

E X E R C I S E

8

Determination of the Cell Cycle

INTRODUCTION

The following exercise is a multi-laboratory, major project. We typically start this exercise at the beginning of the last month of the semester. It requires significant planning. The goal is to determine the 4 stages of the cell cycle: G1, S (where DNA is synthesized), G2 (where the cell grows in size and total protein increases), and M (for mitosis, where cells divide). We precede this exercise with a lecture reviewing the cell cycle. We will quantify DNA and protein by spectrophotometric assay, and cell numbers via hemacytometer. Judging confluency along the way is useful, but is less of an indicator than cell numbers.

We have also collected homogenates from groups and have run a collective immunoblot for the appearance and depletion of cyclins. It would be useful, if sufficient electrophoresis and transfer apparatus exist, to have groups run their own cyclin immunoblot.

The cycle exhibits some variance from group to group. We typically tabulate and compile everyone's data in class after completion of the exercise, and draw a composite graph on the board, and when everyone's points are compiled, and a mean and standard deviation is generated, the graphic result is very impressive.

Simple hemacytometer counts in a growing culture typically display a gradual increase. To observe a "one-step" growth curve, which will reveal the cycle, however, cells must have their mitotic cycle synchronized. Cells are synchronized by treatment with nocodazole, which prevents microtubule assembly (therefore, trapping cells at the G2/M interface). The average length for NB2a cell cycle is 24 hours. Therefore, cells must be treated with nocodazole for 24 hours in order for the entire culture to be arrested at G2/M. Following 24 hours, the cells are removed from the nocodazole treatment by rinsing the plate once with serum-free media, then adding 10% serum media. We then harvest cells at various time points. *The task is not really to harvest cells every hour, which would require a 24-hr. marathon once nocodazole is removed.* Rather, a simple first-run, with observation at intervals of 4-6 hours will provide a "sketch" of when DNA levels are increasing, when cell numbers are increasing, etc. Subsequent time points can refine the onset and cessation of these events. Cells will double in number within approx. 4-6 hrs. after nocodazole is removed (since they are "frozen" at the start of M phase). It is important to catch this point, so we recommend harvest at nocodazole removal, then 2, 4, 6 and 8 hrs. later (8 hrs. should show that this first division has ceased. This doubling signals the start of a new cycle (i.e., the start of G1). This should ideally be referred to as time 0, and the removal of nocodazole as, for example, t -4hrs.)

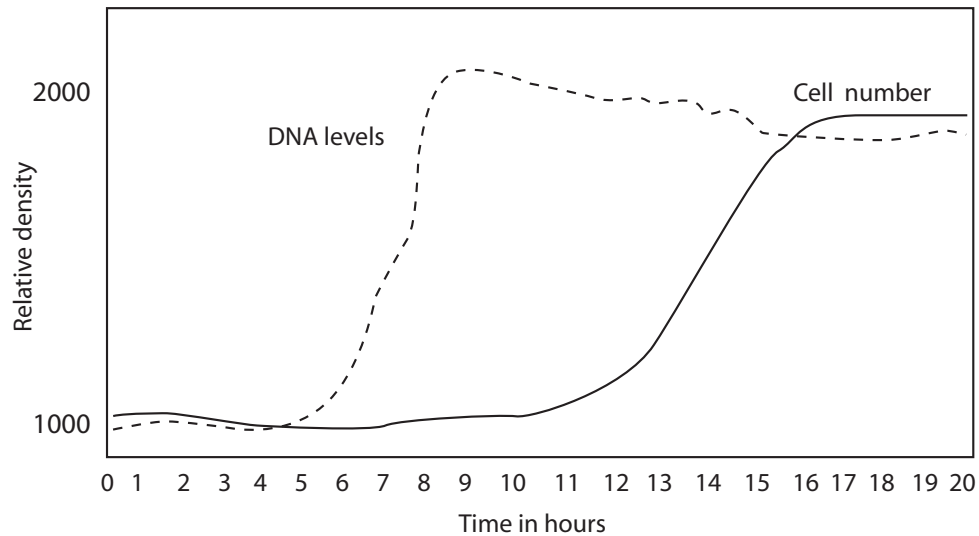
At each time point, the plates are trypsinized (to detach the cells), and a small amount is used for hemacytometer counts. The remaining cells are placed into separate vials to be centrifuged to pellet cells (Exercise 1) and the pellet is resuspended in 1.5 mL of PBS. This is then transferred into a microcentrifuge tube and frozen at -20°C until ready for spectrophotometer analysis of DNA and protein.

For UV spectrophotometer analysis, the tubes are removed from the freezer and warmed by hand, then vortexed gently to evenly distribute the cells within the tube, followed by homogenization for 1-3 minutes. Homogenization is carried out using disposable microfuge homogenizers. Note that the spectrophotometer blank should be PBS, which you can subtract from all other readings or set to “0,” depending upon the capabilities of your spectrophotometer. Read at 260 nm for nucleic acid, and 280 for protein. Note that we will only be obtaining relative levels of DNA and protein, since we are not comparing these with a standard curve. Moreover, the 260 reading will also detect RNA. However, this will still readily yield information on increases and decreases, and therefore, will reveal the time of doubling of DNA and changes in protein levels.

INTERPRETATION

G1 and G2 do not really have indicators of their own. Instead, we observe the onset and cessation of DNA doubling and cell number doubling, which denote the start and end of S phase and M phase, respectively. This is how the G phases, which stand for “Gap,” were first defined. There will be a doubling of cell numbers within 4-6 hrs. after nocodazole removal. (**Note:** If you have been “harsh” with the cells, e.g., passing them too lightly, or they have been having slow divisions, this may be delayed until 7 or 8 hrs. This doesn’t ruin your experiment; just note it, and realize the subsequent doubling may also be delayed. Remember that your data is your data, regardless of what you anticipate.) Regardless of what time this is observed, when cells “level off” after the first doubling following nocodazole removal, this is the onset of G1. When DNA levels start to rise, that marks the onset of S phase. When DNA levels peak, that indicates that S phase has ended, and therefore, cells are now in G2. When cell doubling begins, cells have entered M phase. We recommend taking points after 24 hrs. (e.g., 28, 30) to substantiate the end of M phase by leveling off of cell numbers.

G2 phase is often shorter than G1, and may be difficult to discern. Protein levels classically increase during G2, but we have often found that protein increases parallel DNA increases, or begin to rise while DNA is still increasing. Some groups therefore can discern S phase and M phase, but cannot readily discern G2. Compiling all data from all groups helps, which is essentially like having run the experiment many more times than can be carried out during a semester-long laboratory with multiple exercises.



Above is an example of DNA levels and cell numbers (presented as relative levels) compiled from the data of an entire class. For simplicity, means without standard deviation are presented, and protein levels are omitted. The 4 phases of the cell cycle can readily be determined from this graph. **Note:** Samples were NOT collected every hour; as described above, this is not the intent of the laboratory exercise.

MATERIALS

PBS

Serum-free media

10% serum media

Nocodazole

Trypsin

Microcentrifuge tubes and homogenizers

Plate scraper

UV spectrophotometer

- Serum-free Media:
 - Refer to Introductory Lab.
- 10% Serum Media:
 - Refer to Introductory Lab.
- Nocodazole:
 - Dissolve into DMSO for a final concentration of 330 nM, which can be added to either the media bottle or to the plate directly.

