This PowerPoint presentation accompanies the case study "Group research projects: a framework for providing research experience for students"

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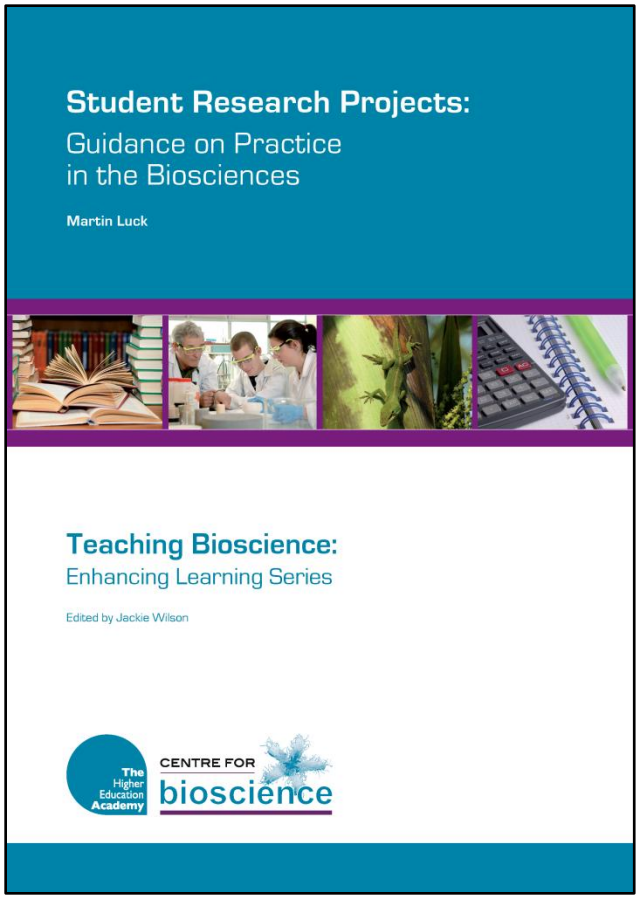


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# Practical Tutorial

**Thursday**

Rationale & intro

Tasks, discussion, report back

Establish key points

**Friday**

Practical advice

Plan experiments

Report back

Calculations

**Paperwork:**

You should all have

- Info on the background, aims and assessment of the practical.
- Background info on what is available for the practical.
- Tutorial summary sheet will be given at the end of today.

For the tasks today and tomorrow you need to split into four groups: Xa, Xb, Ya and Yb, each of 9 people.

You will work together within these groupings on the tasks, and I need one person from each group to report back at the appropriate stages.  It does not have to be the same person each time.

For the planning of the practical tomorrow, however, you will work in your groups of 3 within the larger group.  All three within a group should work together to plan and execute their experiment.

You will write up, submit and be marked on your practical entirely as individuals.

**Aims of practical**
(see handout)

1. Determine the effect of FGF2 and FCS on **proliferation and/or survival** of the **NIH 3T3 fibroblast** cell line, and whether effects seen are mediated via the signaling intermediates AKT (PKB), Erk MAP kinase and p38 MAP kinase.

2. Determine the effect of FGF2 on the **morphology** and **relative numbers** of different cell types present in **primary dissociated cerebellar cultures**.

**Cells**
NIH3T3 fibroblasts (Monday)
Cerebellum (Friday)

**Growth-promoting supplements**
FGF2 (100 µg/ml stock)
FCS (Neat)

**Signalling inhibitors**

| | | | |
|---|---|---|---|
| PD98059 | Inhibits Erk MAPK | Stock 20 mM | “PD” |
| LY294002 | Inhibits AKT/PKB | Stock 50 mM | “LY” |
| SB203580 | Inhibits p38 MAPK | Stock 50 mM | “SB” |

**Culture medium available**
DMEM
(Neat FCS too)

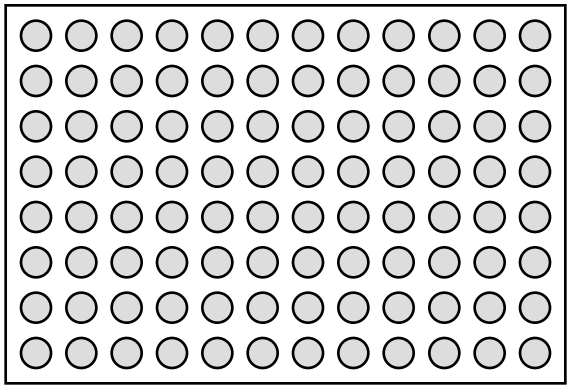
## Shape of practical

| | |
|---|---|
| Monday | **Plate 3T3 fibroblasts** in 96-well plates |
| Tuesday | **Adjust growth conditions** with FGF/FCS/inhibitors etc |
| Wednesday | **MTS** end-point assay if planned |
| Thursday | **MTS** end-point assay if planned |
| Friday 8 | **MTS** end-point assay **AND** **Plate cerebellar cells** in Lab-Tek slides **AND adjust growth conditions**. |
| Monday | **Fix and stain** cells in Lab-Tek slides |
| Tues/Thurs | Slots for fluorescence **microscopy** of Lab-Tek slides |

Hardware

**96-well plates**

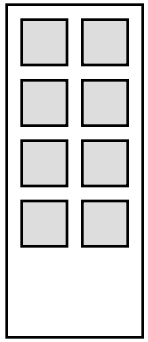
(3T3 fibroblast MTS cell number assay)



**X 4**

**8-chamber Lab-Tek slides**

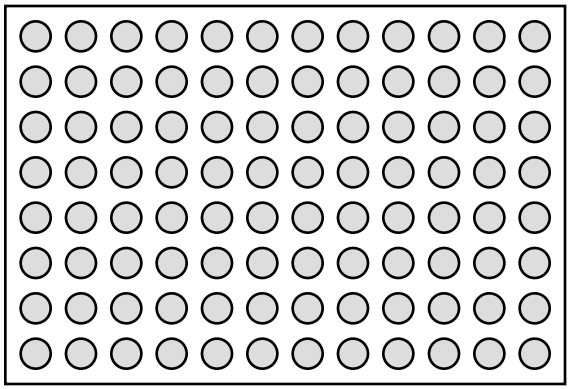
(Cerebellar cell immuno-cytochemistry



**X 3**

**96-well plates**

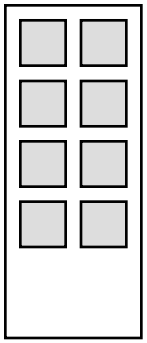
(3T3 fibroblast MTS cell number assay)



Cells plated Monday, growth conditions adjusted Tues, then cultured for up to 3 days.

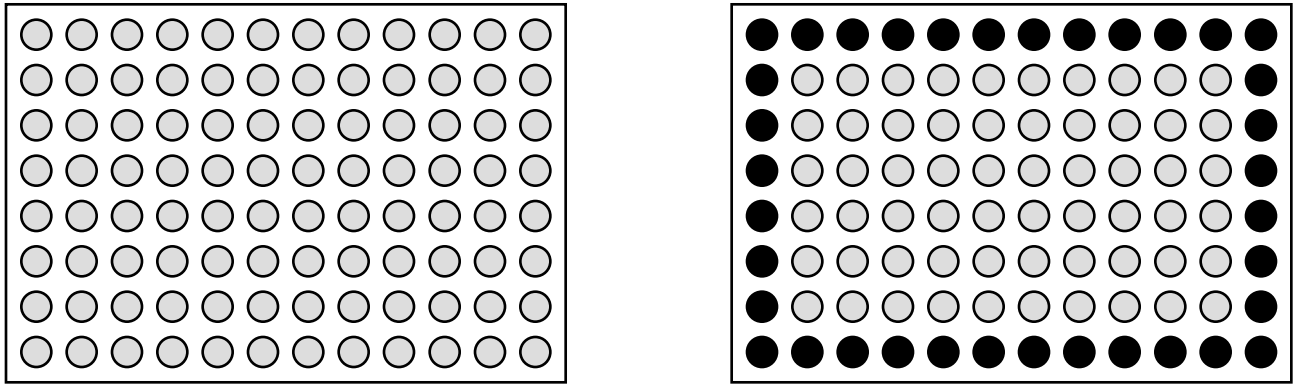
**8-chamber Lab-Tek slides**

(Cerebellar cell immuno-cytochemistry



Cells plated Friday, growth conditions adjusted same day, then cultured for 3 days.

# Evaporation from edge wells



In a 96-well plate cultured at 37 C for some days, the medium from the wells around the edge **evaporates faster** than from the other wells.

Solutes will be more concentrated in these wells.

# Cell plating

On Monday you will be plating your 3T3 fibroblasts into 96-well plates.

On Friday you will be plating your cerebellar cells into 8-chamber slides.

You will need to plate

**50 µl 3T3 cell suspension**, and

**200 µl cerebellar cell suspension**

These are the volumes required to cover the whole bottom of the wells in each case.

If you add your cells in a smaller volume, you will not have an even spread of cells over the bottom of the well, but a concentration of cells in the area covered by the medium, and none at all elsewhere.

You should aim to get cells evenly distributed over the bottom of the plate.

3T3 cells are routinely grown in DMEM/10% FCS, which allows them to thrive and proliferate.

Here, we want to look at the effect of FGF2 (and different concentrations of FCS perhaps) on cell number, so we would prefer not to have 10% FCS in the medium.

However, we cannot remove **all** FCS or the cells will not survive.

We will therefore plate the cells in DMEM containing a **very low** concentration of FCS

**DMEM/0.5% FCS**

This should be sufficient to allow the cells to attach and survive, but be sensitive to the augmenting effect of FGF2 and FCS.

# Cell counts

You will need to plate a **defined number of cells per well** in each case (exact number dependent on your plan):

**7,000 – 15,000** 3T3 cells/**well** of 96-well plate

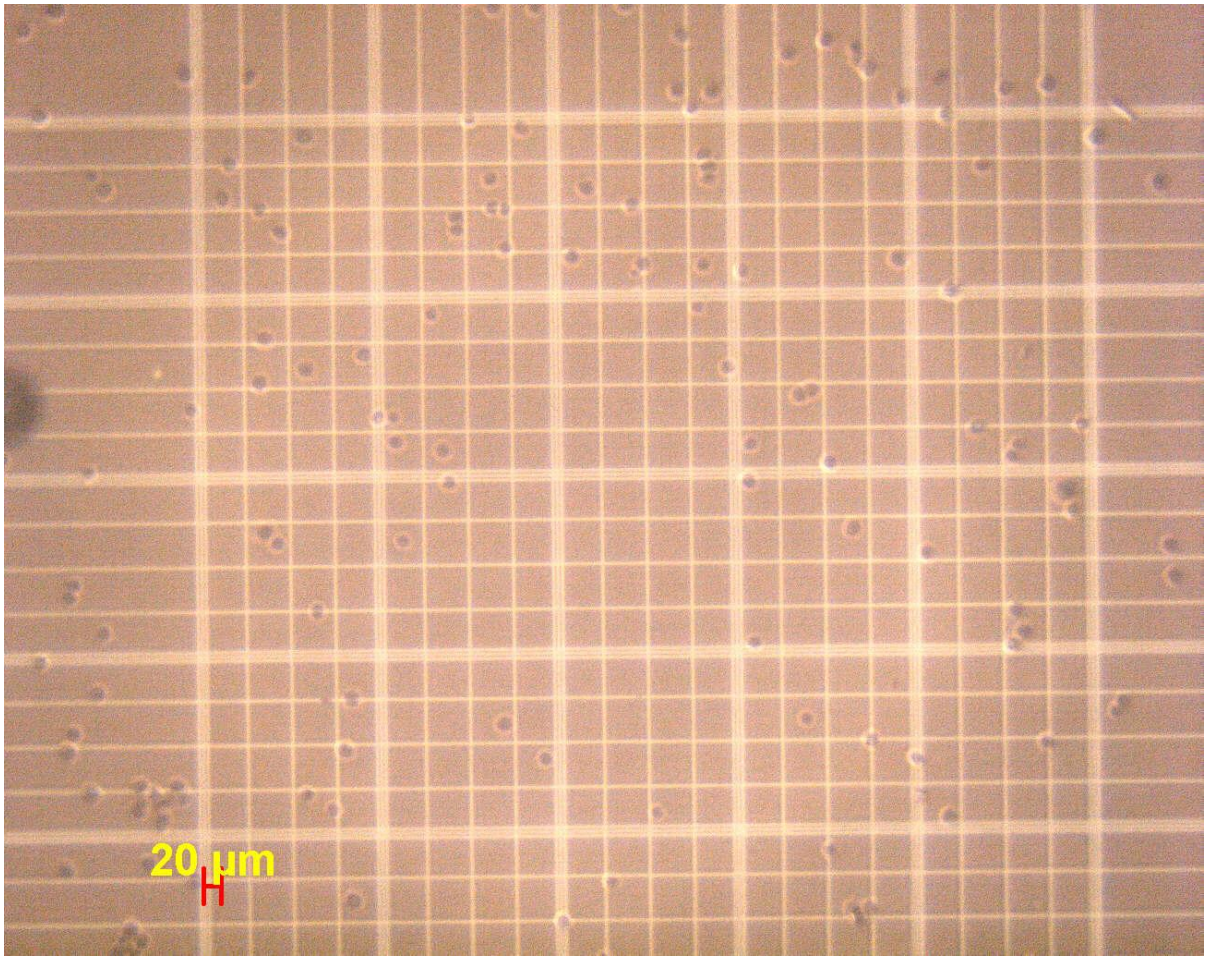
**100,000 – 2,000** cerebellar cells/**well** of 8-chamber slide.

To do this you will have to determine the number of cells **per ml** that you have after trysinising and re-suspending your cells….

…and dilute this to the number of cells you want **per ml** to ensure you have the correct number of cells **in the volume you are adding (50 µl for 96-well plate, and 200 µl for Lab-Tek slide)**

First you need to count the **number of cells/ml in your suspension** using a haemocytometer.

First locate the 25 square grid on your haemocytometer




Count the total number of cells falling within the square made up from the 25 smaller squares.

Multiply this number **by 10,000** to give you the

**number of cells** **per ml** in your suspension

**Note:**

•3T3 fibroblasts are much larger than cerebellar cells (tiny)

•A haemocytometer count of very dilute or very concentrated cell suspensions is undesirable.

•Too few cells (<10) will give you an inaccurate count.

•It is very difficult to count very large numbers of cells (>100).

•If your cells are too concentrated, try again having diluting the suspension as appropriate, e.g. ½ or 1/10, **but remember to factor in the dilution when working out your number of cells/ml.**

## Task 1 – Cell counts

Imagine that you counted **63 cells** within the 25-square haemocytometer grid.

What steps would you take to plate **15,000 (15K) cells per well** into 80 wells of a 96-well plate?

Remember that you need to have your cells plated in **50 µl** DMEM/0.5% FCS.

Get into your 4 groups to work on this task.

(Approx 15-20 mins)

63 cells

X 10,000 = **630,000 cells/ml** is what I **have** in my suspension

**Need** 15,000 cells in 50 µl

How many is this per ml?

15K in 50 µl, so how many in 1000 µl?

$$\frac{(1000 \times 15)}{50} = 300K/ml$$

So, I **have** 630K cells/ml

I **want** 300K cells/ml

What is my **dilution factor**? ***

$$\frac{300K}{630K} = \frac{1}{2.1}$$

i.e., the dilution is 1 in 2.1. I could achieve this by mixing 1 ml cell suspension and 1.1 ml medium

However, I need more than 2.1 mls for 80 wells.

The next thing I need to determine is: **What volume do I need** for 80 wells? ***

80 x 50 µl = 4000 µl = 4 mls.

By proportion, if I need 1 ml of suspension in 2.1 mls total, then how much cell suspension would I need in 4 mls total?

$\frac{1 \times 4}{2.1}$ = 1.9 mls of suspension required in 4 mls total.

Or I could add 2 mls of suspension to 2 x 2.1=4.2 mls total, ie, 2 mls of suspension + 2.2 (4.2-2) mls of medium

Now, when I add 50 µl to each well I know that I am plating 15,000 cells/well.

To re-cap: the two key things you need are

- **Dilution factor** required to get from the cell/ml you have to the cells/ml you want
- **Total volume** required of final cell suspension

Everything else is done by simple proportion

**NB: When you come to do this for real, always make up a slightly larger volume than you need, so that you know you definitely have enough for all the wells you have planned. If you make up exactly the right amount, you will find that you don't have quite the whole volume for the last well.**

## Task 2 – Plate layout

Imagine you are designing an experiment to test the effect on 3T3 cell number of culturing them for 3 days in the presence of 10 ng/ml FGF.

You also want to determine whether AKT (10 μM), Erk MAP kinase (20 μM) or p38 MAP kinase (5 μM) are important for any effects seen.

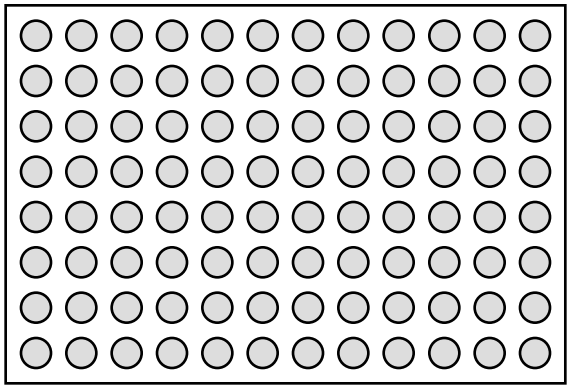
## Discuss and report back:

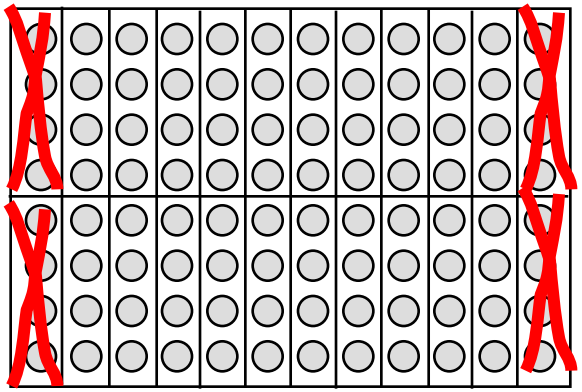
- **How many plates would you use?**
- **What would be your layout? (what would you put in which well?).**

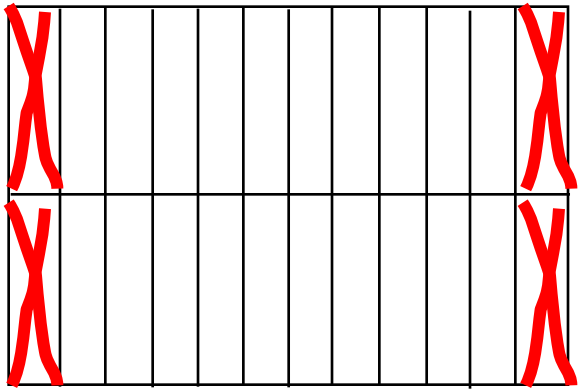
(Don't worry about how you would add FGF etc, just the design of your plate(s))

## Key points

- You need to have an index of **starting cell number** (‘Day 0 Control’).
- You need to have each condition represented in at least **triplicate or quadruplicate**.
- Avoid edge wells, or include equally for each condition.
- To determine the effect of a growth factor, you need to have an index of cell number in its absence.
- To determine the effect of an inhibitor on a growth factor effect, you also need to know the effect of the inhibitor in the absence of the growth factor.







To plan what's going where on my plates, I usually visualise the plate as 24 different 'condition cells.'

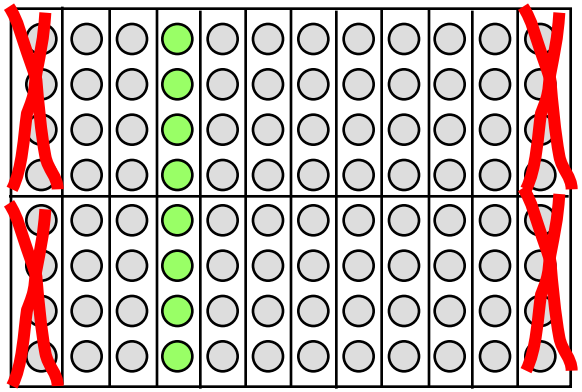
These include one edge well each, to give quadruplicate wells for each condition.

I avoid the strip of wells at either end as these conditions would have all edge wells and would therefore not be comparable to the other conditions.

Thus 80 wells are used, and 20 'condition cells' are available.

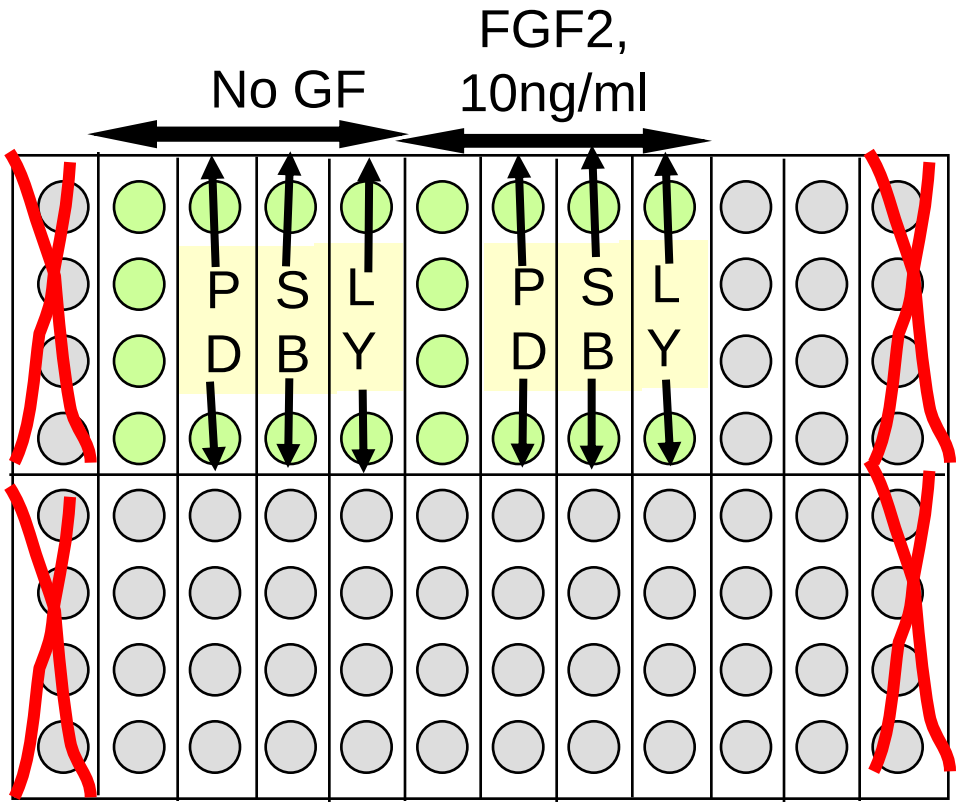
An e.g:

‘Day 0’ control plate



- Cells plated

An e.g.




Cells plated

# Task 3

Imagine you plated your cells yesterday in 50 µl DMEM/0.5% FCS.

Today is Tuesday, the day you need to adjust the growth conditions according to your plan.

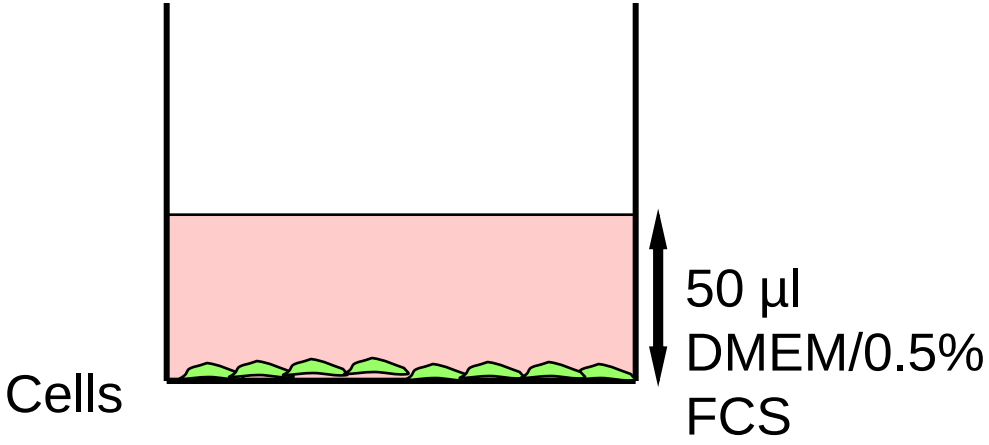
You have stock solutions of FGF2 and the inhibitors (given in your background information, and next slide).

**For the experiment designed in Task 2, how will you go about getting the FGF2 and inhibitors into the wells?**

NB: The final volume of medium in **every well to be assayed** should be **100 µl** at the end.

**Discuss in groups and report back**

| Item | Pathway | Stock | Final |
|---|---|---|---|
| FGF2 | - | 100 µg/ml | 10 ng/ml |
| PD | Erk | 20 mM | 20 µM |
| LY | AKT | 50 mM | 10 µM |
| SB | p38 | 50 mM | 5 µM |




After Monday, you have cells attached to the bottom of the well, and the well contains 50 µl DMEM/0.5% FCS.

To adjust the growth conditions, you could remove all the medium (very carefully), and replace (very carefully) with 100 µl new medium containing factors/inhibitors as per plan.

**Alternatively, you could add the factors/inhibitors in 50 µl to the 50 µl DMEM/0.5% FCS already present to give 100 µl total.**

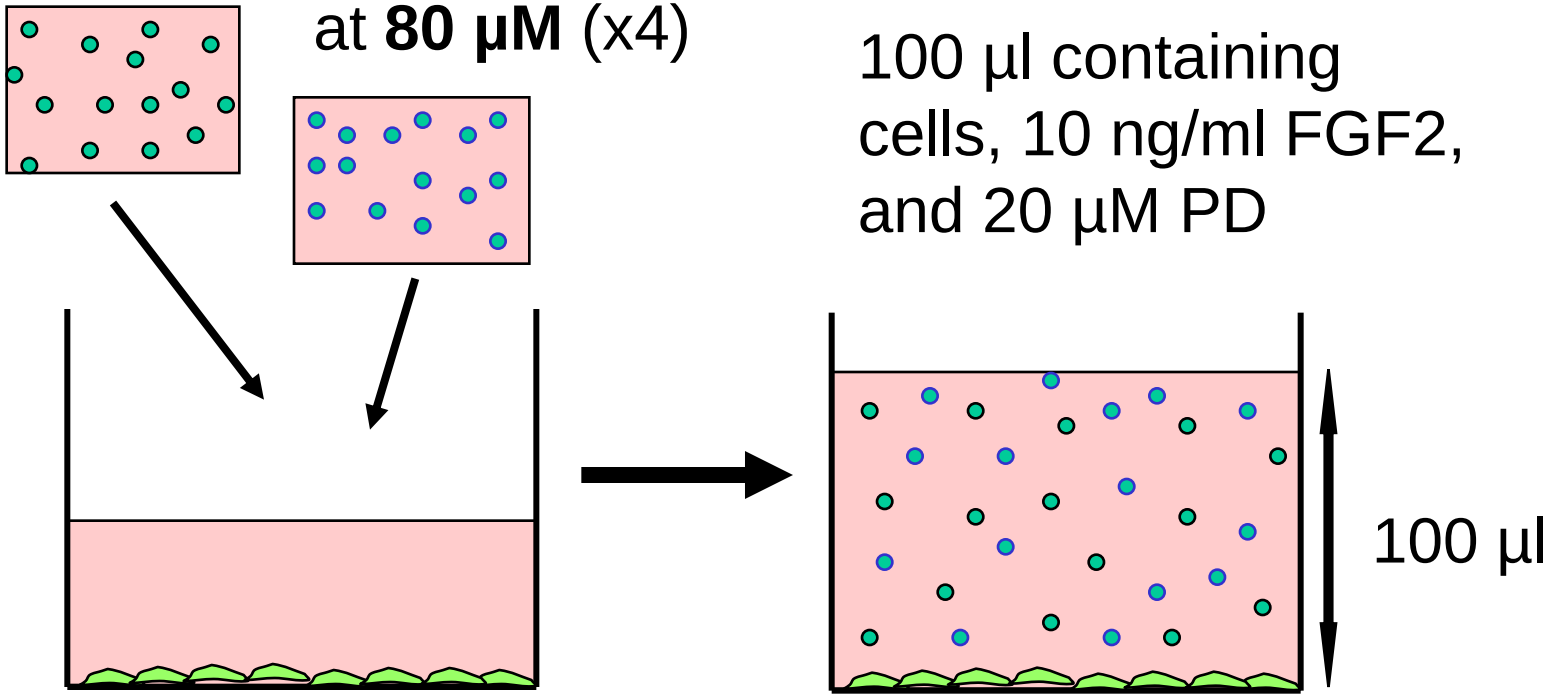
Unless you plan a survival assay tomorrow, and need to add your factors/inhibitors in DMEM without FCS, I would recommend the latter (bold).

**To achieve 10 ng/ml FGF2 with PD 20 µM:**

# Add x4 concentration in ¼ the volume

Add **25 µl** FGF2 at **40 ng/ml** (x4)

And **25 µl** PD at **80 µM** (x4)

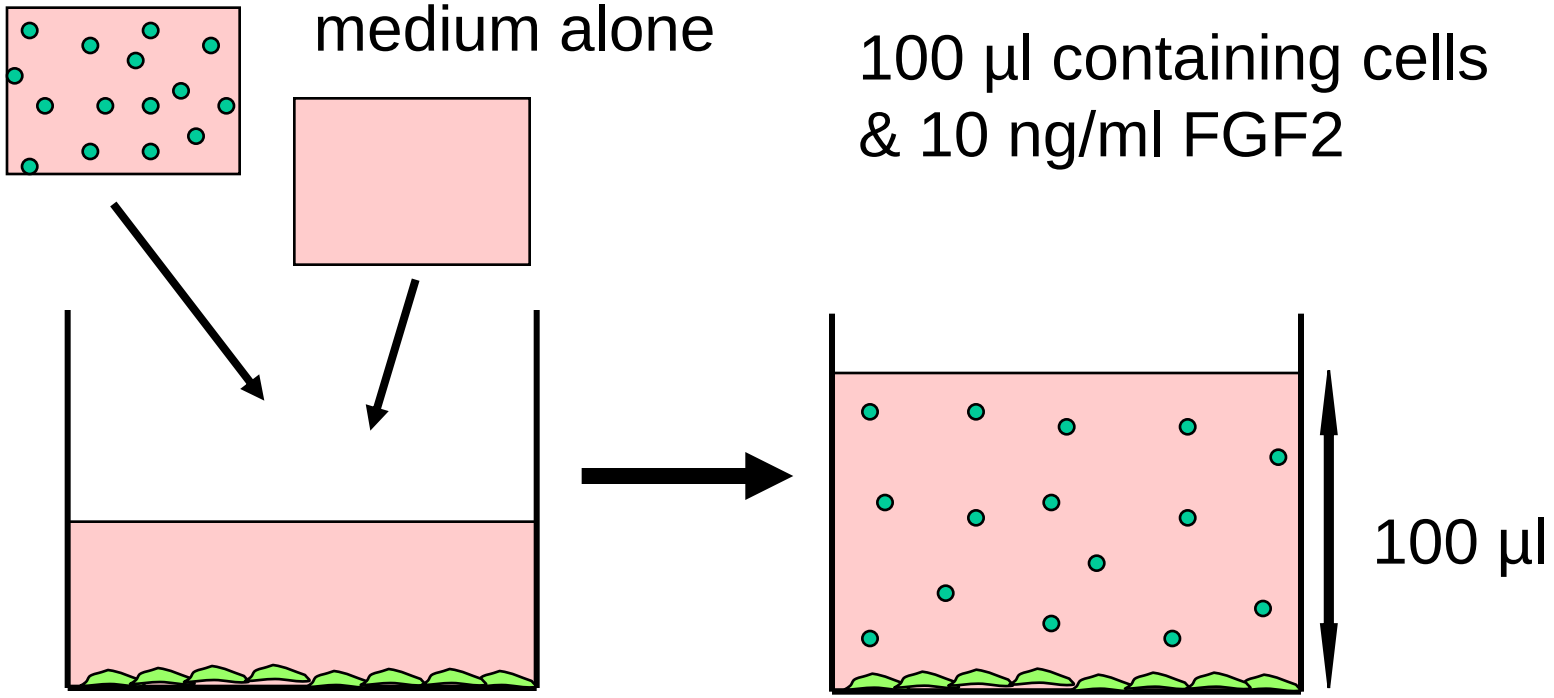


**NB: Ensure that all wells are 100 µl**

For wells containing **only** inhibitor or **only** growth factor, **remember to make up the volume to 100 µl with medium alone**

Add **25 µl** FGF2 at **40 ng/ml** (x4)

And **25 µl** medium alone



**What if you wanted to test the effect on FGF2 response of blocking all three pathways?**

**How would you do that?**

**Add x8 concentration in 1/8 the volume (12.5 µl)**

| Item | | Stock | Final | X4 | X8 | X10 |
|---|---|---|---|---|---|---|
| FGF2 | - | 100 µg/ml | 10 ng/ml | 40ng/ml | 80ng/ml | 1mg/ml |
| PD | Erk | 20 mM | 20 µM | 80 µM | 160 µM | 200µM |
| LY | AKT | 50 mM | 10 µM | 40 µM | 80 µM | 100µM |
| SB | p38 | 50 mM | 5 µM | 20 µM | 40 µM | 50 µM |

**In 100 µl total, add:** 25 µl of x 4 | 12.5 µl of X8 | 10 µl of X10

Makes it easy to add various combinations of factors and inhbitors in complex combinations.

# Task 4 Cerebellar cells

Assume it is Friday and you have plated your cerebellar cells in the 8-chamber slides.

You now need to use these slides to **test the effect of different concentrations of FGF2 on oligodendrocytes, astrocytes and neurons** in the cultures.

## Discuss how you might do this and report back:

- How you might lay out the slides in terms of which wells contain what during the 3 days culture.
- After fixing, which antibodies would you use for each well/slide?
- Any compromises that you might have to make.

**Key points:**

•Since you are adjusting the growth conditions the same day as plating the cerebellar cells, you cannot remove the medium in which the cells were plated.

•Make sure that all the wells to be stained for O4 are together on one slide and that you do not need to stain for anything else on this slide.

**Friday**

Practical advice
Plan experiments
Report back
Calculations

Start thinking tonight about what you might like to test in your experiment.

# Friday Tutorial

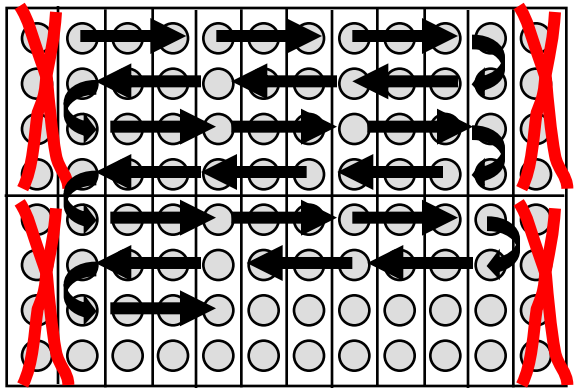
Practical advice
Plan experiments
Report back
Calculations

# Practical Advice

## Cells

•Ensure that you have a **single-cell suspension.** Re-suspend the cell pellet vigorously.

•Plate the cells in an order perpendicular to the arrangement of your ‘condition cells’



•This ensures that, if your cells settle slightly in the tube during plating, all wells in a certain condition do not get all the less dense or more dense plating. It would be worth re-suspending cells periodically during plating.

**Tubes** (demo) - Sterile

**Universal tube**

Capacity 30 mls.

Conical, but with skirted base.

Use for centrifuging cells, making up medium.

**Bijou tube**

Capacity 7 mls

Flat base

Use for making up solutions of growth factors and inhibitors – including interim stocks (no eppendorf tubes available)

**Sterile technique**

Do not open medium bottles etc outside the hood.

Do not open sterile pipettes outside the hood.

Do not touch the pipette tip on anything. If you do, throw it away and get another one.

Do not touch the inside of any plate or tube.

Do not open your tip boxes outside the hood.

Do not open the Pasteur pipette box outside the hood.

**NB:** Place tube caps, slide lids, 96-well plate lids, 10 cm plate lids **the right way up** when you take them off in the tissue culture hood (the same orientation they had when they were on). **Do not put them upside-down**.

**Inhibitor solutions**

•The three inhibitor stocks are in DMSO. DMSO is an organic solvent that is much denser than your culture medium. **When you add a few microlitres of inhibitor stock to medium, it will sink straight to the bottom**.

After adding the inhibitor, therefore, you must triturate the **whole volume** up and down several times to ensure thorough dispersal of the inhibitor throughout the solution.

If you do not do this, the wells you plate first will contain no inhibitor and the wells you plate last will contain very high concentrations.

(Even interim stocks need to be made up Bijou tubes, even though the volume may be very small. Just use a region of the bottom and ensure that the inhibitor is thoroughly mixed with the medium.)

## Growth factor and inhibitor dose-response concentrations

Guidelines on possible dilutions. Red are recommended if only using one concentration.

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| FGF2 | | 0.1 | 1 | **10** | 100 | | | ng/ml |
| | **OR** | 2.5 | 5 | **10** | 20 | 40 | 80 | ng/ml |
| PD | | | 10 | **20** | 40 | µM | | |
| LY | | | 5 | **10** | 20 | µM | | |
| SB | | | 2.5 | **5** | 10 | µM | | |

- Set out dilution calculations and plate/slide plans clearly so you can easily follow them on Tues/Fri.
- Label tubes clearly & organise hood.
- Ensure your plates and slides are clearly labelled, not only with your name, but with a means of distinguishing them from one another.

Recognise each others strengths and weaknesses.

There are distinct phases of this practical that require varying degrees of

Imagination

Planning

Calculation

Project co-ordination

Calmness

Manual dexterity

Analysis

Decision making

We are not all good at everything.

**You need to decide what are the aims of YOUR research. The types of things studied in the past include:**

Different concentrations of growth factors

Different concentrations of inhibitors

Effect of combined inhibitors.

Effect of x on cell number over 1, 2, 3 days

Effect of different cell density.

Effect of different basal medium – survival? Proliferation?

???? What do you want to find out?

**Design your experiment in your group of 3, then return and present your plan to the class, for discussion and comment.**