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Experimental Design

There are 7 steps to experimental design:

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1. Design

Experiments in StarCellBio generally involve treating either whole organisms or group of cells with various drugs and/or altering their growth conditions. The goal of a StarCellBio experiment is to determine if a treatment affects the whole organism or cells being treated. There are many ways in which a treatment can affect an organism or a cell. Scientists use various methods to diagnose the effect of a particular treatment on a cell/organism. Some

treatments result in a change in morphology while others do not. A lack of change in morphology does not mean a treatment has not affected an organism or a group of cells. Some changes lead to internal changes within an organism or cells without resulting in visible changes.

The first step is to think about the question or objective that you are trying to answer and then design a hypothesis. The user should also start to think about the experimental technique that will be best suited to analyze the samples from the experiment.

2. Set Up

During the experiment set up step, students will think of the different treatments and conditions they would like to test on a strain or set of strains during a particular experiment. StarCellBio contains information regarding the assignment and the necessary background information that is required for a student to design, perform, and analyze experiments within StarCellBio.

Within StarCellBio, these are all the possible variables that a student will be able to specify:

1. strain(s) used during an experiment. The instructor will provide all of the available strains in the software.
2. available treatment(s) and their associated conditions. Possible treatments include drugs and/or media conditions to be used during the experiment. The instructor will provide all of the available treatments in the software. Each treatment has the following conditions associated with it:

    a. concentration of treatment

    b. start of treatment

    c. duration of treatment

    d. temperature

3. time of sample collection from the start of treatment. The instructor will provide all of the possible options for collection time in the software.

In addition to the variables mentioned above, a user can also treat a particular sample with multiple treatments in StarCellBio. These treatments can either be simultaneous or sequential, depending on the treatment start time and treatment duration variables selected by the user.

At this point in the experimental design, it is critical for a user to set up positive and negative controls. A positive control is a sample in which a specific result is expected. A negative control is a sample in which no result is expected. These serve to demonstrate that the experiment worked as expected and also as comparisons to which a user can compare the results of the experimental samples.

3. Run Experiment

The experiment is performed during this stage and the samples are generated.

4. Select Technique(s)

Now the researcher selects the experimental technique that will be used to analyze the samples. In StarCellBio, there are three available techniques: western blotting, flow cytometry and microscopy.

A. Western blotting detects overall changes in the amount or chemical modifications of a particular protein.

B. Flow cytometry is used to count and analyze the size, shape and properties of individual cells within a heterogeneous population of cells.

C. Microscopy is used to study the shape, morphology and properties of cells, tissues or organisms that otherwise cannot be observed with the human eye.

For more information regarding the specific experimental techniques, see the appropriate section below.

5. Run Technique(s)

The experimental technique is performed at this time and the experimental output is created. For more information regarding the experimental techniques, see the appropriate section below.

A. Western blotting detects overall changes in the amount or chemical modifications of a particular protein.

B. Flow cytometry is used to count and analyze the size, shape and properties of individual cells within a heterogeneous population of cells.

C. Microscopy is used to study the shape, morphology and properties of cells, tissues or organisms that otherwise cannot be observed with the human eye.

6. Analyze

A student now analyzes the data that has been generated from the experimental output. For example, in western blotting, the final blot will be analyzed. In flow cytometry, students will analyze either a histogram or a dot plot to determine the number of events of a specific parameter and the size of subpopulations in their sample. With microscopy, students will study images obtained from the microscope.

For more specific information regarding the analysis of each experimental technique, reference the appropriate experimental technique below.

7. Conclude

To construct an overall conclusion for an experiment, a user brings together all of the data that they have generated – possibly from multiple western blotting and/or flow cytometry and/or microscopy techniques – to form a conclusion about the original hypothesis proposed. At this point, the user will need to decide whether the original hypothesis should be revised and/or if additional experiments should be set-up to better address the hypothesis.

Experimental Techniques

A. Western Blotting

General Information

Western blotting is a technique that allows scientists to detect overall changes of a particular protein of interest in an organism or cell. These changes can be:

1. changes in protein concentration.
2. addition or subtraction of small chemical modifications to a protein that can alter its behavior.

Proteins are the workhorses of a cell. Proteins are tightly regulated by changes in the environment so that they are only “expressed” (present) or can only be “activated” at the right time and place. Proteins are regulated by altering their concentration in the cell and/or by adding a chemical modification to the protein that “activates” or “deactivates” it. Either or both of these types of protein regulation can occur for a given protein.

Any particular process in a cell is controlled by the action of many proteins which form complex regulatory networks with some “activating” and others “repressing” the expression or activity of other proteins within the pathway. In addition, most proteins are involved in more than one cellular process, so the whole system looks like an interconnected network of protein pathways. This means that often times a particular treatment will result in a change in the amount of a protein or a change in the activity of a particular protein that is important for a specific cellular process. For example, carcinogens often cause alterations or mutation in DNA sequence, which can have detrimental effects on an organism in the long run. When you treat cells with carcinogens, cells increase the production of proteins that help them deal with the damage incurred by the treatment. In addition, cells will also suppress the production of proteins involved in promoting growth and cell division to allow for the repair of the damage before cell division, and therefore propagation of the damage to the next generation can take place. One can diagnose what type of cellular response the cells are experiencing after treating them with a carcinogen by looking at (1) how the overall concentration of a particular protein involve in repair or cell division, for example, changes after treatment and/or (2) how the particular concentration of the activated or deactivated form of such said proteins changes after treatment.

Western Blotting Experimental Technique

After the experiment is set up and performed, the protein samples need to be prepared. Proteins in the sample are then separated using a method called “gel electrophoresis”,

transferred to a cellulose membrane and then the specific protein(s) of interest are detected and visualized on a piece of photographic film.

Western Blotting Video Resources

This 'In the Lab' feature highlights how a western blot is performed in a real laboratory at the Whitehead Institute at MIT. The link is: http://www.youtube.com/watch?v=u7VwmJw9Gbc.

An overview of the western blotting procedure can be accessed in the video article entitled: "*Western blotting: Sample preparation to Detection*"[1] on www.jove.com. The link is: http://www.jove.com/video/2359/western-blotting-sample-preparation-to-detection.

The 7 steps to perform a western blotting experimental technique are detailed below.

1. Sample Preparation

Once an experiment is run and the samples are collected, the samples need to be prepared in a specific way to analyze the proteins within the sample. Once a sample has been prepared for analysis it is called a lysate.

To analyze the proteins within your sample by western blotting, you first need to prepare your lysates. Lysates can be prepared from whole tissue (made of cells as well as connective materials, etc.) or cells. If tissue is being used, then the tissue first needs to be broken down in a mechanical manner by cutting or crushing the sample. The sample can then be processed using different combinations of salts and detergents to isolate the proteins. In addition, the sample preparation is usually performed in the cold to prevent the proteins from being degraded or denatured (un-folded). Enzymes can also be added to the lysate preparation to prevent proteins from being digested or chemically modified by other enzymes contained within cells.

Proteins are found within several cell compartments: the cytoplasm, the nucleus or embedded within a membrane (either surrounding the cell or an internal membrane that surrounds an organelle within the cell). Depending on where a particular protein is located within the cell, different conditions will need to be applied to isolate the protein(s) of interest. All protein preparation protocols involve using detergents and/or salts of various strengths. Detergents cause the membrane that surrounds and protects the cell to fall apart, providing access to the proteins within the cell. Depending on where the proteins that the researcher is studying are localized within the cell, the researcher will use either weak or strong detergent to break apart only the outer membrane or all the membranes within a cell.

There are several different types of protein samples that a researcher can prepare:

1. One type of lysate sample is called a whole cell lysate because it contains all proteins that are located in a cell.
2. Another type of lysate that a researcher may want to prepare is a nuclear & cytoplasmic lysate. In this type of lysate preparation, the researcher uses increasing concentrations of detergent to first isolate proteins that are found in the cytoplasm and then a stronger detergent to isolate proteins that are found in the nucleus. The researcher will end up with two samples: one will contain all of the proteins from the nucleus and the other will contain all the cytoplasmic proteins.

3. A third type of protein sample is called an immunoprecipitate or IP. In some experiments, a researcher will want to determine whether a particular protein is associated with another protein (proteins often time found large complexes – sometimes binding to a complex is the direct result of a particular cellular event and can alter the properties of a particular protein). In this experiment, the researcher would first isolate, or immunoprecipitate (IP) a specific protein, along with anything else it is bound to, from the lysate. To do this, the researcher will use an antibody that will bind and isolate the protein from the lysate sample (see the Blotting section for information about antibodies). Then in later steps of the western blotting procedure, the researcher will probe whether this protein is associated with another protein of interest.

Once the protein sample is prepared – either whole cell, cytoplasmic/nuclear fractionations, or IP – the amount of total protein that is in each sample should be quantified. In particular experiments, it is critical to do a western blot with the same amount of total protein in each sample in order to draw conclusions from the experiment. This is extremely important in experiments that are trying to determine whether a particular treatment has an effect on the amount of a protein in a sample. StarCellBio currently has the functionality to make whole cell as well as cytoplasmic and nuclear fractionations. The capability to make immunoprecipitates will be added in the future.

Now the samples are ready to be prepared to load in the polyacrylamide gel. The appropriate amount of protein is mixed in a specific buffer, and a dye (blue color) is added to the mixture of lysate and buffer. The dye will help the researcher visualize the protein mixture in the gel, prior to and during movement of proteins through the gel. It is important to note that the dye does not actually bind to the proteins; instead, it provides a visual sense of where the protein mixture front is within the clear polyacrylamide gel. If a denaturing gel is being used, then a chemical to denature the proteins is also added and the samples are heated at 95 ºC for 5 minutes prior to being loaded in the gel's well.

2. Gel Preparation

Proteins are separated by size and shape by applying an electric field to them in a "gel". The word, gel, refers to a mesh-like matrix made of an acrylamide polymer through which different proteins will travel at different speeds depending on their size and shape (once the electric field is applied).

The gel itself is created by pouring a solution of a chemical, acrylamide, at a specific concentration into a framework that is provided by two vertical plates. Under the appropriate treatment conditions, the acrylamide will polymerize to form a solid, mesh-like matrix that is only 1-2 millimeters thick. The polymerized acrylamide is now called polyacrylamide.

Figure 1. *Separation of proteins by SDS-PAGE at varying concentrations of acrylamide.* The images show the resulting separation of proteins following electrophoresis when separated on 3%, 6%, 9%, and 12% (from left to right) acrylamide gels. Figure Credit: Ernst Hempelmann (Produced by Ernst Hempelmann) [CC-BY-3.0 (http://creativecommons.org/licenses/by/3.0)], via Wikimedia Commons.

Depending on the size of the protein of interest, the concentration of acrylamide can be adjusted, usually in the range of 6-15%. Lower gel concentrations result in large holes in the matrix and are used to obtain good separation of large proteins. In contrast, high concentrations result in the creation of small holes and crevices through which small proteins can travel and are used to obtain good separation of small proteins (Figure 1). In StarCellBio, users may have the option of selecting 10%, 12% or 15% acrylamide.

Western blotting gels usually contain 10 – 15 wells, or pockets that serve to contain the protein samples prior to the application of an electric field. In StarCellBio, the gels have 15 wells, and one well should be reserved for the protein marker.

There are two types of gels that are used in the research lab. Both of these are polyacrylamide gels (PAGE or polyacrylamide gel electrophoresis).

1. The first kind of gel that is run is a denaturing gel, also called “SDS-PAGE” gel. This gel contains a very strong negatively charged detergent (SDS) that causes the proteins to be denatured (lose their three-dimensional shape) and also coats the proteins with a negative charge. A denaturing gel separates proteins based solely on size.
2. The second kind of gel is a native gel. As the name suggests, this type of gel preserves the native conformation of a protein (no detergent is used) and separates the proteins based on shape and size.

StarCellBio currently only simulates western blotting with denaturing gels. In the future, we may add the capability to run native gels.

Video Resource

Benchfly.com has a great video resource on preparing gels. In particular, the video details how to pour an acrylamide gel and set up the gel for running. The link is: http://www.benchfly.com/video/106/pouring-acrylamide-gels-for-sds-page/. There are two additional notes to make about this video:

1. This video will provide you with a visual of what the gel actually looks like. The gel is a polyacrylamide gel. The names of the other chemicals (TEMED, etc.) mentioned are used to solidify the gel and the names of each of the chemicals are not relevant for our purposes.
2. In the description above, we have not described the two layers (upper and lower) that are contained within the gel. Because the goal of running the gel is to separate proteins based on size (and sometimes also shape), you need to have the proteins enter the gel at the same time. However, the well that holds the protein prior to the application of the electric field is relatively tall. As a result, we use gels with two layers. The sole purpose of this is to ensure that all of the proteins contained in the well enter the lower portion of the gel at the same time.

3. Load Gel

Once the gel has solidified, it is placed in a transparent box surrounded by a liquid buffer, which will help conduct the electric field once it is applied. Each of the samples can now be

pipetted into a well in the gel. Once all of the samples of interest are added to their own individual wells, the gel is ready to run.

In StarCellBio, all of the samples are loaded into the gel for the user. Users can organize their samples into by dragging and dropping the samples in the Samples Window into the particular order in which they should appear in the gel.

In order to determine the actual size of a protein, we always run a sample that is composed of proteins of known sizes in one of the gel's wells. We refer to this sample as a protein marker or ladder and it usually consists of a wide range of protein sizes, from approximately 10-250 kDa. Kilodaltons (kDa) is the term of measurement for the molecular weight of proteins.

Video Resource

Benchfly.com has a great video resource on loading a gel. In particular, the video provides a nice visual of the wells and how they hold the protein sample prior to applying the electric field. The link is: http://www.benchfly.com/video/120/how-to-load-and-run-sds-page-gels/

4. Run Gel

To separate the proteins in the gel, a uniform electric field (usually around 120-200V for about an hour) is applied. The electric field is applied in such a way that proteins will be drawn into the gel and will travel through the gel at various speeds depending on their size and shape. For the first iteration of StarCellBio, the protein samples are denatured, which means that the proteins are no longer found in their natural shape in which they are found inside a cell. Instead, they are in a linear form and they will be separated in the gel only by size, and not by shape or charge.

If multiple samples have been loaded into separate wells in the gel, then each sample will run in a parallel fashion in their individual lanes. A "lane" refers to the physical vertical space that is located beneath the well. Larger proteins will take longer to run through the gel and as a result will remain closer to the starting point (the wells) of the gel. Smaller proteins will be able to squeeze and move through the gel matrix much more easily and will travel farther through the gel matrix. As a result, smaller proteins will move closer to the bottom of the gel (farther away from the wells). Proteins in different lanes that travel the same distance will be approximately the same size.

Depending on the proteins of interest that are being examined, the electric field will need to be shut off after different lengths of time for different experiments. There are two visual indicators to alert the researcher to when the electric field should be turned off.

1. The protein samples all contain a blue dye (see sample preparation). This blue dye travels through the gel at the same rate as the smallest proteins. When the dye front gets to the bottom of the gel, this indicates to the researcher that the smallest proteins in each sample will be running off of the gel and into the buffer (lost forever!).
2. The protein ladder creates samples of known sizes that are all marked in various patterns and/or colors. Unlike the proteins in a researcher's samples that are not dyed, the proteins in the protein ladder are visually apparent. The protein ladder can

be used as a visual guide to tell the researcher where proteins of specific sizes are present in the gel as the gel is running.

If the researcher would like to visualize ALL the proteins in the gel, and not a specific protein of interest, then the gel can be stained at this point with a dye that will bind all proteins in a non-specific manner. The dye that is most commonly used is Coomassie blue, which dyes all of the proteins in the gel blue (Figure 2). It is important to note that the stain binds all proteins in a non-specific manner and as a result, a researcher cannot tell at which proteins he/she is looking. This step is not always necessary and it is often skipped during a western blotting experiment. Up to this point, this procedure can be called just “gel electrophoresis”. It is the detection of specific proteins of interest that is at the heart of a western blot. StarCellBio does not currently have the capability of performing Coomassie blue staining, but it will be added in the future.

Figure 2. *Coomassie Blue staining of two gels.* Coomassie blue staining is used to visualize all of the proteins in the gel, but does not allow for detection of specific proteins in a sample. Figure Credit: Stephen Helms from Dallas, TX, United States (My Pretty Gels) [CC-BY-SA-2.0 (http://creativecommons.org/licenses/by-sa/2.0)], via Wikimedia Commons.

5. Transfer

In this step, the proteins are transferred from the gel to a membrane. After the gel has run for an appropriate length of time to allow for proteins to separate sufficiently on the gel, the electric field is turned off. At this point, although you can’t see all the proteins, all of the proteins in a given sample are separated based on their size and are present at different places in the gel. We then immobilize the proteins by transferring them from the gel to a membrane. We do this for three reasons:

1. The gel is very thin and fragile and will not hold up well to further manipulations.
2. The proteins are contained within the gel, and are not accessible to detection by antibodies, which is the next step of the experiment. Once the proteins are transferred to a membrane, the proteins will be localized on the surface of the membrane and they will be accessible to antibody detection.
3. The proteins are not immobilized in the gel. Although the electric field is no longer applied, proteins will continue to slowly diffuse through the gel, and the diffusion will no longer be in a specific direction. As a result, we cannot use proteins in the gel to differentiate between proteins of different sizes.

To transfer the proteins from the gel to the membrane, we set up a “sandwich” in which the gel is placed next to a membrane and protected by filter paper and sponges. The membrane is a type of cellulose (looks like a thick, flexible and impermeable piece of paper) and is usually made up of nitrocellulose or PVDF (polyvinylidene difluoride). The sandwich is placed in a box and again surrounded by a liquid buffer that will help to conduct the electric field.

Once the transfer apparatus or “sandwich” is assembled, we again utilize an electric field to pull the proteins from the gel and onto the membrane. The proteins are still negatively

charged from the use of the strong detergent in the sample preparation and gel running steps. As a result, the proteins will migrate out of the gel and move toward the positive charge and, due to the membrane's properties, the proteins will "stick" to the surface of the membrane.

This step can either occur at room temperature for approximately an hour at around 100 V or can be done overnight at 4 ºC at 20-30 V.

6. Blot

Following the transfer of the proteins from the gel to the membrane, we are now ready to detect a specific protein(s) of interest that is bound to the membrane. In this step, we probe, or "blot", the membrane for our protein of interest using antibodies. Antibodies are naturally made by our immune systems in response to infection to specifically bind proteins directly. Foreign invaders contain proteins that are not recognized by our bodies as our own. The immune system then uses the foreign proteins to produce antibodies that recognize them and therefore will recognize the same foreign agent in other cells within our bodies in a current and future infection. Researchers utilize this natural process to isolate antibodies that specifically recognize proteins of interest. Companies now make antibodies commercially available and can isolate antibodies that specifically bind a protein of interest.

Antibodies are a Y-shaped protein (Figure 3). The tips of the two "arms" of the Y structure vary between antibodies and specifically recognize and bind different proteins. The "stem" portion of the Y structure is the same between antibodies of the same organism. All antibodies made in a particular organism, for example, will have a similar stem structure.

To detect proteins on the membrane, we use multiple steps designed to specifically recognize and bind a protein of interest and then amplify this detection through a second detection step and a chemical reaction, which allows the visualization of protein of interest.

Figure 3. *Antibody structure.* An antibody is a Y-shaped protein that recognizes and bind specific proteins or protein fragments, called antigens. Figure Credit: Fvasconcellos 19:03, 6 May 2007 (UTC) [Public domain], via Wikimedia Commons.

We call an antibody that recognizes a specific protein of interest a primary antibody[2]. A secondary antibody[3] is an antibody that recognizes the primary antibody. More specifically, it recognizes the stem portion of the antibody. If you use a secondary antibody that does not bind to the primary antibody, then no signal will appear on the western blot at the end of the experiment.

The secondary antibody that is used in a western blot experiment is bound to an enzyme that will then catalyze a chemical reaction to create a light signal upon the addition of a chemical substrate. This light signal can be detected using photographic film. Alternatively, the secondary antibody can be bound to a fluorescent molecule, which eliminates the need for the chemical reaction to detect the protein. StarCellBio currently simulates detection of proteins through a chemiluminescent reaction.

The multiple steps of blotting the membrane to detect proteins using antibodies are as follows:

1. The membrane is washed with a “blocking” solution that is designed to block non-specific binding of an antibody to the membrane or proteins. Although antibodies will bind specific proteins tightly, then can also bind nonspecific proteins weakly. The blocking solution will prevent weak nonspecific binding.
2. The membrane is incubated in a dilute solution of the primary antibody. The first (primary) antibody will specifically bind to target proteins that are bound on the membrane.
3. The membrane is then washed in a very low concentration of detergent to remove any weakly bound or non-specifically bound antibody to the membrane or the proteins bound to it that were not successfully blocked by the blocking solution. Any primary antibody that is tightly bound to the protein of interest will remain.
4. A second antibody incubation step is performed in which the membrane is incubated in a dilute solution of the secondary antibody. The secondary antibody will specifically bind the primary antibody. The secondary antibody is also bound to an enzyme that will be used to detect the protein. Many secondary antibodies can bind to each primary antibody, which serves to amplify the light signal that will be created in a later step.
5. The membrane is then washed in a very low concentration of detergent to remove any weakly bound or non-specifically bound antibody to the membrane or proteins bound to it. The antibodies that are tightly bound will remain.

The blot is now ready to be exposed to film to detect the protein of interest.

7. Develop

After blotting the membrane with primary and secondary antibodies, the blot is now ready to be exposed to film to detect the protein of interest. The secondary antibody is bound to an enzyme that will catalyze a chemical reaction to create a light signal that will be detected on film. There are two steps to develop a western blot:

1. To catalyze the chemical reaction to create a light signal, the necessary substrate of the reaction is added on top of the membrane. This will create a light signal wherever there is a secondary antibody bound to the primary antibody, which is bound to the protein of interest.
2. A piece of photographic film is applied to the membrane in a dark room to visualize the light signal because the light signal is not visible to the eye. The location of the protein will be marked by a light signal, which manifests itself as a dark band on the photographic film. The membrane will typically be exposed to film for a length of time that varies anywhere from seconds to hours. The amount of luminescent signal is proportional to the amount of protein that is present on the membrane. Additionally, the luminescent signal appears in the physical location of the protein on the membrane and can be used to determine the size of a protein by comparing its location with that of the protein ladder.

At this point, the researcher will have a piece of photographic film that (hopefully!) has black bands on it (Figure 4). The black bands represent the protein of interest.

If a researcher would like to look at another protein(s) of interest, then the blot (the membrane) can be erased, or “stripped”, of the antibodies (first and secondary) that are bound to the first protein of interest.

Figure 4. *An example western blot experimental result.*The black bands on the photographic film represent antibody detection of the protein(s) of interest. Figure Credit: Magnus Manske on en.wikipedia [GFDL (http://www.gnu.org/copyleft/fdl.html) or CC-BY-SA-3.0 (http://creativecommons.org/licenses/by-sa/3.0/)], via Wikimedia Commons.

Following the stripping of the blot, the new blotting conditions can be selected to detect the second protein of interest utilizing a different set of antibodies. This process can potentially be performed several times, although usually the clarity of protein detection decreases with each stripping procedure. In StarCellBio, select **RE-PROBE** to detect another protein.

Western Blot Analysis

The user will analyze the presence, position and intensity of the bands in all the lanes in order to determine the effect of a specific treatment on the protein of interest. The location of the protein bands on the membrane relative to the protein ladder will enable the researcher to determine the size of the protein. The comparison of the presence, position and intensity of the bands in the experimental samples to those for the positive and negative controls is critical to the analysis of the western blot. While the film depicts the location of the protein of interest, it does not depict the proteins that were present in the protein ladder. This is because the antibody used to detect the protein of interest does not also detect the proteins in the protein ladder (although there are cases when an antibody non-specifically binds to proteins in the protein ladder). As a result, the researcher will need to align the membrane with the film to determine the size of the proteins relative to the known sizes of the proteins in the ladder.

The analysis of the western blot is a critical educational experience and as a result, students are required to interpret their particular western blot on their own.

B. Flow Cytometry

General Information

Flow cytometry is used to count and analyze the size, shape and properties of individual cells within a heterogeneous population of cells.

There are two main categories of flow cytometry experiments that a researcher may perform. Flow cytometry may be performed to:

- Analyze the size, shape and properties of individual cells.
- Analyze and sort cells based upon the size, shape and properties of individual cells.

The reference library will focus on the analysis of the size, shape and properties of individual cells because StarCellBio currently only supports this type of flow cytometry analysis.

Flow cytometry is utilized to analyze many aspects of the cells within a population including: cell size, cell shape, DNA content, RNA content, protein expression – surface or intracellular, protein modifications, and cell death/viability, amongst others.

Flow Cytometry Experimental Technique

After the experiment is set up and performed, the samples need to be prepared for flow cytometry analysis. Each sample is prepared for the specific type of flow cytometry analysis that will be performed, run through the flow cytometer, and then the populations within the sample are analyzed.

The 3 steps to perform a flow cytometry experimental technique are detailed below.

Video Resource

To learn more about flow cytometry, see the following comprehensive reference material at Life
Technologies: http://probes.invitrogen.com/resources/education/tutorials/4Intro_Flow/player.html

1. Sample Preparation

Once an experiment is run and the samples are collected, the samples need to be prepared to analyze the individual cells within the sample. To analyze the individual cells within your sample by flow cytometry, you first need to prepare your samples to create a sample that contains only single cells, and not clumps of cells. Samples of cells can be prepared from whole tissue (made of cells as well as connective materials, etc.) or cells growing in tissue culture. If whole tissue is being used, then the tissue first needs to be broken down in a mechanical manner by cutting or crushing the sample. The sample can then be processed using different combinations of enzymes to dissociate the cells within the sample. If tissue culture cells are being used, then the cells simply need to be removed from the tissue culture plate and dissociated to create an individual cell suspension. In addition, further sample preparation is usually performed in the cold to prevent the continued cellular processes that may confound the analysis.

Depending on the particular flow cytometry analysis the researcher would like to perform, the researcher will choose one or more of the following analyses:

1. Analyze the size and shape of the cells in a sample,
2. Analyze cell viability and/or DNA content using fluorescent dyes, and/or
3. Analyze different cell properties including: protein expression, protein modifications or the cell cycle, amongst others, using antibodies.

While flow cytometry can be used to analyze the properties of cells within a population and sort the cells based on those properties, StarCellBio currently only supports using fluorescent dyes to analyze a cell's DNA content (Analysis B).

A. Size and shape

If a researcher would like to examine the size, shape and internal complexity of the cells within their sample, the researcher does not need to perform any additional steps once a single cell suspension has been prepared.

This type of analysis is beneficial for all flow cytometry analyses to identify and remove debris and dead cells from further analyses. The analysis of a cell's size, shape and complexity is at the core of quantifying the populations of different blood cell types, such as red blood cells, neutrophils, and lymphocytes, amongst others.

B. Fluorescent dyes to analyze viability and/or DNA content

To analyze cell viability within a population, the researcher incubates live cells with a fluorescent dye, such as propidium iodide (PI) or 7-amino-actinomycin D (7AAD). Performing this analysis with a population of live, in comparison to fixed, cells is critical because only the cells that have died, and therefore contain holes in the cell membrane, will allow the fluorescent dye into the cells. Therefore, following incubation of the cells with the fluorescent dye, only the dead cells will have, or be positive for, the dye. The researcher will easily be able to detect the cells that are positive for the dye and if desired, remove them from future analyses.

To analyze a cell's DNA content, the researcher incubates fixed cells with a fluorescent dye, such as propidium iodide (PI), that binds, or intercalates, DNA. While PI can also be used to analyze cell viability, PI can also be used to analyze DNA content, but must be used with fixed cells. The fixation process will enable the dye to enter all of the cells and the researcher will be able to analyze the amount of DNA in all of the cells within the sample. The amount of fluorescence signal that is detected by the flow cytometer will be proportional to the amount of DNA that is present in the cell and the researcher will use that information to determine the cell cycle stage of a particular cell.

C. Antibody-detection of protein expression, protein modifications and/or the cell cycle

We use antibodies to analyze the amount of a specific protein(s) of interest and/or protein modifications on the surface of or in the intracellular space of the cells within a population. Antibodies are naturally made by our immune systems in response to infection to specifically bind proteins directly. Foreign invaders contain proteins that are not recognized by our bodies as our own. The immune system then uses the foreign proteins to produce antibodies that recognize them and therefore will recognize the same foreign agent in other cells within our bodies in a current and future infection. Researchers utilize this natural process to isolate antibodies that specifically recognize proteins of interest. Antibodies are now commercially available and companies can isolate antibodies that specifically bind a protein of interest.

Antibodies are a Y-shaped protein (Figure 3). The tips of the two "arms" of the Y structure vary between antibodies and specifically recognize and bind different proteins. The "stem" portion of the Y structure is the same between antibodies of the same organism. All antibodies made in a particular organism, for example, will have a similar stem structure.

To detect protein expression and/or modification in individual cells, we use multiple steps designed to specifically recognize and bind a protein of interest and then amplify this detection through a second detection step using a fluorescent-label to allow the detection and analysis of the protein of interest.

We call an antibody that recognizes a specific protein of interest a primary antibody[4]. A secondary antibody[5] is an antibody that recognizes the primary antibody. More specifically, it recognizes the stem portion of the antibody. If you use a secondary antibody that does not recognize and bind to the primary antibody, then a fluorescent signal will not be detected by the flow cytometer at the end of the experiment because the primary antibody is not fluorescent by itself. The secondary antibody that is used in a flow cytometry experiment is bound to a fluorescent molecule, or fluorophore, that produces a light signal when excited at the correct wavelength of light by a laser in the flow cytometer. This light signal can be detected by the flow cytometer.

The multiple steps of detecting proteins and/or modifications using antibodies are as follows:

1. The cells are first washed with a “blocking” solution that is designed to block the non-specific binding of an antibody to the protein of interest. Although antibodies will bind specific proteins tightly, then can also bind non-specific proteins weakly. The blocking solution will prevent weak non-specific binding.
2. The cells are incubated in a dilute solution of the primary antibody. The first (primary) antibody will specifically bind to target proteins that are located on the cell surface or inside the cell.
3. The cells are then gently washed in buffer alone, not containing any antibodies, to remove any weakly or non-specifically bound antibody to the protein of interest. Any primary antibody that is tightly bound to the protein of interest will remain.
4. A second antibody incubation step is performed in which the cells are incubated in a dilute solution of the secondary antibody. The secondary antibody will specifically bind the primary antibody. The secondary antibody is also bound to a fluorescent molecule, or fluorophore, that will be used to detect the protein. Many secondary antibodies can bind to each primary antibody, which serves to amplify the fluorescent light signal that will be detected by the flow cytometer following excitation by the laser light.
5. The cells are then gently washed in buffer alone to remove any weakly or non-specifically bound antibody to the protein of interest. The antibodies that are tightly bound will remain.

Following these steps, the cells are now ready to be run through the flow cytometer to detect and analyze the protein of interest.

2. Run

Once the samples are prepared, they are ready to be run through the flow cytometer. A tube containing the suspension of individual cells is placed in the machine. The flow cytometer aspirates the solution of cells and draws the cells into the machine in such a way that the cells form a single line within a stream of liquid. In this manner, each cell within the suspension can be analyzed independently.

At a particular point in the machine, each cell will pass through a laser beam. Additionally, if the researcher is using fluorescent dyes and/or fluorescently-tagged antibodies, then additional laser(s) with the appropriate light wavelength to excite the fluorescent molecule will be utilized. A detector within the machine will collect and report the amount of light that 1) is scattered in the forward direction (forward scatter, FSC), 2) is scattered to the side (side scatter, SSC), and 3) corresponds to the emission spectra of the fluorescent molecule(s), if

applicable. Detectors will read the amount of scattered light and the amount of fluorescence detected for each cell, which is then translated into a single data point on the computer. The first two types of data, FSC and SSC, are utilized to determine the size, shape and complexity of each cell (Analysis A above), while the third type of data is used for Analyses B and C.

To learn more about how flow cytometry works, see the following comprehensive reference material at Life
Technologies: http://probes.invitrogen.com/resources/education/tutorials/4Intro_Flow/player.html

3. Analyze

As the samples are analyzed by the flow cytometer, the computer will display plots of the amount of light detected by each of the detectors in the flow cytometer for each single cell. The amount of fluorescence signal detected by the flow cytometer equates to the presence and quantity of a fluorophore, corresponding to a protein or DNA, in a cell. Each individual cell will be depicted as a single dot on the graph. The researcher can format the data to display it either as a histogram, which represents the number of cells with particular emission levels for one fluorophore or as a graph depicting the emission levels of two different fluorophores.

By examining the properties of each cell within a sample, populations or groups of cells within a sample that are expressing the same relative amounts of fluorescence will become apparent. Once the researcher has identified the appropriate populations for further analysis, the researcher can use the flow cytometry software program to divide the plot into appropriate segments to determine the percentage of cells that are within each segment of the graph.

Currently, StarCellBio only supports the analysis of DNA content by flow cytometry. When a researcher is analyzing the amount of DNA in a cell using a fluorescent dye, such as PI, the amount of PI fluorescence will be proportional to the amount of DNA in a cell. Based on knowledge of the relative amounts of DNA in a cell at various stages of the cell cycle, the researcher can determine the cell cycle stage of a particular cell.

To learn more about analyzing flow cytometry experiments, see the following comprehensive reference materials:

1) http://probes.invitrogen.com/resources/education/tutorials/4Intro_Flow/player.html

2) http://probes.invitrogen.com/resources/education/tutorials/5Data_Analysis/player.html

C. Microscopy

GENERAL INFORMATION

Microscope analysis is used to study (1) the shape and morphology of the samples (cells or tissue slices), (2) protein expression, localization and/or modification, and (3) in counting the number of objects per unit volume (e.x. counting the number of cells or bacteria) that can otherwise not be seen by the normal eye. Microscopy is also used in determining the

presence and absence of certain type of cells or organelles in a sample or specimen of tissue.

While there are many types of microscopy, this resource is only going to cover optical microscopy, which is modeled in StarCellBio. In optical microscopy, light is focused through a lens or series of lenses in order to provide magnification for a sample. There are many types of optical microscopy, including bright field, dark field, phase contrast, confocal, and fluorescence. Within the microscopy experimental technique, StarCellBio currently models phase contrast, bright field, and fluorescence microscopy.

Online Resources about microscopy:

1) Open University's OpenLearn. *Introduction to microscopy*. http://www.open.edu/openlearn/science-maths-technology/science/biology/introduction-microscopy/content-section-0

2) Wartburg College's Virtual Phase Contrast & Fluorescent Microscope. https://www.wartburg.edu/biology/fluorescentmicro/

MICROSCOPY EXPERIMENTAL TECHNIQUE

After the experiment is set up and performed, the samples may need to be prepared for microscopy analysis. Each sample is prepared for the specific type of microscopy analysis that will be performed, loaded onto the microscope, and then the samples are analyzed.

The 3 steps to perform a microscopy experimental technique are detailed below.

1. Sample Preparation

Once an experiment is run and the samples are collected, the samples may need to be prepared prior to viewing them under the microscope. Samples of cells can be prepared from whole tissue (made of cells as well as connective materials, etc.) or cells growing in tissue culture.

To analyze tissue samples, the samples will need to be collected and are often also fixed. Samples are then embedded in paraffin, or a wax substance, that provides stability to the structure during the cutting process. μM-thin sections of the paraffin-embedded tissue are then cut and adhered to slides. The slides of the unstained, cut tissue sections are now ready to be prepared for microscopy analysis.

Depending on the purpose of the analysis the researcher would like to perform, the researcher will choose one or more of the following microscopy analyses:

1. Analyze the size, shape, and quantity of cells in a sample using phase contrast microscopy,
2. Analyze a protein, nucleic acids, and/or cellular feature using dyes or stains to provide contrast in an otherwise colorless sample using bright field microscopy, and/or
3. Analyze protein expression, modification, and/or localization using antibody labeling via:

i. immunofluorescence (IF), or

ii. immunohistochemistry (IHC)

StarCellBio currently supports using microscopy in all of these ways.

A. Analyze the size, shape, and quantity of cells in a sample using phase contrast microscopy

If a researcher would simply like to view a sample of cultured cells under the microscope to examine the size, shape, and/or quantity of the cells within their sample, the researcher will use a phase contrast microscope. The sample, which is either a tissue culture plate or dish of cells, simply needs to be placed on the microscope.

A phase contrast microscope is required in this case to provide a sufficient amount of contrast to the sample to allow it to be visualized under the microscope. Other samples, including a tissue sample, will be translucent and will lack sufficient contrast to be viewed under the microscope without a dye/stain or antibody labeling.

B. Analyze a protein, nucleic acids, and/or cellular feature using dyes or stains to provide contrast in an otherwise colorless sample using bright field microscopy

Dyes and stains are used to visualize specific features including nucleic acid, subcellular structures and proteins in the sample. These features would otherwise not be visible or have sufficient contrast to view under a microscope. The use of most stains or dyes requires that the samples be killed and fixed, instead of using live cells. In these cases, it is critical that the cells have died, and therefore contain holes in the cell membrane, to allow the dye or stain into the cells. A dye or stain can be used on cultured cells or slices of tissue samples.

If the dye or stain is not fluorescent, then the sample can be viewed under a simple bright field microscope. Some of the dyes or stains may be fluorescent in which case fluorescence microscopy will be required to view the location and amount of staining.

Some commonly used dyes or stains are:

Hematoxylin and eosin (H&E). This is one of the most commonly used stains in medical histology. After a sample or tissue is H&E stained, it can be viewed on a simple bright field microscope. Hematoxylin stains nucleic acid structures blue and eosin stains basic structures, such as a cell's cytoplasm, various shades of pink, red, and orange.

Phalloidin. Phalloidin binds F-actin. It is usually conjugated to a fluorescent dye, or fluorophore, that enables the signal to be visualized using fluorescence microscopy.

DAPI. DAPI is a fluorescent dye that binds to DNA and can be visualized using fluorescence microscopy. DAPI can be used to stain live or fixed cells as it is able to pass through the cell membrane on its own. DAPI can either be used on its own or together with fluorescent antibody labeling.

C. Analyze protein expression, modification, and/or localization using antibody labeling via immunofluorescence or immunohistochemistry

We use antibodies to analyze the amount, localization and/or modifications of a specific protein(s) of interest located on the surface or in the intracellular space of the cells within a sample. Antibodies are naturally made by our immune systems in response to infection to specifically bind proteins directly. Foreign invaders contain proteins that are not recognized by our bodies as our own. The immune system then uses the foreign proteins to produce antibodies that recognize them and therefore will recognize the same foreign agent in other cells within our bodies in a current and future infection. Researchers utilize this natural process to isolate antibodies that specifically recognize proteins of interest. Antibodies are now commercially available and companies can isolate antibodies that specifically bind a protein of interest.

Antibodies are a Y-shaped protein (Figure 3). The tips of the two “arms” of the Y structure vary between antibodies and specifically recognize and bind different proteins. The “stem” portion of the Y structure is the same between antibodies of the same organism. All antibodies made in a particular organism, for example, will have a similar stem structure.

We call an antibody that recognizes a specific protein of interest a primary antibody[2]. A secondary antibody[3] is an antibody that recognizes the primary antibody. More specifically, it recognizes the stem portion of the antibody. If you use a secondary antibody that does not recognize and bind to the primary antibody, then a signal will not be visible under the microscope at the end of the experiment because the primary antibody is not visible or fluorescent by itself.

i. Immunofluorescence (IF)

To detect protein expression, modification, and/or localization by immunofluorescence, we use multiple steps designed to specifically recognize and bind a protein of interest and then amplify this detection through a second detection step using a fluorescent-label to allow the detection and analysis of the protein of interest. One of the benefits of using immunofluorescence over immunohistochemistry is that the researcher can label several different proteins of interest, each with its own color. In StarCellBio, we model using three-color immunofluorescence, with up to three different proteins being labeled in the red, green, and blue channels on the fluorescence microscope. DAPI, which appears blue on a fluorescence microscope can also be used to stain DNA instead of one of the proteins.

In immunofluorescence, the primary antibody will bind specifically to the protein of interest. The secondary antibody is bound to a fluorescent molecule, or fluorophore, that produces a light signal when excited at the correct wavelength of light by a laser in the microscope. This light signal can be detected by the user through the microscope’s eye piece and camera.

The multiple steps of detecting proteins and/or modifications using antibodies by immunofluorescence are as follows:

1. The samples or slides are first washed with a “blocking” solution that is designed to block the non-specific binding of an antibody to the protein of interest. Although antibodies will bind specific proteins tightly, then can also bind non-specific proteins weakly. The blocking solution will prevent weak non-specific binding.

2. The samples or slides are incubated in a dilute solution of the primary antibody. The first (primary) antibody will specifically bind to the target proteins.
3. The samples or slides are then gently washed in buffer alone, not containing any antibodies, to remove any weakly or non-specifically bound antibody to the protein of interest. Any primary antibody that is tightly bound to the protein of interest will remain.
4. A second antibody incubation step is performed in which the samples or slides are incubated in a dilute solution of the secondary antibody. The secondary antibody will specifically bind the primary antibody. The secondary antibody is also bound to a fluorescent molecule, or fluorophore, that will be used to detect the protein. Many secondary antibodies can bind to each primary antibody, which serves to amplify the fluorescent light signal that will be visible under the microscope following excitation by the laser light.
5. The samples or slides are then gently washed in buffer alone to remove any weakly or non-specifically bound antibody to the protein of interest. The antibodies that are tightly bound will remain.

Following these steps, the samples or slides are now ready to be viewed under the fluorescence microscope to detect and analyze the protein(s) of interest.

ii. Immunohistochemistry (IHC)

To detect protein expression, modification, and/or localization by immunohistochemistry, we use multiple steps designed to specifically recognize and bind a protein of interest and then amplify this detection through a second antibody detection step and then through a third detection step that provides a colorimetric signal to allow the detection and analysis of the protein of interest. One of the benefits of using immunohistochemistry over immunofluorescence is the extra labeling step, which serves to help amplify the signal or detection of the protein of interest. In addition, analyzing immunohistochemistry does not require a fluorescence microscope, but only a bright field microscope. While immunohistochemistry can be performed to label two proteins at the same time, it is typically only used to label one protein at a time.

In immunohistochemistry, the primary antibody specifically binds to the protein of interest. The secondary antibody that is used in immunohistochemistry specifically recognizes the primary antibody and is also bound to an enzyme, horseradish peroxidase (HRP) that will catalyze the reaction in the next step to produce a visible signal that will be visible under the microscope. This color signal can be detected by the user through a bright field microscope's eye piece and camera. Immunohistochemistry typically has a multi-step process of detection that serves to amplify the signal that will be visible under the microscope.

The multiple steps of detecting proteins and/or modifications using antibodies by immunohistochemistry are as follows:

1. The samples or slides are first washed with a "blocking" solution that is designed to block the non-specific binding of an antibody to the protein of interest. Although antibodies will bind specific proteins tightly, then can also bind non-specific proteins weakly. The blocking solution will prevent weak non-specific binding.

2. The samples or slides are incubated in a dilute solution of the primary antibody. The first (primary) antibody will specifically bind to target proteins that are located on the cell surface or inside the cell.
3. The samples or slides are then gently washed in buffer alone, not containing any antibodies, to remove any weakly or non-specifically bound antibody to the protein of interest. Any primary antibody that is tightly bound to the protein of interest will remain.
4. A second antibody incubation step is performed in which the samples or slides are incubated in a dilute solution of the secondary antibody. The secondary antibody will specifically bind the primary antibody. The secondary antibody is also bound to biotin, which will be important in the next steps. Many secondary antibodies can bind to each primary antibody, which will serve to amplify the signal that will be visible under the microscope.
5. The samples or slides are then gently washed in buffer alone to remove any weakly or non-specifically bound antibody to the protein of interest. The antibodies that are tightly bound will remain.
6. The samples or slides are then incubated in the ABC reagent that contains avidin, a protein found in egg whites and the binding partner of biotin, bound to horseradish peroxidase (HRP). HRP will catalyze a colorimetric reaction in the next step to produce a color signal at the location of the protein of interest.
7. To produce a color signal where the protein of interest is located, the sample or slides are incubated with DAB, the substrate that HRP requires to produce a brown signal. Note that other protocols use different enzymes and different substrates to generate different color signals on the sample or slide.
8. Lastly, if desired, the samples or slides are counterstained with hematoxylin, which is a stain that stains nucleic acid structures blue, to lightly stain the nuclei of each cell blue.

Following these steps, the cells are now ready to be viewed under the microscope to detect and analyze the protein of interest.

2. Load

Once the samples are prepared, they are ready to be visualized under the microscope. The sample of cultured cells or a prepared sample or slide is placed on the microscope stage.

If the researcher is simply viewing a sample under the microscope then the researcher will use a phase contrast microscope. If the researcher is using a non-fluorescent dye or stain, then the researcher will use a phase contrast or bright field microscope. If the researcher is using fluorescent dyes/stains and/or fluorescently-tagged antibodies, then the researcher will require a fluorescence microscope equipped with a laser and multiple emission filters that can be changed to specifically detect fluorescently-labeled proteins.

3. Analyze

A typical microscope has many settings and controls to ensure proper visualization of the sample or slide. Not all of the controls will be relevant for every sample or microscopy analysis.

When a phase contrast or bright field microscope is being used, then the researcher will turn on the light, adjust the brightness, focus the sample, move the sample or slide around the stage, and view the image through the eye piece.

When a fluorescent microscope is being used to visualize a fluorescent dye/stain or immunofluorescence, the researcher will turn on the laser and turn off the light, use the appropriate filter for the fluorophore, focus the sample, move the sample or slide around the stage, and view the image through the eye piece.

The sample or slide on the microscope stage may need to be moved around the objective to visualize the desired portion of the sample through the eye piece.

In StarCellBio, we model the following microscope controls:

- *Move the image.* Use the navigation arrows or keyboard to move the image around underneath the objective.
- *Light on/off switch and brightness adjustment*.
- *Laser on/off switch*.
- *Filters*. Select the appropriate filter for the emission level of the fluorophore in the sample. There are four options in StarCellBio: red (R), green (G), blue (B), and all (A) filters.
- *Course and fine focus*. Use the up and down arrows to adjust the course and fine focus on the left and right, respectively.
- *Objective*. Depending on the assignment in StarCellBio and the available images, you may be able to select a microscope objective to use. If there are no options for changing the objective, then the current objective will be displayed.

Acknowledgements

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[1] Eslami, A., Lujan, J. Western Blotting: Sample Preparation to Detection. *J. Vis. Exp.* (44), e2359, doi:10.3791/2359 (2010).

[2] If a primary antibody is made in a mouse to detect protein X, then it will be called “mouse anti-protein X”.

[3] If a secondary antibody is made in a rabbit to detect a mouse primary antibody, then it will be called “rabbit anti-mouse”.

[4] If a primary antibody is made in a mouse to detect protein X, then it will be called “mouse anti-protein X”.

[5] If a secondary antibody is made in a rabbit to detect a mouse primary antibody, then it will be called “rabbit anti-mouse”.