

EXERCISE 2

Influence of Serum, Coated Surfaces, and Alteration in Signal Transduction on Cell Division and Differentiation

Note to the Instructor: While this exercise can be modified for any line that undergoes morphological differentiation, we utilize a neuroblastoma line since neurite elaboration provides an excellent microscopic index of differentiation. These cells undergo morphological differentiation in the presence of 1 mM dibutyryl cyclic AMP (this is cyclic AMP modified such that cells cannot break it down, which maximizes cyclic AMP-dependent processes). PC12 cells can also be utilized, but note that they require nerve growth factor (which is expensive) for differentiation. Myoblasts are also useful, since they will form myotubes (fused multi-cellular muscle cell precursors) when prompted to differentiate.

You will use the NB2a plates you made last week as stocks.

You will be provided with 4 poly-L-lysine-coated plates, and several uncoated plates. Coating of plates simply requires spreading a small amount of filter-sterilized poly-L-lysine then letting it dry. Should the poly-L-lysine not be sterile (or should some other coating be desired), one can simply leave the plates opened within the laminar flow hood overnight with the UV light on.

YOUR OBJECTIVE

Create a growth curve under various conditions with time points of 0, 24, 48, 72, and 96 hrs.

TASKS TO COMPLETE THIS OBJECTIVE

- *Generate many identical plates from your stock NB2a plate.*
- *Alter the medium in some plates after the cells have attached.*
- *Record morphology and cell numbers at $t=0$ and at 2 subsequent points.*
- *Tuesday lab will record on Wednesday and Thursday, which = 24 and 48 hrs.*
- *Friday lab will record on Monday and Tuesday, which = 72 and 96 hrs.*
- *Analyze the effect of different conditions on cell growth and differentiation.*

THE CONDITIONS WILL BE

- Regular serum “Control” (10% serum)
- Low serum (1%)
- 10% serum + dbcAMP
- 10% serum on poly-L-lysine

METHODOLOGY

For accurate cell counts, you must count each plate twice.

For accurate growth curves, you must have duplicate plates.

Each group will be counting cells on two days, and therefore must have two sets of cells.

Figure out how many plates you will need. Knowing this, you can determine how much media you will need for dilution of cells from your stock plates.

With this in hand, trypsinize your stock plates and dilute them.

Some Basics for Your Calculations:

- NB2a cells double at least once a day.
- Therefore, if your stock plate is 80% or more confluent, a 1:30 passage allows 4 days of growth.
- If your stock plate is only 50% confluent, a 1:20 would also allow 4 days of growth.
- Plate enough plates for all necessary conditions.
- Wait 1-2 hrs. for cells to attach. This is a good time to count what was in the dilution from which you plated. This means 1 mL extra of media will be needed (this will provide your time 0 point).
- *After* cells have firmly attached (to test: gently shake the plate while looking through microscope), replace media on 4 uncoated plates with 1% serum-medium and replace media on 4 uncoated plates with 1 mM dbcAMP-containing media (There is a 100 mM dbcAMP stock solution; how much will you need per 2 mL plate?).

NOTE: You must design a protocol BEFORE coming to the lab. This must be written out (NOT in your lab notebook, but on a separate paper) and checked by the instructor before beginning your experiment. You will enter each step into your notebook AFTER you have performed it.

Everyone provides a time 0; different lab sections, meeting on different days of the week, can provide complimentary time points, which will add up to a complete profile. For example, the Tuesday lab section will count at 24 and 48 hrs. (i.e., Wed. and Thurs.). The Friday lab section will count at 72 and 96 hrs. (i.e., the following Mon. and Wed.). *Obviously, these times vary when laboratory sessions are held on different days; this is just an example to allow two different laboratory groups to generate complementary data sets.*

Note: The Friday lab section is, of course, aware that they must re-feed their cultures (with the proper respective media formulations) on Monday since the “96 hr.” plates will be around for >3 days.

Calculate the average \pm standard deviation of your cell counts at $t=0$, calculate the average \pm standard deviation of your cell counts at your other days, and calculate a percent change at each day versus day 0 (average of day x / average of day 0).

Email these data to the lab instructor (or provide them in the manner specified, if different). Only one person per group should send the data. Of course, remember to include your partner’s name on the submitted material.

Your laboratory instructor will pool all data. This will provide a large number of data sets, which should help generate a curve that would resemble one that you would generate had you repeated the experiment 2 or 3 times. Once the data are pooled, we can generate a growth curve under various conditions at 24, 48, 72, and 96 hrs. The growth curve will be posted on the website. This exercise will require a formal lab report (do NOT write the words “formal lab report” as the title for your report). The combined class data must be included, as well as your own.

SAMPLE PROTOCOL

Objective:

To create a growth curve under various conditions by recording changes in morphology (shape) and cell numbers.

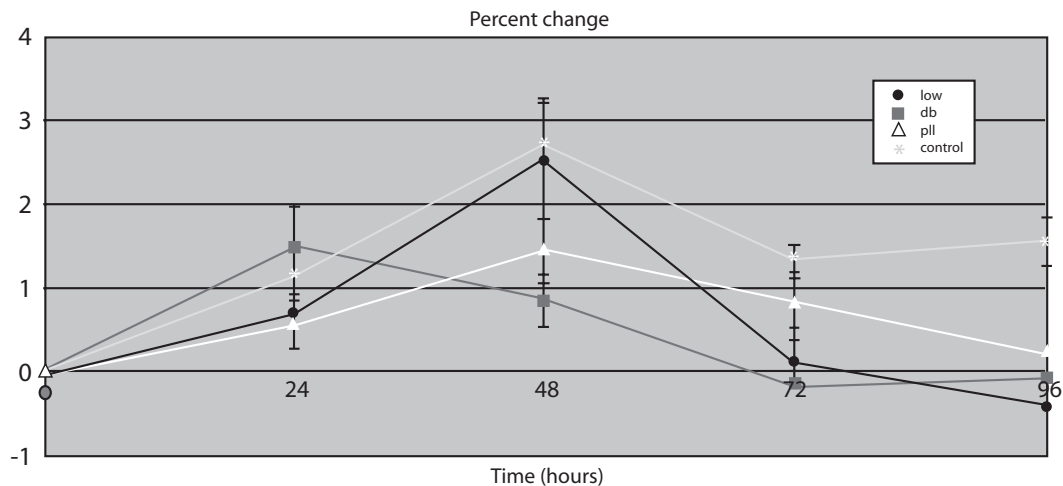
Protocol:

1. Determine the confluency of stock NB2a culture plates using a microscope.
 - a. Make observations of confluency and morphology.
2. Trypsinize NB2a stock plates.
 - a. Pipette media from each plate and empty into waste beaker.
 - b. Pipette 1 mL of serum-free medium into each stock plate and gently swirl plates.
 - c. Remove serum-free medium from plates.
 - d. Pipette 1 mL of trypsin into each stock plate.
 - e. Incubate plates for 5 minutes at 37°C.
3. Remove each plate from incubator and examine under the microscope.
 - a. Gently tap the side of each plate to detach any remaining attached cells.
 - b. Pipette trypsin and detached cells into sterile centrifuge tubes.
 - c. Centrifuge cells at 1,000 \times g for 3-5 minutes.
4. Remove tubes from the table-top centrifuge and carefully decant the supernatant.
5. Resuspend in 10% serum-containing media—add an appropriate amount to create the necessary dilution.
6. Create new plates, pipette 2 mLs of the cell suspension into each plate, and incubate at 37°C. Allow 1-2 hours for cells to adhere to the plate.

- a. Add an extra 1 mL of 10% serum-containing media so that it can be used for a cell count for each condition. Each plate of each condition must be counted twice for an accurate growth curve.
 - b. For cells to be plated onto poly-L-lysine coated plates, the cell suspension must be plated directly onto the plate.
7. Use the microscope to confirm that a majority of the cells have attached.
 8. For the various serum conditions, the media can be changed at this time, e.g., from 10% to 1% serum-containing medium. Also, dbcAMP can be added at this time to each plate directly.

HINTS

- dbcAMP is an analog of the second messenger, cAMP. This synthetic compound mimics the function of endogenous camp, but is not as susceptible to hydrolysis by cAMP phosphodiesterases.
- In order to be effective, it can only be added once the cells have attached. It also must be re-added each time the media is changed.
- Coating plates with poly-L-lysine must be done the day of the experiment.
- It is possible when looking under the microscope that the poly-L-lysine will crystallize. Simply swirl the plate around to put the poly-L-lysine back into solution.
- When using trypsin to passage cells, not every cell will detach. To determine the efficiency of trypsin, a majority of the cells must be detached.
- When centrifuging, cells have various speeds at which they will pellet, but not lyse. Be sure to note the optimum speed at which this occurs.
- Oftentimes, students will not be able to detect a visible pellet. Instruct them to handle this as a “theoretical” pellet and leave approximately 0.5 mL of liquid in the tube to ensure that no cells are accidentally removed along with the supernatant.



The above is a sample growth curve, compiled from the data from all student groups.

MATERIALS

10% serum-containing media

1% serum-containing media

dbcAMP

Plates coated with PLL

- To make 10% serum-containing media:
 - Refer to Exercise 1, Introductory Laboratory.
- To make 1% serum-containing media (500 mL):
 - Thaw the following: 1 L bottle of DMEM-only media (this may or may not contain L-Glutamine), antibiotic/antimycotic solution, and serum (either horse serum or iron-supplemented calf).
 - Make a 500 mL aliquot.
 - From the 500 mL aliquot, remove 5 mL.
 - Then add 5 mL of serum.
 - Remove 10 mL and dissolve into vial of L-Glutamine.
 - Then add back the dissolved L-Gluamine into the media bottle.
 - Add 5 mL of antibiotic/antimycotic solution.
 - Make aliquots and store at 4°C.
- To make 100 mM stock dbcAMP:
 - For 10 mL stock: dissolve 491.37 mg (.49137 g) into 10 mL DMEM-only.
 - Filter through a 0.2 µm filter.
 - Store at -20°C.
 - ** Using stock: add 20 µL per plate of stock for a final concentration of 1 mM.
- To make 0.1 mg/mL poly-L-lysine:
 - Dissolve 5 mg into 50 mL of sterile ddH₂O water.
 - Using a transfer pipette, coat an untreated 2-mL plastic Petri dish with approx. 1 mL of pLL solution.
 - Incubate at 37°C for 2 hours.
 - Rinse twice: once with DMEM-only and once with sterile ddH₂O.
 - **Mixture can be reused several times once stored in 4°C.

