Combining Activities 3 and 4

The Scaffold Diffusion and Biochemical Assay exercises can be readily combined into a more comprehensive and meaningful activity. Students can select varying types of paper to simulate potential scaffolds. These scaffolds are then placed in the diffusion apparatus, wherein food coloring is allowed to migrate across the surface of the paper scaffolds. The food coloring represents a growth factor/reporter molecule conjugate. After the selected time interval, the paper scaffolds are removed and cut horizontally into equivalent size sections. The coloring is then leached out of the sections within test tubes. By inspection, it is usually observed that the sections vary in their absorption (affinity for) of coloring.

The variation in paper scaffold affinity for coloring (growth factor) can be quantified by utilization of a standard curve. Students can be presented with a stock solution of coloring (growth factor/reporter molecule conjugate) of known concentration. It is recommended that teachers assign the stock a concentration of 100%. For classes familiar with other units of concentrations, 100 nanomolar or 100 ppm is more reflective of actual concentrations correlated with tissue engineering research. It is recommended that teachers prepare the stock to have an absorbance of approximately 1.0. Students can then prepare dilutions of this stock and record the absorbance of each sample by use of a spectrophotometer (page 70). Students should then plot their data (concentration of coloring = x-axis, absorbance = y-axis), and generate a best-fit line. This relationship can then be used to determine the concentrations of coloring (growth factor) extracted from each section of their paper scaffolds.

With this information now available, students can assess the probable success of each scaffold in regenerating new bone tissue. It is thought that stem cells, either endogenous to the host or seeded from an external source, must be induced to develop into cells capable of forming new mineralized bone tissue. Cells that are most capable of performing this function are known as osteoblasts. Thus, the ultimate success of this scaffold/growth factor combination will depend upon a critical range of growth factor concentration, one that favors the development of osteoblasts from less committed ancestral cells. A table revealing a correlation between growth factor concentration and cell developmental status can be presented to the students. Teachers can use the table on page 72 as is, or modify the numbers to better fit student data. Student reports can thus contain a similar table, allowing the reader to readily understand the relationship between the raw data of the experiment and the predicted consequence (success) of the engineered tissue. Below is a sample table:

Scaffold section	Absorbance	[Growth Factor]	Cell Status
top	0.09	12.2	Pre-osteoblast
middle	0.51	44.5	osteoblast
bottom	0.68	60.1	osteocyte

To review, the students can allow food dye (representing growth factor) to diffuse across paper scaffolds, as described in Activity 3. They then can determine the concentration of dye (growth factor) in various sections of their scaffolds. This can be

done by leaching the dye out of the paper scaffold sections, followed by spectrophotometry. The obtained absorbance values can then be converted to a concentration of growth factor by comparison to a standard curve (described in teacher section of Activity 4). This value of growth factor can then be utilized to predict the projected status of stem cells that can be seeded onto the original scaffold. This is done by means of a chart (page 72) that correlates growth factor concentration with the developmental outcome (fate) of stem cells.

If teachers choose this option of combining activities 3 and 4, it is highly recommended that students be given a copy of this description to serve as a guide for both procedure and lab report.

Student Background

Tissue-engineering researchers often face the challenge of constructing a suitable framework for the cells of a particular lab-derived tissue. It is often overlooked that cells, in the context of a tissue, are imbedded in a matrix of living and non-living components. In the past few decades, great insights into the nature of this matrix and its relation to the health and normal functioning of tissue cells have been revealed. Scientists now recognize the many indispensable roles played by this matrix, and they realize that their efforts to mimic bodily tissues may well depend upon their ability to construct a suitable artificial matrix that may serve similar functions.

There are a number of characteristics of the normal extracellular matrix that researchers would like to mimic. Immediately, one can envision the importance of the mechanical properties of the artificial scaffold, especially in light of those tissues that must resist various types of physical forces or stresses. For example, blood vessels must maintain their integrity and pliability in the face of both exterior and interior pressures. Cartilage, tendon, ligament, and bone face obvious, and sometimes powerful, mechanical stresses. Skin, the first FDA-approved tissue-engineered product, also is subjected to a number of deforming stresses, as well as chemical and radiation stresses. Many of the scaffolds (artificial matrices) are designed to be biodegradable, with the hope that the body will slowly replace the artificial scaffold with a natural extracellular environment.

It is also easy to understand that the scaffold must allow normal cellular and physiological processes to occur. This requirement can involve quite complex and subtle phenomena, leading many researchers to a number of different types of investigations. The following types of questions should serve to illustrate the diversity of scaffold concerns: (1) Will the cells seeded in the scaffold behave normally or as designed? For example, will the cells differentiate into the proper specialized cells and do so within the proper time-frame or sequence? Even simpler, can the scaffold support living cells for an extended period, or is it somehow toxic to life? Will the cells be able to adhere properly to the scaffold components? Will the cells migrate appropriately and assume proper relationships with each other? (2) Will the cells be able to communicate appropriately with each other and the new physiological environment into which they have been transferred? For example, will the scaffold permit the adequate exchange of biochemicals between cells or between the vascular system and cells? Likewise, can the appropriate regulatory molecules be effectively dispersed throughout the scaffold? Remember that a critical component of tissue engineering involves coordinating the activities of cells within the scaffold construct.

This last question is the topic for this activity. Using simple materials, students can evaluate the diffusion characteristics of simulated scaffolds and regulatory molecules. This experiment is to serve as a simulation of the types of studies performed by tissue engineers concerned with the proper delivery or diffusion of biochemicals through a scaffold. Hormones, especially those regarded as growth factors, are thought to play an integral role in the development and maintenance of tissue, either natural or engineered. In this lab scenario, dyes will be used to simulate the diffusion of biochemicals. The scaffolds will be represented by various types of paper or, as an extension of the exercise, by polymer gels.

For this exercise, it is imperative that the student teams determine the exact focus of their investigation. There are many possible options to study, and it will be necessary to focus the analysis on a defined area that lends itself to a testable hypothesis. For example, a given team may wish to compare the diffusion properties of one particular dye across various paper-product scaffolds. Even this narrow focus presents options such as the measurement of diffusion rate, the absolute diffusion distance, or the quantification of the relative concentrations of dye across the scaffold.

Purpose

Life science researchers are often faced with the challenge of identifying biochemicals within their experimental system. In addition, it is often advantageous to develop a technique for measuring the amount of that biochemical. One common strategy for quantifying a chemical is based on a specific interaction between the chemical of interest and a 'reporter' molecule. A 'reporter' molecule is usually designed to interact only with the target chemical, resulting in some type of physical change that can then be detected by the investigator. These types of analyses are employed in many settings, and you may have experienced them in your daily life. Diabetes monitoring kits, pregnancy tests, urine test strips, water test kits, and soil test kits often make use of measurable physical changes resulting from specific chemical interactions. Usually, the 'measurement' involves a color change that can be readily compared to a reference chart, allowing one to estimate the amount of the particular substance of interest.

Tissue engineers are often required to quantitate a number of biochemicals within a tissue construct. Some of these molecules may have been deliberately seeded into a tissue matrix or scaffold, perhaps to serve as growth or differentiation promoters for the cell population. For example, in the case of bone engineering, molecules such as Transforming Growth Factor (TGF) or Bone Morphogenic Protein (BMP) are utilized to induce the differentiation of bone precursor cells into osteoblastic cells. Much work focuses on the effective concentrations and appropriate localization of these growth factors within the engineered scaffold. Other chemicals may be secreted by the tissue itself, offering researchers clues pertaining to the health or developmental status of the cells. For example, a quantitative assay for alkaline phosphatase is commonly used to reveal the developmental status of a number of tissue types. Commonly, cells that have begun to differentiate will begin to secrete this enzyme. In the bone cell differentiation cascade, mesenchymal stem cells (relatively unspecialized cells) do not produce this enzyme. However, as the cells mature into pre-osteoblasts and begin to differentiate into osteoblasts, they characteristically manufacture alkaline phosphatase. This product can be measured in an indirect fashion. Scientists can add a colorless substrate (the 'reporter' molecule) to these cells. The enzyme has the ability to catalyze the cleavage of this substrate, yielding a colored product. Researchers measure the intensity of the color produced, which then can be used to assess the developmental state of the cells. This type of analysis, using color to quantitate a process, is called a colorimetric or **chromogenic assay**.

Most of these assays require a set of standards for direct comparison. Generally, a series of specific concentrations of the chemical to be measured are created. These are then subjected to the same measuring technique to be employed in the study. The resulting data can be plotted on a graph, providing the investigator with a correlation between the dependent and independent variable. From this standard curve, researchers can measure one characteristic in the relationship and deduce the other characteristic. For example, let's assume that the velocity of a species of fish can be measured. After timing many fish, one might plot the relationship of fish size to swimming velocity.

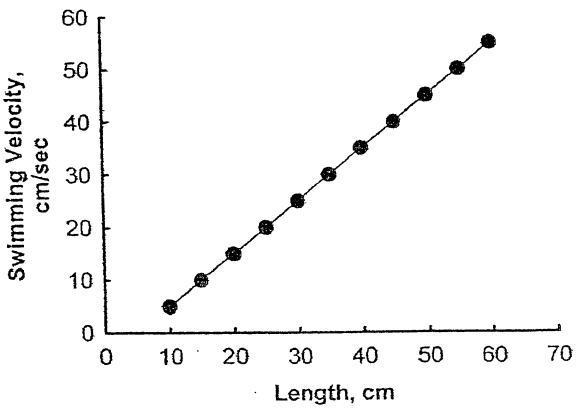

Figure 4.20: A standard curve.

Assume that the data is plotted and the following graph results (Figure 4.20):

Suppose now you capture a fish and house it in a small tank. Without renting elaborate equipment at great cost, you can still predict the swimming velocity of the specimen. How can the graph enable you to determine this quantity?

In this exercise, you will be challenged to determine the concentration of biochemical 'x' in a sample of tissue extract (the liquid juice comprising the extracellular matrix). This information will then be utilized to assess the developmental status of the cell population. Food coloring will be used to simulate the 'reporter' molecule, and a clear solution will represent the tissue extract that should contain the biochemical of interest.

Activities 3 and 4 Overview – Scaffold Diffusion, Biochemical Assay, and Cell Response

A. Scaffold Diffusion

1. Place the simulated scaffolds (papers) in the diffusion trough. Remove when the dye (simulated growth factor) front reaches the top of one of the scaffolds. Let hang over a beaker or hold by the straw to avoid touching wet scaffold paper to table or other object (this will avoid leaching problems).
2. Pipet 5 ml. of tap water into each of 6 culture tubes (spectrophotometer tubes). Steps 1 and 2 can be completed simultaneously by different group members.
3. Cut scaffolds horizontally into 3 equal sized strips. Transfer strips (avoid touching to a surface) to the culture tubes containing 5 ml. of water. Label tubes accordingly (e.g. scaffold A-top, bottom, middle).
4. Invert tubes gently 10-20 times (be consistent with all tubes).
5. Remove the scaffold paper or tamp to bottom to avoid interference during spec reading.
6. Record the absorbance of each solution. Remember to read absorbance at the appropriate wavelength. For red dye, set to 500. For others, ask instructor.
7. Refer to standard curve. Determine the concentration of growth factor that was imprinted onto each section of the paper scaffolds.
8. Determine the resulting developmental status of stem cells in each section of scaffold by referring to the chart below. Evaluate the relative potential (efficacy) of each scaffold/growth factor construct.

[Growth factor] in ppm	Cell Developmental Status
0-10	Mesenchymal (stromal) stem cell
11-20	Pre-Osteoblast
21-50	Osteoblast
51-70	Quiescent Osteocyte
71-100	Dying cell

B. Standard Curve

1. Utilizing the provided stock (100 ppm) of food dye (growth factor) and tap water, generate at least six tubes containing known concentrations of dye. For example, you might wish to create solutions having the following concentrations (in ppm): 0, 10, 20, 30, 50, 70, and 100. It is suggested that most of your concentrations be between 0-50ppm. The following equation might assist you in determining your desired concentrations: [ml. of stock/ total ml. of solution] x 100 ppm = [solution]. For example: suppose you add 2 ml. of stock to 3 ml. of tap water. Total volume of solution = 5 ml. Using the equation: [2 ml. / 5 ml.] x 100 ppm = 40 ppm. Recommendation: the culture tubes just hold 10 ml. of fluid; thus it might be easier to mix the solutions if the final volume is in the range of 5-8 ml.
2. Record the absorbance of each solution.
3. Plot a graph of the results (use a best fit line); x-axis = concentration, y axis = absorbance.

Scaffold Diffusion Assay
Standard Curve

Tube number	mL of 100 ppm GF stock	mL of water	Final Concentration of Solution	Absorbance