Do not allow your cells to dry out at any stage.

FIXED STAINING of astrocytes and neurons:
(NB: to stain oligodendrocytes, follow alternative LIVE staining protocol)

1. Take slides from incubator and briefly observe cells using inverted phase microscope. Label your slides clearly with your name and information that will distinguish them from each other.

2. Take slides and tube of 4% formaldehyde in PBS to fume hood and fix cells by adding 400 µl 4% formaldehyde in PBS per well to the 400 µl growth medium already present. Leave in the hood for 15 min with the lids off (the volume exceeds the well capacity).

3. Gently tip the medium/formaldehyde onto a wad of blue tissue and press down gently several times. Add 200 µl 4% formaldehyde in PBS per well and leave as before in the fume hood for a further 15 min, this time with the lid on.

4. Invert and dab as before to remove formaldehyde solution, then wash twice by adding PBS provided, flicking down sink and dabbing on blue tissue wad.

5.** Working quickly to avoid letting the cells dry out, take the slides back to your bench and permeablise the fixed cells by adding 200 µl per well of 0.2%Triton X100 in PBS. Incubate at RT for 5 min, then wash twice with PBS.
    (Carry out step 5 and onwards to co-stain for GAP-43 or GFAP a slide already stained with O4)

6. Block non-specific binding sites by adding 200 µl per well of 10% FCS in PBS and incubate at RT for 45 min.

7. Flick down sink, dab on tissue wad and wash twice with PBS.

8. Ask a demonstrator for the volume and type of antibody you require (supplied as a x1 solution diluted in PBS/0.05% BSA solution). Add 150 µl antibody solution per well. Leave at RT for 1 hour. Wash three times with PBS as before.

9. Ask a demonstrator for the volume and type of secondary antibody you require (supplied as a x1 solution diluted in PBS/0.05% BSA. Add 150 µl antibody solution per well and leave at RT for 45 min. Wash four times with PBS as before.

10. Before flicking off final wash, ensure you have everything ready for mounting.

11. Flick off final wash, dab thoroughly on blue tissue to remove as much PBS as possible. Remove the well assembly and silicon gasket from the LabTek slide (as per demonstration). Without allowing the cells to dry out, carefully place drops of moviol mountant (obtained from a demonstrator) containing DAPI, as demonstrated, onto the well intersection points and gently lower a coverslip over the slide area. Place the slide between a sheet of blue tissue folded in half and gently press down to remove excess mountant (as per

demonstration). **Be very careful not to press too hard (or your cells will be squashed!), and do not move the coverslip once it is lowered.**

12. Give slides to a demonstrator for observing over the next few days, ensuring they are clearly labelled with your name and information that will allow you to distinguish your slides from one another.

LIVE STAINING of oligodendrocytes:

(Remember that the cells are LIVE so you need to be very careful not to wash them off during this procedure. Success depends on being very gentle with your washing and pipetting.)

1. Ask a demonstrator for the O4 antibody solution (O4 diluted in DMEM/20mM Hepes) and some DMEM/Hepes medium for washing. Place on ice and allow to become ice-cold.

2. Take your slide from the incubator. Carefully and gently pour off the growth medium onto a wad of blue tissue and dab very gently.

3. Very carefully add 200µl O4 antibody solution per well and place slide on ice. Leave for 1h.

4. After 1 h, carefully pour off the antibody solution onto blue tissue and dab very gently. Very carefully add 400 µl per well of ice-cold DMEM/Hepes medium and incubate slide on ice for 5 minutes.

5. After 5 minutes, pour off the DMEM/Hepes onto blue tissue and dab very gently.

6. Repeat with a further 400 µl ice-cold DMEM/Hepes medium, so that the cells have been very gently washed twice x 5 min. Add a final 400 µl DMEM/Hepes per well.

7. Take slide and tube of 4% formaldehyde in PBS provided to fume hood and fix cells by adding 400 µl 4% formaldehyde in PBS per well to the 400 µl DMEM/Hepes already present. Leave in the hood for 15 min with the lids off (volume exceeds the well capacity).

8. Gently tip the medium/formaldehyde onto a wad of blue tissue and press down gently several times. Add 200 µl 4% formaldehyde in PBS per well and leave as before in the fume hood for a further 15 min, this time with the lid on.

9. Invert and dab as before to remove formaldehyde solution, then wash twice by adding PBS provided, flicking down sink and dabbing on blue tissue wad.

10. Block non-specific binding sites by adding 200 µl per well of 10% FCS in PBS and incubate at RT for 45 min.

11. Flick down sink, dab on tissue wad and wash twice with PBS.

12. Ask a demonstrator for the anti-mouse IgM secondary antibody you require (supplied as a x1 solution diluted in PBS/0.05% BSA). Add 150 µl antibody solution per well and leave at RT for 45 min. Wash four times with PBS as before.

13. If you are staining **only** oligodendrocyes on this slide, before flicking off the final wash, ensure you have everything ready for mounting and proceed to point 14 below.

(**Alternatively**, if you now want to **additionally** stain the slide with an astroycyte- or neuron-specific antibody, flick off final wash, dab on blue tissue and continue with Point 5 (asterisked) onwards.)

14. Flick off final wash, dab thoroughly on blue tissue to remove as much PBS as possible. Remove the well assembly and silicon gasket from the LabTek slide (as per demonstration). Without allowing the cells to dry out, carefully place drops of moviol mountant (obtained from a demonstrator) containing DAPI as demonstrated onto the well intersection points and gently lower a coverslip over the slide area. Place the slide between a sheet of blue tissue folded in half and gently press down to remove excess mountant (as per demonstration). **Be very careful not to press too hard (or your cells will be squashed!), and do not move the coverslip once it is lowered.**

15. Give slides to a demonstrator, ensuring they are clearly labelled with your name, and information for yourself to distinguish one slide from another.

COSTAINING

N.B. For co-staining, ensure that your two primary antibodies are raised in different species and that you are using species-specific antibodies conjugated to two different fluorophores.

Possible combinations:

Neuron & astrocyte costain	**Yes**
Neuron & oligodendrocyte costain	**Yes**
Astrocyte & oligodendrocyte costain	**No**

1. If you choose to do **co-staining** of your cultures this will take an additional ~2 hours (neurons and astrocytes) or ~3 hours (oligodendrocyes and neurons).

2. For neuron/astrocyte costains, proceed with the protocol for the first antibody up to the end of the secondary antibody incubation. After washing off the secondary antibody, incubate with the next primary antibody (no need to block again) and follow the protocol to the end.

3. For oligodendrocyte/neuron costains, you will obviously need to do the **live staining with O4 first.** Proceed with the live staining protocol up to Point 13, then switch to the protocol for staining astrocytes or neurons, beginning from Point 5 (asterisked), as indicated in the protocol.