CELL BIOLOGY MODULE: SESSION 1

Location: 3110 Digital Computing Laboratory (DCL)

Lead Instructor: Joanne Manaster, School of Integrative Biology
Lab Assistant: Meghan McCleary, School of Integrative Biology

Cell Biology Lab Exercise 1a: Aseptically Preparing Media

Supplies

You and your partner, aseptically prepared to enter the biosafety hood

70% ethanol in spray bottle

Kimwipes

Automatic pipettor in the Biosafety hood

10 ml and 5 ml pipettes

Orange cap pyrex bottle with 43ml DMEM-

Eppendorf tube with .5ml penicillIan/streptomycin

15ml tube with 5ml NCS (newborn calf serum)

Eppendorf tube with .5ml Sodium pyruvate

Cell Biology Lab Exercise 1a: Aseptically Preparing Media

Procedure

1. Wash your hands and spray them with 70% ethanol.
2. Wipe down the surface of the Biosafety hood with 70% ethanol and a large kimwipe.
3. Obtain all bottles and tubes from the refrigerator. Spray (with 70% ethanol) and wipe the bottles and tubes you will be using. Place them in the Biosafety hood, and loosen the bottle and tube caps before beginning. Get the pipettes you will be using out of the drawers. Spray, wipe and place these in the Biosafety hood.
4. Note: your media bottle currently contains the red solutions and is labeled DMEM- for now.
5. With a 5 ml pipette and the automatic pipettor, remove 5 mls of NCS from the tube. Add it to your media bottle. Leave the empty tube in the Biosafety hood, but throw the pipette away. Loosely replace your media lid.
6. Place a new 5 ml pipette on the automatic pipettor. Add 0.5ml sodium Pyruvate to your bottle of media. Leave the empty tube in the Biosafety hood, but throw the pipette away. Loosely replace your media lid.
7. Place a new 5 ml pipette on the automatic pipettor. Add 0.5ml penicillin/streptomycin to your bottle of media. Leave the empty tube in the Biosafety hood, but throw the pipette away.
8. Tightly close your media bottle. Remove it from the Biosafety hood, and label it with a piece of tape with your group member's initials, today's date and add "DMEM 10% NCS++" . Ask Joanne or Meghan to place your media in the water bath for the next exercise.
9. Remove and throw away all empty tubes and other trash from the biosafety hood. Spray the work surface of the Biosafety hood with ethanol and wipe it down with a kimwipe.

Cell Biology Lab Exercise 1b & c: Lifting and Seeding Cells

Supplies

You and your partner, aseptically prepared to enter the biosafety hood

70% ethanol in spray bottle

Kimwipes

Automatic pipettor in the Biosafety hood

10 ml and 5 ml pipettes

Your media from exercise 1a*

A bottle of PBS*

A tube of TRED*

Water bath set to 37^{O}C

Flask of cells from Meghan or Joanne

Glass Pasteur pipettes in sterile square metal box

Vacuum pump

Incubators with and without CO_2

Inverted phase microscope

New, sterile T-25 flask

*Can be found in the water bath. Label the PBS with your group's initials.

Cell Biology Lab Exercise 1b: Lifting Cells

Procedure

1. Wash your hands and spray them with 70% ethanol.
2. Wipe down the surface of the Biosafety hood with 70% ethanol and a large kimwipe.
3. Take warmed media, PBS and thawed TRED out of the water bath. Spray (with 70% ethanol) and wipe the bottles and tubes you will be using. Place them in the Biosafety hood, and loosen the bottle and tube caps before beginning. Get the pipettes you will be using out of the drawers. Spray, wipe and place these in the Biosafety hood.
4. Remove your flask of cells from the incubator. View the cells under the inverted phase microscope to observe their shape and confluency. Take the flask to your Biosafety hood, and spray it with 70% ethanol. Wipe it dry with a Kimwipe; place in the Biosafety hood.
5. Gently shake one glass Pasteur pipette from the metal container. Place the large end of the pipette into the vacuum tube, found on the right side of the Biosafety hood. Draw off the media from your flask. Throw away the glass pipette in the sharps box.
6. Place a new 5 ml pipette on the automatic pipettor. Obtain 5 ml PBS (replace cap back on bottle) and dispense PBS into your flask. Loosely replace the cap on the flask. Throw the pipette away. Rock the flask back and forth to spread the PBS across the surface of the cells.
7. Using a new glass Pasteur pipette and the vacuum pump as before, draw off the PBS from the flask. Throw away the Pasteur pipette into the red sharps box.
8. Using a 5ml pipette and the automatic pipettor, remove all 2 ml of TRED from the tube and dispense into the flask. Rock the dish for 5-10 seconds to distribute the TRED. Use the same pipette to remove excess TRED and replace in the tube.
9. Place the flask in the “non CO2” incubator for 1-5 minutes. During that time, check to see if your cells have detached , and ask Joanne or Mike to help you.

Cell Biology Lab Exercise 1c: Seeding Cells

Procedure

1. When your cells have detached, use a 5ml pipette and automatic pipettor to obtain 4ml of media and dispense into the flask. Gently pipette the liquid up and down.
2. Ask a lab assistant to calculate how many cells are in your flask.
3. Write the volume of cells you need to seed in the new flask _____ml____ (1)
4. A flask holds 7-8 mls of solution. Calculate how much media you need to add to the flask. Write it here: _____ml ___(2)
5. Get a new T-25 flask from the center table. Spray, wipe and place it in the Biosafety hood. Get more pipettes ready to use.
6. In the Biosafety hood, remove the lid from the sterile, new flask.
7. Using a new glass Pasteur pipette and the vacuum pump as before, draw off the PBS from the flask. Throw away the Pasteur pipette into the red sharps box.
8. Using a 5ml pipette and the automatic pipettor, pipette the cell volume (1) out of your first flask and into your new one. Throw away the pipette.
9. Using a new pipette and the automatic pipettor, pipette the media volume (2) out of the media bottle and into your new flask, quickly replace the cap of the bottle. Throw away the pipette.
10. Replace the lid on all your bottles. Using a Sharpie, label you r flask with your group member's initials on the frosted section on the side of the flask. Place your flask in the 5% CO_2 incubator.

Cell Biology Lab Exercise 1: Media prep and cell handling

Information

Equipment in Cell Culture

There are several pieces of equipment and tools used commonly by those who practice cell culture.

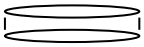
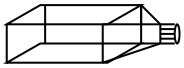
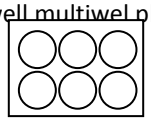
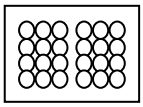
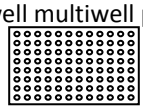
1. **Pipet aid or automatic pipettor**: A tool used to aspirate and dispense volumes of fluid quickly. Care must be taken to not pull fluid into the mouth of the pipet. Top button will draw fluid up into the pipet; Bottom button will eject fluid from the pipet. Doing either at too fast a rate or past the fluid level will result in the formation of bubbles that can disrupt your cells.

2. **Sterile serological pipets**: Come in various sizes, wrapped individually with a cotton plug at the top. Carefully open the top of the package, insert end into mouth of pipet, turn the pipet so the numbers are facing you and then remove the rest of the package.

3. **Various serological or conical tubes and bottles:** Most commonly used are 4ml and 15ml round bottom tubes with a snap cap. The snap cap has two stops. To fully close the tube, press past the second stop. Conical tubes come in 15ml or 50ml size with a screw cap. Bottles have been pre-sterilized for this course and have a screw cap. For ease of performing your techniques, it is best to loosen all lids in the hood before beginning. If you can, hold the lid in your hand when bottle or tube is open. If your dexterity does not allow for this, you may set the cap down either facing up or down (it is a point of argument as to which is more sterile)

4. **Micropipets:** Come in various sizes. A more detailed account follows. Used for dispensing very small volumes of liquid.

5. **Culture dishes and flasks:** Come in various sizes (see table below). These are sterile packaged and the surfaces are usually treated in a way that the cells will adhere. Typical bacterial Petri dishes are not suitable for mammalian cell adhesion.

Tissue culture vessel	Surface growth area	Volume of media	Volume of trypsin
60mm (6cm) dish 	21.0cm^2	4.0ml	0.5-0.6ml
100mm (10cm) dish 	78.5cm^2	10.0-ml	1.0ml
T-25 flask 	25cm^2	7.0-8.0ml	0.5-0.8ml
6 well multiwel plate 	9.6cm^2 per well	2.0ml per well	0.2-0.3ml per well
24 well multiwell plate 	2.0cm^2 per well	0.8-1.0ml per well	0.08-0.10ml per well
96 well multiwell plate 	0.32cm^2 per well	0.1-0.2ml per well	0.01-0.02ml per well

Using Micropipettors

There are several different size micropipets. Each has a range of usefulness. The button at the top of the pipettor indicates the volume range it can accommodate.

Pipettor	**Range**	**Tips** (*always use tips!*)
2-20 (red ring)	2 - 20 µl	natural or yellow tips
20-200 (green ring)	20 - 200 µl	same as above
100-1000 (blue ring)	100 - 1000 µl	large blue or natural tips

Micropipet plungers have two stops. Before doing anything else with the micropipet, practice with the plunger to sense the two stops you can feel when it is depressed with your thumb or forefinger. The first stop, when released, will draw up, into the tip, the volume you have dialed. The second stop is used to force the entire volume of liquid out of the pipet. If you depress to the second stop to draw liquid into the pipet, you will have an unknown volume in your tip.

Dial the correct volume on the micropipet (more on this below). Place a tip on the end of the pipettor by plunging pipettor into tip in tip box. You do not need to jam the pipettor repeatedly into the tip. Hold pipettor in vertical position and using thumb or forefinger, depress the plunger to the **FIRST STOP** and submerge the tip below the liquid surface.

To draw up this volume…..	**…..submerge your tip approximately this far beneath the surface of the liquid**
volumes up to 10 µl	1mm
volumes up to 100 µl	2-3mm
volumes from 100 µl to 1000µl	2-4mm
volumes over 1000 µl	3-6mm

Carefully and slowly, release the pressure on the plunger to draw the liquid into the tip.

Move the micropipette tip to the desired tube or dish and dispense the liquid by pressing the plunger to the **SECOND STOP**. Release the pressure from the plunger. Eject the tip if changing to a different solution.

To **properly set the volume**, wind the micrometer 1/3 revolution above the desired volume setting and slowly wind down to the desired volume. Never wind up to a desired volume.

Using Micropipettors, continued

The place value of the numbers varies by the pipet. Refer to the chart below. In addition, there are 5 tick marks between the numbers at the bottom of the dial. On the 20-200µl pipet, if you want to pipet 71.5 µl, the top number would be 0, the second number would be 7, the third would be halfway between 1 and 2 (each tick mark would be .2) and the bottom number will appear offset.

	2-20µl	20-200µl	100-1000µl
Top # value	10s (up to 20)	100s (up to 200)	**1000** (red digit)
Middle # value	1s	10s	100s
Bottom # value	**1/10** (red digit)	1s	10s
Tick marks	1/100(each tick worth .02µl)	1/10 (each tick worth .2µl)	1 (each tick worth 2µl)

Aseptic Technique

One of the most important concepts central to performing tissue culture is that of **aseptic technique**. The media the cells grow in is very nutrient rich. Bacteria and fungi (present on every surface) as well as the cultured cells will proliferate in the media. The presence of bacteria and fungi will compete with the cultured cells for the nutrients and dissolved CO_2 and O_2. Even bacteria and fungi that would not harm humans can contaminate the culture.

The goal of aseptic technique is to avoid introducing bacteria and fungi into the culture. Doing so takes some practice and attention to detail, but for the most part is common sense. The following is a list of certain things to be aware of and guidelines to follow.

1. Work in a culture hood set-aside for tissue culture purposes. Most have filtered air that blows across the surface to keep microbes from settling in the hood.

2. Wear short sleeves or roll your sleeves up. Turn your baseball caps back if you MUST wear them, tie long hair back and remove rings and watches.

3. Wash hands with soap and water before beginning the procedure and rewash if you touch anything that is not sterile or within the hood.

4. Spray down your hands, work surface, and anything that will go into the hood with 70% ethanol. Rewipe at intervals if you are working for a long time in the hood. This will reduce the numbers of bacteria and mold considerably.

5. Do not breathe directly into your cultures, bottles of media, etc. This also means to keep talking to a minimum. No singing or chewing gum. No texting!

6. Work as quickly as you can within limits of your coordination. Also, keep bottles and flasks closed when you are not working with them. Avoid passing your arm or hand over an open bottle.
7. Use only sterilized pipets, plates, flasks and bottles in the hood for your procedures.

8. Remove sterile pipets from the package just before use. Set up the numbers on the pipet so that they face you. Never mouth-pipet, use the pipetting aid. Change pipets for each manipulation. If the tip of the pipet touches something outside of the flask or bottle, replace with a new one. Never use a pipet twice.
9. When handling flasks and plates, take care to not allow media to slosh near the rims or openings. This is one common way for microbial contamination to get into the dish.

Discard all used items immediately in the proper receptacle. Glass goes into a "sharps" box, plastic pipets go into the trash bag, and excess cells are treated with a diluted bleach solution for a few minutes and then discarded down the drain.

Solutions Used in Media

1. DMEM (Dulbecco's Modified Eagle Media**)** - Buffered (at pH 7.2) media used to grow cells *in vitro*. Contains glucose, glutamine, and essential salts, which are necessary as energy sources and for proper balance for cell growth. It also contains a pH indicator (phenol red) to indicate when the media is too acidic (orange) or too alkaline (purple).

2. Pen/Strep (penicillin/streptomycin) - two antibiotics added to the DMEM to keep the levels of bacterial contamination to a minimum. Usually added at a 1% of total solution.

3. FBS (Fetal Bovine Serum) or Newborn Calf serum (NCS) - serum added to the media as a nutrient source for the growing cells. Serum is the fluid obtained when blood is allowed to clot and the clot (containing cells and clotting factors) is discarded. The substances in FBS are undefined and vary from batch to batch so is not used by some researchers and is avoided for industry purposes. The components of serum are also the items that make the media "bubble". Care must be taken to avoid bubble formation in the culture as it will disrupt the cell layer or possibly introduce microbes. Added at 5-10% of total media solution.

4. Sodium Pyruvate- a component of the Kreb's cycle and is needed by most cells to grow in culture. Sometimes included in the DMEM. Added at 1% of total solution

5. Sodium Bicarbonate- to help buffer the solution. Helpful for some cell lines grown at higher CO_2 concentrations.

Cell type classification

Cells come from many sources and can be classified depending upon that source. Below are a few common definitions.

1^{O} Culture- cells taken directly from a tissue (i.e., fibroblast from an embryo chick tendon) and grown *in vitro*.

2^{O} Culture- these are cells taken from a primary culture and passed or divided *in vitro*. Therefore, once a primary culture has divided and proliferated it can be termed a secondary cell culture. These cells normally have a limited number of divisions or passages. Once they reach their limit they will undergo *apoptosis* (programmed cell death).

Cell Line- some cells in a passage may undergo a genetic mutation, thus altering them in such a way as making them immortal. They will grow beyond their limit: therefore, will not undergo apoptosis. Such cells will grow indefinitely. This is the definition of the cells you are using in lab today.

Transformed Cell Line- this is a cell line that has been transformed by a tumor-inducing virus or chemical. These cell lines often grown without attaching to a surface and will proliferate to a much higher density in a culture dish. Transformed cell lines have the ability to cause tumors (benign or cancerous) when injected into a susceptible animal.

Hybrid Cell Line (Hybridoma)- A cell line where two different cell types have been "fused' together creating a single cell which often confers the ability of both individual cell's characteristics into one.

More cell culture terminology

Contact Inhibition- A normal cell (primary, secondary or cell line) culture will exhibit this phenomenon, where the cells that come into contact with other cells will cease their growth and arrest in G_0 of the cell cycle. Transformed cell lines tend not to be constrained by this density-dependent inhibition of cell division, and will "pile up" in layer upon layer as they proliferate.

Anchorage Dependence- The majority of cell cultures require that the cells bind to an extracellular surface (matrix) for growth. Transformed cells lack this dependence for anchorage and thus can grow freely in the culture/media solution. Cells that float freely *in vivo* such as blood cells also are anchorage independent (also known as suspension culture).

Passage Number- The number of time the cells have been removed from the culture plate and then replated. Sometimes, the passage can refer to the completion of a cell cycle, but this definition is not pertinent to our lab today.

Additional Equipment used in Cell Culture

CO_2 incubator-a humid chamber kept at 37^{o} C and usually between 5%-10% CO_2 where cells can grow.

Non-CO_2 incubator- a heated dry chamber without additional gas, used to expedite the trypsinization process.

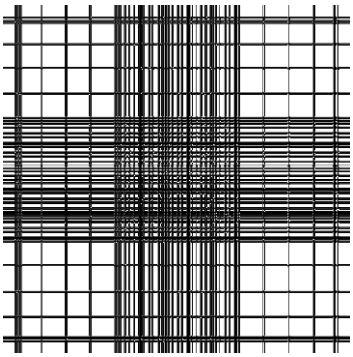
Vacuum aspirator-near the hood, used to permanently aspirate liquids from the plate or flask.

Inverted phase microscope- The primary obstacle of biological microscopy is that most objects have poor contrast (most everything is primarily water). Phase microscopy exploits differences in refractive indices in different areas of cells to create contrast, thus no stain is needed. The objective lenses are below the sample and the oculars are above, thus the term 'inverted'.

Hemacytometer -a specially designed counting chamber with a precisely etched counting grid for counting cells. Each square of the hemacytometer represents a total volume of 0.1 mm^3 (10^{-4} cm^3) under the coverslip. Since one cubic cm is approximately 1 ml, you can determine the cell concentration and the total number of cells using the following calculations:

$$\text{Cells/ml} = \frac{\text{total number of cells}}{\text{number of squares} \times \text{dilution factor} \times 10^4}$$

DIAGRAM OF ONE CHAMBER OF A HEMACYTOMETER



Other Useful Solutions

PBS-CMF (phosphate buffered saline-calcium magnesium free)- A buffered solution used to "wash" or dilute cells. It is buffered at 7.2 so as not to disrupt the cells when rinsing. It is warmed to 37^{o} C before use.

TRED (trypsin EDTA)- used to detach the cells from a culture dish. Trypsin cleaves a peptide bond (lysine or arginine) in the fibronectin (of the extracellular matrix) while EDTA chelates calcium ions (on which cell-cell adhesion depends) in the media. This is also a reason we use PBS that is free of calcium ions, since it is such a vital component in extracellular binding.

Since trypsin is an enzyme, it works better at higher temperatures so the plate with a thin layer of TRED is set into a non-CO^2 incubator for a few minutes to increase the speed of enzyme function.

Extra cautions while using TRED:

- When warming to place on cells, don't leave in water bath too long or it will self digest.
- When initially adding TRED, remove the extra fairly quickly or you will aspirate all of your cells.
- Trypsinizing cells for too long (greater than 10-15 minutes) will reduce cell viability

Trypan blue- an exclusion dye. Cells that are living have intact membranes and cannot take up the dye. These cells will appear bright and refractile under the phase microscope while dead or membrane damaged cells will take up the dye and appear dark. Useful in conjunction with the hemacytometer to cound cells and determine their viability.

Bleach- used to kill any remaining living cells in the dishes and tubes before disposal. This is an acceptable method for disposing of Biosafety Level 1 materials.

Observing Cultured Cells

While maintaining your cultures, you will want to assess their health before you work with them. You will want to do this everyday under normal circumstances to determine if your aseptic technique is good and your media is appropriate. This is easy to do by making the following observations:

Check the color of the media. It indicates the pH of the media and the health of the

cells. After a day or two of growth, it is normal for the media to appear slightly orange (acidic) since the cells waste products will lower the pH. However, a yellow color may indicate microbial growth. If the media turns dark red or purple, this is a danger sign because there is a problem with the CO_2 concentration (Usually too low, or you forgot to loosen the cap of your flask). Your cells are most likely dead at a high pH. Cells can tolerate slightly acidic media.

Observe the cells under the inverted phase microscope (how does this scope provide contrast without the need for staining the cells?) Healthy 3T3 fibroblast cells will be spread out and adhere to the surface of the plate. Dead or dying cells will be rounded and appear highly refractile (bright). Dividing cells will also appear round and refractile because they must detach from the dish in order to divide (look for mitotic figures). If most of the cells are floating or are in an uncrowded dish, your culture is most likely dead.

- After passaging, healthy cells will adhere to the plate within an hour or so. Cells are susceptible to damage from rough treatment including the addition of cold media, over- trypsinization, foaming the media with the pipet, and exposing the cells to air for too long. Treat your cells gently.

Observe the *confluency* of your culture. A fully confluent plate has cells covering most of the surface with very little space between cells. If about 60% of the plate is covered with cells with 40% of the plate visible, the culture is considered 60% confluent. Best results are obtained with a plate of about 70-90% confluency (a 20% confluency will not give good cell counts and most of the cells may be lost during passaging).

Once anchorage dependent cells are growing, what can you do with them to keep them growing?

1. **"Feed them"**: Remove old media and replace with fresh warm media. Usually done when confluence is low and culture is less than 4-5 days old.

2. **Lift and replace on same plate**: Remove old media, rinse plate with warm PBS-CMF and using trypsin EDTA, lift the cells from the plate and then feed. Again, this is for low confluence but when culture is more than 4 days old. Secreted factors remain on the plate to facilitate growth. Detaching the cells may keep them from spontaneous "transforming" into an unusual phenotype.

3. **Subculture, pass (passage) or split them**: Different terms for the same process. Remove all media, rinse plate with warm PBS-CMF, trypsinize to remove cells from plate, resuspend cells in warm media and transfer some cells to a new plate (or plates) with fresh warm media.

CELL BIOLOGY MODULE: SESSION 2

Location: 3110 Digital Computing Laboratory (DCL)

Lead Instructor: Joanne Manaster, School of Integrative Biology
Lab Assistant: Meghan McCleary, School of Integrative Biology

Cell Biology Lab Exercise 2: Transfecting Cells

Supplies

You and your partner, aseptically prepared to enter the biosafety hood

70% ethanol in spray bottle

Kimwipes

6 well plate with cells in each well from instructor

Serum free media- 1ml in eppendorf tube

3 micropipettors, set to dispense 100 µl, 97µl and 94µl

0.5µ -10µl micropipettor and tips

Gloves

6 0.5ml eppendorf tubes

FuGENE reagent

DNA 1µg/µl

Incubators with and without CO2

Inverted phase microscope

Cell Biology Lab Exercise 2: Transfecting Cells

Procedure

1. Wash your hands and spray them with 70% ethanol.
2. Wipe down the surface of the Biosafety hood with 70% ethanol and a large kimwipe.
3. Get an eppendorf tube of serum free media out of the water bath. Take it to your hood, and spray and wipe it with 70% ethanol before placing it in the hood.
4. Take 6 small eppendorf tubes out of the beaker (in the Biosafety hood). Set them up in a tube rack. With a Sharpie, label them A, B, C, D, E and F.
5. Using the pre-set micropipettors with yellow tips, pipet up the following volumes and place them in the appropriate small eppendorf tubes.

Tube	Volume of serum free media
A	100 µl
B	94 µl
C	100 µl
D	97 µl
E	97µl
F	94 µl

6. Mix the room temperature FuGENE reagent by tapping (1 second).

   The next few steps can be tricky because you are working with tiny volumes, if you feel uncertain, Joanne or Meghan will be more than pleased to step in and assist!

7. Using the 0.5-10µl pipettor and specialized tips, carefully add the FuGENE reagent to the serum free media in your eppendorf tubes as follows:

Handling FuGENE 6

Avoid contact of FuGENE with the sides of the tubes

Submerge pipet tip into the media to avoid a layer of FuGENE reagent on the top of the media

FuGENE is very volatile. Recap the tube immediately after use.

Procedure continued

Tube	FuGENE
A	0μl
B	6μl
C	0μl
D	3μl
E	3μl
F	6μl

8. Once the FuGENE reagent is added, immediately flick or tap the tube for 1 second to ensure adequate mixing of the components.
9. Incubate at room temperature for 5 minutes.
10. Using a 0.5-10μl pipet and the special tips, add DNA (1μg/μ) to each tube as follows:

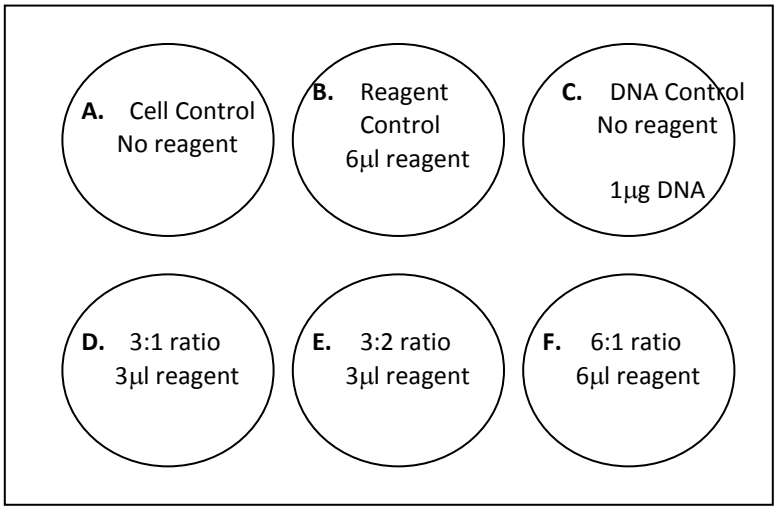
Handling DNA

- Wear gloves to prevent DNases from contacting the sample and destroying the DNA
- DNA is used in small amounts. Practice using a 0.5-10μl pipet today before beginning the experiment. Specialized tips are required.
- DNA must stay on ice. Enzymes that could destroy the DNA will be less active when cold.

Tube	DNA
A	0μl
B	0μl
C	__μl
D	__μl
E	__μl
F	__μl

11. Mix by tapping the tube for 1 second.
12. Incubate for at least 15 minutes at room temperature
13. Remove the cells from the incubator and place in the Biosafety hood

Procedure, continued



14. Using a micropipettor, add 100μl of the complex drop-wise to its designated well as outlined above. Swirl the plate to ensure mixing of the complex in all of the wells.
15. Return the plate to the incubator. Incubate the cells for 1-3 days.
16. **The next time you come to this lab**, you will observe your cells under the fluorescent scope, switching between phase contrast and fluorescence to compare how many cells have begun expressing GFP-tubulin.

Cell Biology Lab Exercise 2: Transfecting Cells

Information

Today's cells

The **COS-7 cell line** developed from the standard CV-1 African green monkey kidney line by transforming the normal cells with an origin defective mutant of simian virus 40. COS-7 cells exhibit typical fibroblast morphology and are often used in transfection experiments and in research focusing on SV40 (simian virus 40). Due to the presence of portions of DNA from SV40, COS-7 cells are officially classified as Biosafety level 2. Your cells will already be grown and no harm should come from adding the transfection reagent and DNA to them. Additionally, gloves will be used to protect the DNA and also to protect the participants.

Transfection

Transfection is a process by which foreign DNA is purposefully introduced into mammalian cells such as those in cell culture. **FuGENE 6 Reagent** is a transfection reagent that interacts spontaneously with DNA to form a lipid-DNA complex and the fusion of the complex with tissue culture cells results in the efficient uptake and expression of the DNA. It is a proprietary formulation of a nontoxic cellular protein and a small amount of a novel polyamine optimized for maximal transfection efficiency, ease of use and minimal cytotoxicity.

GFP

Green Fluorescent Protein is a single chain of 238 amino acids. The light-producing part of the protein is the chromophore, which is formed by the amino acids Serine, Tyrosine and Glycine. In nature, the chromophore emits light through oxidation of these amino acids.

The DNA which encodes the GFP gene is located on one of the jellyfish chromosomes. Biotechnologists have isolated this gene using genetic engineering techniques and have inserted it into a bacterial plasmid. The recombinant plasmid can be placed into bacteria or mammalian cells, the GFP protein can be expressed and harvested or used to trace cellular movement through the organism or view overexpressed proteins also on the plasmid.

GFP is used to monitor gene expression and protein trafficking within intact cells. GFP fusion proteins are easily visualized by standard fluorescence microscopy. The plasmid for transfection today contains DNA coding for GFP and tubulin (a cytoskeletal protein that polymerizes to form microtubules). While most cells have plenty of tubulin, this plasmid will cause the cell to over-express tubulin. This will be seen as glowing microtubules within the cell.

CELL BIOLOGY MODULE: SESSION 3

Location: 3110 Digital Computing Laboratory (DCL)

Lead Instructor: Joanne Manaster, School of Integrative Biology
Lab Assistant: Meghan McCleary, School of Integrative Biology

Cell Biology Lab Exercise 3: Fluorescent Staining of Actin

Supplies

One per group:

Box of Kimwipes:

1 piece of Parafilm

Lab Tek slide with four chambers, seeded with fixed fibroblasts

1 glass microscope slide

Foil box

10 ml PBS

1ml glycine in tube

1ml triton-X in tube

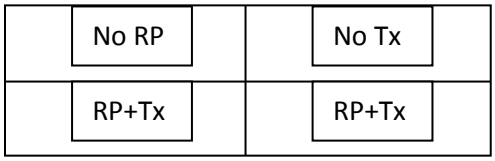
Liquid waste container

Micropipettor (1000µl) with tips

Cell Biology Lab Exercise 3: Fluorescent Staining of Actin

Procedure

1. You will receive a slide with four chambers mounted on the top. Each chamber has fibroblast cells fixed to the slide with formaldehyde (4%). They have also been rinsed with PBS and glycine.
2. Aspirate the glycine from each chamber with a 1000μl micropipettor. Place the glycine in your "liquid waste" container. Using a new pipette tip, add fresh glycine to each coverslip dropwise (gently). Let it sit at room temperature for 5 minutes.
3. Aspirate the glycine as before, with a new pipette tip, rinse each chamber 5 times with PBS. Remember to add the liquid dropwise.



4. Aspirate the last PBS wash and add Triton-X 100 to all chambers except the one that says "no Tx" in the diagram above. Keep PBS in chamber with "no Tx". Let it stand for 10 minutes (set your timer)
5. Aspirate the Triton X-100 and rinse 5 times with PBS. Place a wet paper towel under the Parafilm.
6. Aspirate the last of the PBS, Ask Joanne or Meghan to place 250 μl of Rhodamine phalloidin in each section of the chamber except the one that says "No RP". Keep PBS only in chamber that says "No RP" Cover the slide that is sitting on the wet paper towel with the foil box.
7. Aspirate the Rhodamine phalloidin and place it in the liquid waste container. Rinse each chamber 5 times with PBS. Leave last rinse of PBS on chamber and take the slide to the culture room where we will use the inverted phase fluorescent scope to view the samples. Keep slide "in the dark" at all times to prevent photobleaching.

Cell Biology Lab Exercise 3: Fluorescent Staining of Actin

Information

The cytoskeleton

Virtually all eukaryotic cells contain an intracellular support system known as the cytoskeleton. Microtubules (approximately 20-25 nm in diameter), intermediate filaments (7-11 nm in diameter) and microfilaments (3-7 nm in diameter) are the three components of this web-like mesh that inhabits the cytoplasm and gives dimension and shape to the cell. Microfilaments, named because of their relatively small diameter, will be the focus of this exercise. The primary component of a microfilament is filamentous actin (F-actin). These actin filaments consist primarily of globular subunits (G-actin) which are actually single polypeptides about 375 amino acids long.

When cultured cells are faced with a novel environment such as new ECM proteins or artificial scaffolding made of metal, plastics or ceramics, the receptors on the cells sense this and send the information into the cell via integrins to the cytoskeletal components (particularly actin). Current research investigating cellular response to artificial surfaces has begun to discover that the cytoskeletal response to topography is transmitted to the nucleus and may determine the upregulating of genes that will eventually to choose whether the cell will adhere, migrate, proliferate, differentiate or undergo apoptosis.

Fluorescence as a research tool

Fluorescence microscopy is used to detect structures, molecules or proteins within the cell. Fluorescent dyes can be a molecule that is a direct stain or probe for specific structures or it can be bound to a non-fluorescent probe (an antibody or other molecule) that recognizes specific structure.

The fluorescent microscope makes use of two highly specialized filters called **barrier filters**. These filters assure that light only of specific wavelengths reach the observer's eye. In the case of rhodamine, "red" light is emitted by the dye when it is excited by "green" light. Essentially, the first barrier filter only lets light with wavelengths of 520-560 nm (green) pass through and this light is cast upon the stained object which then emits light at a much longer wavelength. This emitted light then passes to the second barrier filter, which allows only the red light (650-720 nm) to pass through to the viewer's eye. Thus, through the fluorescent microscope, we see rhodamine stained F-actin as red fibers against a black background.

This exercise highlights a prominent technique that is commonly used to visualize F-actin before and after specific manipulation. This technique involves the use of the fluorescent stain **rhodamine phalloidin** in conjunction with a fluorescent microscope. Phalloidin is a small bicyclic peptide of seven amino acids derived from the deadly mushroom *Amanita phalloides*. Rhodamine phalloidin is a fluorescent conjugate that readily binds to F-actin filaments, but unlike antibodies to actin, is unable to bind to monomeric G-actin. This provides a high level of staining precision resulting in an extremely large contrast between stained F-actin and non-stained background.

You will learn about the functions/modes of action of formaldehyde, Triton-X 100 and glycine in the class lecture.