	SKILLS CENTER **STANDARD OPERATING PROCEDURE**	**A BIOFIZZ** **PRODUCTON**
Tissue Cell Culture: Sterile practice and cell culturing **Module Hours: 4**	**Effective Date: 1/9/2025** **PRQs:** - **Bacterial Transformation** - **Brightfield Microscopy** - **Fluorescence Microscopy**	**Revision 1.0** **Author: Melody A. Conde** **Checked by Editor: M. Stowell**

Background

1. Foundations of Tissue Culture Basics

Tissue culturing, also referred to as cell culturing, is the process in which cells from an organism are taken and then cultured in variable conditions. These conditions can either simulate a normal environment or abnormal; using different culturing medias, growth factors, and serums can mimic or create differing conditions. These conditions influence cell growth and behavior to help researchers understand and explore the relationship between the cell and its environment, its behavior, and for investigating more clinical applications by culturing diseased models. Cell culturing is an invaluable skill in all scopes of research as sterile technique and proper culturing practices are the foundation to not only good research, but by allowing you to even start your research. Poor efforts can hinder research timelines and quality of research costing time and money.

Cell culturing provides researchers the flexibility of growing cells in two main types of conditions: adherent and suspension cells. Adherent cells are normally grown in monolayer conditions, which means the cells grow flat in “one” layer. These cells may depend on a matrix which helps the cells adhere to the bottom of your culture vessel for growing primary cell lines or other cell lines. The U2OS cell line (depicted to the left) comes from human osteosarcoma cells and are considered immortalized[3] (Fig. 1). These types of cells can divide indefinitely, which makes sense considering cancerous cells divide uncontrollably. Cells can also still be grown without matrix using only media and additional serums as long as

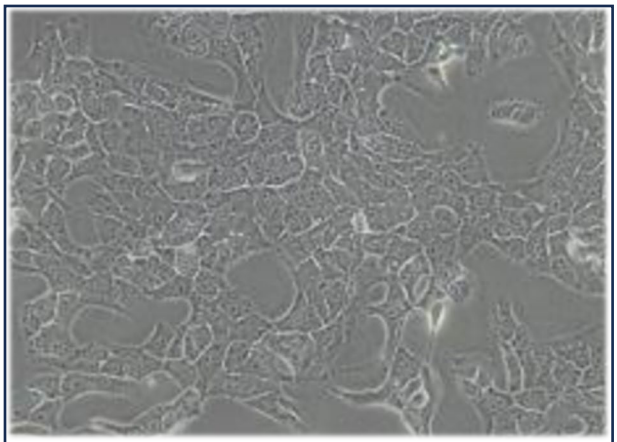


Figure 1- Adherent monolayer U2OS cells at 75-80% confluency

https://www.civicbio.com/product/u-2-os-u2os-cell-line-ep-cl-0236/

the plate used is "Tissue Culture Treated" which allows cells to still adhere. Those grown in suspension have circular morphology and "float" around in the media, so they do not need matrix. These are grown in a way in which they are shaken (or agitated) in a circular motion at certain speeds, and the cells are able to grow in the media into spheres in which they can then be referred to as spheroids (Fig. 2). Adherent 2D-monolayer conditions do not allow for 3D models like suspension cells do, but one method can be more advantageous than the other depending on your research needs. Monolayer conditions are great for expanding and subculturing or passaging cell lines for basic research models where you don't need too much complexity, whereas spheroids provide more complexity to organization and behavior between growing cultures and can even be further grown into organoids which serve as "mini-organs" or tissues – you could grow a mini brain!

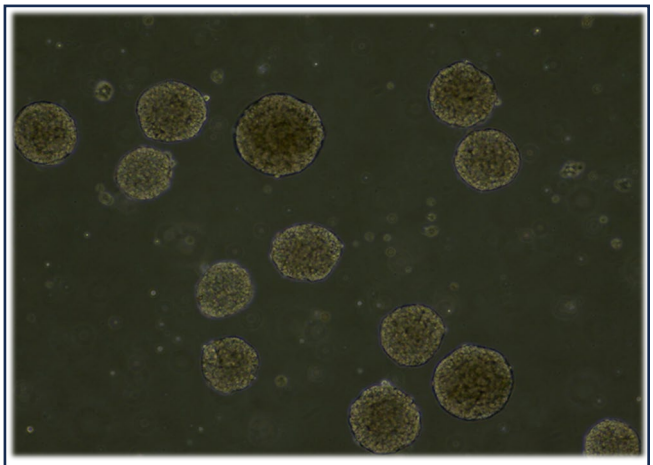


Figure 2- Spheroids suspended in media

Figure 3- Aspirating media using sterile technique

https://www.thermofisher.com/us/en/home/references/gibco-cell-culture-basics/introduction-to-cell-culture.html

The process for cell culturing applies many methods from sterile technique (arguably one of the more critical aspects), cell counting, and microscopy skills. Sterile technique ensures you are working in a manner that is effective in preventing contamination[2]. This is essential to research as you cannot continue to culture your cells if your media is contaminated, your cell cultures are contaminated, your working surfaces have not been disinfected, or you

forget to sanitize your hands- there are many variables that tie into sterile technique. Once you are successful in growing cell cultures that are not contaminated and have established normal cell morphology, you can then move forward in research endeavors remembering to be consistent in sterile technique. Cell counting is also an important aspect of tissue culture as you need to ensure you have an adequate concentration of cells to move forward with, but also potentially how many are alive and dead out of this concentration. Additionally, if your cell line also happens to have the ability to express fluorescent protein to visualize specific parts of the cell, fluorescent microscopy is the only way to visualize this.

Tissue culturing is a skill that translates into many niches of science, but it provides researchers with the ability to grow dimensionally different cell cultures for various applications making it an important skill. Quality science begins with the basics, and having the ability to culture cells with great sterile technique and in an efficient manner that keeps your cells happy is important. Being able to do this will then allow you to layer additional skills involved such as cell counting and applying knowledge of microscopy use.

2. Foundations of Tissue Culture Basics – Sterile Technique

Good cell culture practice is critical in ensuring your experiments are successful and your results are the best they can be. Lack of proper cell culture practice can impede your research timeline and even lead to contamination of your cells which ultimately would void further experimentation. Proper cell culturing practices begin with how clean/sterile you keep your environment to reduce the probability of contamination in tandem to the physical aspect of how you interact with your materials. Some good rules of thumb are shown below to mitigate potential problems common with poor culturing practice:

- **Always** wear gloves
- Before entering **anything** into your lab hood, wipe down all surfaces in the hood thoroughly with 70% ethanol (EtOH)

- All items (with the exception of your plates/flasks containing your cells) that are placed into the hood should also be thoroughly sprayed + wiped down using a KimWipe with 70% EtOH
- Prior to entering your hands— **every time**— inside the lab hood, you should be spraying your gloves with 70% EtOH
- Clean hood BEFORE and AFTER USE

Anytime anything enters the lab hood that is not your cell culture, you should always be spraying and decontaminating. This includes pipette tip boxes, serological pipette tips (yes, the surrounding paper packing can be sprayed with ethanol), serological pipette itself, normal pipettes, microcentrifuge tubes, conical tubes, tube racks, quite literally all consumables needed for experimentation- even the bottles of media that you use.

When using tips (serological/pipette), it is critical that these tips do not touch anything rather than the media or intended cell culture. Also, don't double dip in different medias. This is because outside of the sterile environment of the bottle, even though you have sanitized the surrounding area there is still the potential for bacteria to remain. Touching a tip with media into your media bottle, then into media containing your cells is okay as long as you discard that tip before reentering to retrieve more media.

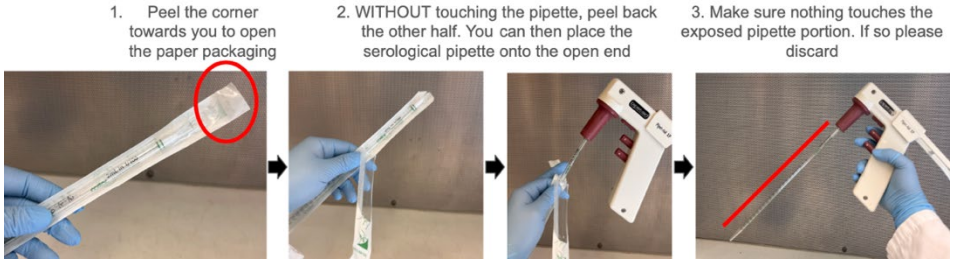


Figure 4- Demonstration of sterile technique via serological pipette

→ EX: I am pipetting media into my serological pipette, but when I pull this out my tip accidentally touches the lab bench for a brief second. I must discard the entire serological pipette tip (even if it has media) because if I were to reintroduce this into my cell culture, I could also be introducing bacteria into my cell culture. This would create contamination. The same concept applies to normal pipette tips.

Another good rule of thumb is when uncapping media/conical tubes, do not set down the cap on the side that comes in contact with the tube body. Lay this upside down far away from where you are working within the lab hood. This goes for the lids of your cell cultures as well. When you are not using your media or working with your cultures, try and keep these capped while not in use to avoid airborne bacteria potentially entering your cultures/media. Try to avoid passing your arms/hands/instruments over open caps and open media, especially your plates. This is why it is important, when they are exposed, to place these away in a section of the hood where you won't have to worry about this.

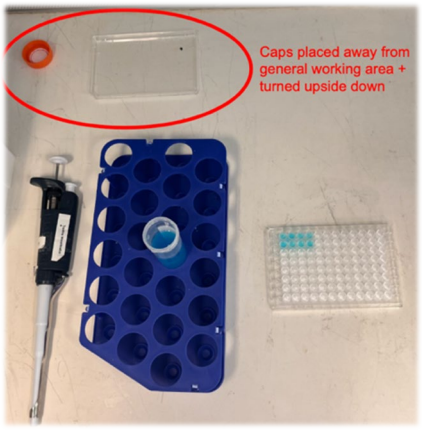


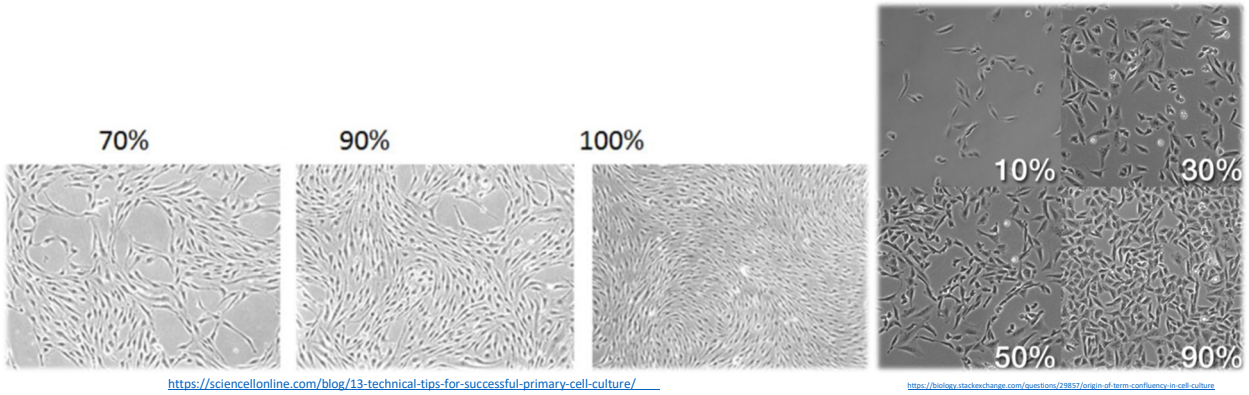
Figure 5- Demonstration of sterile technique

SHORT N' SWEET - HOW YOU SHOULD OPERATE WHEN USING CELL CULTURE HOOD

1) Put on gloves and a lab coat. Spray your hands with ethanol
2) Before opening your hood and entering anything, wipe down the glass first
3) To open the hood, push the metal handles on the glass up gently until you are met with resistance and the beeping stops. The glass should be aligned with the protruding grooves on the left/right of the hood.
4) After opening the hood, spray everything inside with ethanol generously. Wipe down your working surfaces with a large KimWipe
5) Anything being introduced into the hood should be sprayed and wiped down with ethanol (minus your cell cultures)
   → anything going in = spray with ethanol
   → brought something out of the hood and need to put back in? = spray with ethanol
6) Done using the hood? Spray everything inside with ethanol generously. Wipe down your working surfaces with a large KimWipe. Pull down the glass and wipe down too.
7) Turn on UV light. This automatically turns off by itself

3. Foundations of Tissue Culture Basics – Confluency

Figure 6- Confluent examples



https://sciencellonline.com/blog/13-technical-tips-for-successful-primary-cell-culture/

https://biology.stackexchange.com/questions/29857/origin-of-term-confluency-in-cell-culture

Confluency in simplest terms is what percent of the culture vessel is covered by cells; how "full" is your plate of cells (Fig. 7). It is important to identify how confluent your culture is for several reasons. One reason is that before moving to the next steps of your experiment, you need to see if you have actually grown enough cells to begin with. Too much or too little of confluency can be a bad thing; over confluent cultures can lead to loss of normal cell behavior and even cell death. Too little confluency and you won't have enough cells to work with. The rate at which your cultures become increasingly confluent is important to pay attention to as well. If you know the general rate at which your cell line reaches 80% confluency and you have a cell culture failing to reach confluency at appropriate rates, cell morphology looks off, etc. this may be a sign of poor cell culture viability. This would indicate that your cells are struggling to survive, they aren't able to signal to each other to grow, and potential user error in plating cells or some other variable. Consistent application of sterile technique may help mitigate potential errors attributed to the researcher in poor cell cultures.

4. Foundations of Tissue Culture Basics – Contamination

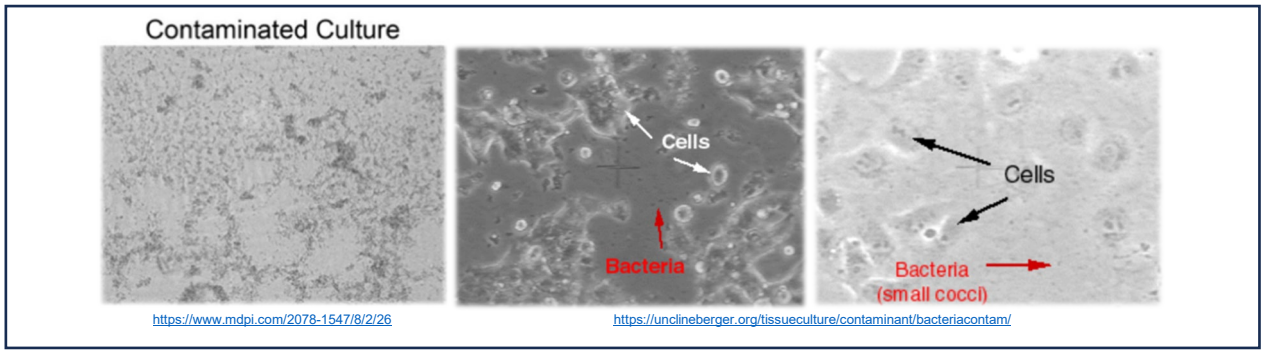


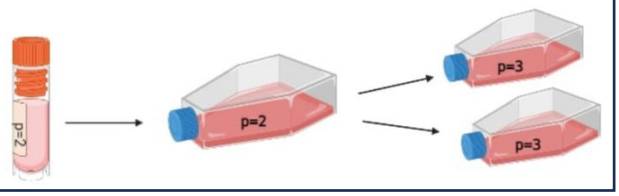
Figure 7- Examples of cell culture contamination; small "dots" or patches of brown/dark brown indicate poor culture

https://theory.labster.com/biological-contamination/

Cell culture contamination is very likely to occur if researchers aren't carefully to thoroughly and consistently disinfect surface areas with 70% EtOH and a guaranteed result of poor sterile technique. If you can't tell whether your culture is contaminated or if it's cell debris, start by changing the media to fresh media to eliminate any cell debris. When viewing your cultures under the microscope if you see tiny fragments "wiggling" or moving even when your cell culture is stable, this is likely contamination. Bacteria in cell culture look like they "pepper" your culture and may come in and out of focus if you look long enough signifying movement.

5. Foundations of Tissue Culture Basics – Passaging/Plating/Freezing

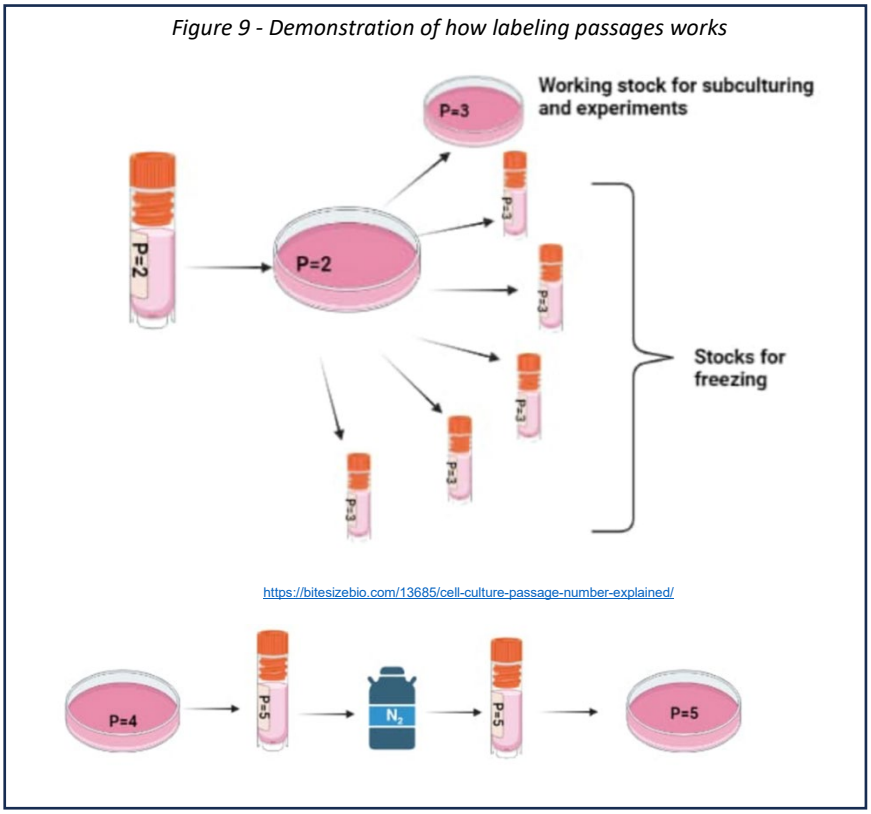
Figure 8- Determine passage number starting from thawed vial



It is important to keep track of how many times you passage a cell line, as some cells lines are less tolerant to being constantly subculture; this can lead to abnormal cell behavior over time. When retrieving cells, these are kept frozen and are labeled with their cell line, passage number, date, and person who froze down the cells. The individual may also add the media the cells were grown with and media the cells were frozen with, as keeping media types consistent may be important in stringent protocols. If you are going to plate cells in adherent monolayer conditions from

a frozen vial labeled P2, you are going to establish this P2 colony so you would label your vessel P2 as well. If wanting to expand your P2 culture, this would mean you need to passage your cell culture. Passaging cells occurs by removing your adhered cells from your P2 vessel, collecting, centrifuging, and splitting your cells into another flask. These newly plated cells are the start of a new cell colony indicated as passage 3 (P3) (Fig. 9). If you decide you want to save some cells for stock during that passage, the vial used would be labeled as one more than the current passage number. This is because if you were to then plate that vial, you would then be plating that new passage of cells.

Figure 9 - Demonstration of how labeling passages works



6. Foundations of Tissue Culture Basics – Cell Count/Cytometry

Cell count is important to corroborate that you have enough cells to move forward with experimentation. You can also do a LIVE/DEAD cell count to see out of the total concentration of your cells, how many of these are actually alive. It is important to have the right number of viable cells, not just enough on the basis of concentration. This helps you know that you have enough cells to continue experiments with, if you have enough cells to use in an assay, etc. When mechanically/enzymatically collecting your cells, it is common to do a cell count starting by collecting a small sample of your harvested cells.

Purpose

The purpose of this Standard Operating Procedure (SOP) is to instruct and familiarize students on the concept and techniques of tissue culturing technique, sterile practice, and basic principles associated with cell culture.

Scope

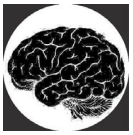
- This procedure applies to qualified skills center users

Responsibility

- It is the responsibility of the user to understand and perform the procedure described in this document
- It is the responsibility of the user to fully document any deviations from the written procedure
- It is the responsibility of the user to become trained on and display mastery of the procedure

Definitions

Morphology – Describes the form (shape, structure, size) of cells
Confluent/Confluency – Describes how “full” your culture vessel is
Cytotoxic – Toxic to your cells
Aspirate – To suck out

	SKILLS CENTER **STANDARD OPERATING PROCEDURE**	**A BIOFIZZ**  **PRODUCTON**
Tissue Cell Culture: Sterile practice and cell culturing **Module Hours: 4**	**Effective Date: 1/9/2025** **PRQs:** - **Bacterial Transformation** - **Brightfield Microscopy** - **Fluorescence Microscopy**	**Revision 1.0** **Author: Melody A. Conde** **Checked by Editor: M. Stowell**

Materials/Equipment

- T-25 ventilated flask
- U2OS Cells → -80*c freezer
- McCoy 5A Medium + FBS → -20*c freezer chest
- TrypLE → Room temp; 1.5mL MCT aliquots in cell culture room (drawer)
- (1X) DPBS (-/-) → Room temp; 1.5mL MCT aliquots in cell culture room (drawer)
- 1.5mL Microcentrifuge tube (MCT)
- 15mL Conical tubes
- Centrifuge
- Serological Pipette
- CO2 incubator
- Tissue culture hood
- Millipore Sigma Scepter Cytometer + tip
- Fluorescent microscope
- Phone (for pictures)

Recipes

- McCoys Complete Medium (McCoy 5A + 10% FBS)
  + (450mL) McCoy 5A Medium (CAT#: 30-2007)
  + (50mL) FBS (CAT#: A52568)

1. ***This SOP can ONLY be started on a Tuesday – NO exceptions due to timing of cell cultures***

Expected Timeline:

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
Week 1		**(START)** Plate cells	→ → → →	Media change	Passage + plate into new flasks	→ → → →
Week 2	**(END)** Check cells via fluorescent microscope					

2. *Be **EXTREMELY** careful how many times you pipette – be gentle with them! Pay close attention to the protocol where it is explicit in number of times to pipette. Over pipetting will kill your cells or cause severe stress impeding survivability. Avoid pipetting up + down more than 3x for any step*

1. Procedures

6.1. Put on gloves and sterilize your work environment. Remember to practice sterile technique and spray anything that enters the hood. It is recommended to have everything you need ready to go in your tissue culture hood prior to starting.

6.2. Retrieve a frozen 45mL aliquot of McCoy's media and leave at room temp (2hr) to thaw. Spray this with ethanol and place into your tissue culture hood.

→ Media that will be used within the week after thawed can be placed in the 4˚C fridge. Make sure you have thawed enough media ready for a full week of experimenting. Label all of these clearly with your initials.

6.3. Prepare a 15mL tube with 9mL McCoy media.

→ If starting with media pulled from 4˚C, you should allow this to warm up for 45mins

6.4. Retrieve a vial of U2OS cells from the -80˚C fridge. Thaw your cells in a bead bath that is at 37˚C. Avoid the beads touching or getting close to the top of the cryovial to avoid contamination. Once you notice a medium chunk of ice (~4mins), immediately bring to the cell culture hood.

6.5. Using a 1000µl tip, aspirate the cell solution from the thawed cryovial into your 15mL conical tube in a SLOW drop-wise motion (total volume = 9.5mL)

6.6. Centrifuge your 15mL conical tube at 37˚C, 300 x g for 5 minutes. Make sure to properly balance the centrifuge using the appropriate counterbalance.

6.7. Retrieve a 15mL conical tube, and label this as “MEDIA WASTE”. You will place your aspirated media into here. Once your cells are done centrifuging, using a 1000µl tip aspirate the supernatant 1mL at a time making sure to remove as much media as possible while leaving your cell pellet undisturbed.

→ You will likely NOT see your cell pellet as there is less than 1 million cells in your vial. Aspirate the media and leave around ~100µL in the bottom of the conical tube

6.8. Retrieve a T-25 ventilated flask. You do not have to spray this down – just make sure this is directly and immediately placed from the bag into the cell culture hood. Label this with “U2OS mito-GFP, PASSAGE #, YOUR INITIALS, DATE” somewhere on the edge of the flask.

6.9. Place 2mL of warmed McCoy media into the 15mL conical tube containing your cell pellet. Once you have placed 2mL media, resuspend your cell pellet in your media up and down 2 – 3x MAXIMUM. Using the same tip you resuspended your pellet with, place the cell suspension from the 15mL tube into your T-25 flask.

6.10. Once you have your 2mL cell suspension placed into your flask, add another 2mL warmed media inside for a total volume of 4mL.

6.11. Go to the fluorescent microscope and take a picture IN BRIGHTFIELD of your freshly plated cells in 20X (take picture of computer screen displaying cells). Moving forward, all pictures should be taken in 20X. Then, place your cell culture into the cell culture room incubator. Leave undisturbed for 24hrs. **This is considered Day 0**. You are aiming for 70% confluency in 2 – 3 days.

6.12. After 24hrs, bring your cell culture back to fluorescence microscope. Take a picture of your cell culture. **This is considered Day 1.** Take notes of cell confluency and justify why you think so, if contamination is present or not with justification, or anything else you deem important. This will be turned in for the MMT. If contaminated, you will need to restart this MMT next Tuesday.

6.13. On **day 2** of cell culture, do a media change. You will need to let your media sit at room temperature for at least 45mins – 1hr. Prepare another 15mL conical tube labeled as “MEDIA WASTE”.

6.14. Grab your cell culture from the incubator and place inside your hood. When ready to remove your old media, tilt your flask so all the media goes to a corner (refer to Fig. 3). Aspirate the old media.

6.15. Place 4mL of fresh media into your flask. Take a picture of your cell culture, and place back into the incubator. Come back in 24hrs.

6.16. On **Day 3**, your culture should be 70 – 75% confluent. Remember, you should start by immediately warming your cell media for 30mins. Take a picture of your cell culture PRIOR to starting any next steps.

6.17. Retrieve 2 aliquots of TrypLE – each aliquot is 1mL and you need a total of 2mL. Prepare a media waste tube.

6.18. Aspirate the old media out of your cell culture flask into your media waste container.

6.19. Retrieve 2 aliquot of (1X) DPBS - each aliquot is 1mL and you need a total of 2mL. Add 2mL into the flask, gently agitate your flask for a few seconds by moving back and forth on the surface it’s on. Aspirate the (1X) DPBS.

6.20. Pipette 2mL of TrypLE into your flask (total 2mL). Let this sit undisturbed for 5 minutes in your incubator at 37˚C. Once 5 minutes is up, remove from incubator and gently tap the flask against your palm to dislodge cells for 1 minute.

6.21. Add 3mL of your McCoy’s media into the flask. Then, take the entire volume containing your media and TrypLE (5mL total) into a new 15mL conical tube. Centrifuge at 300 x g for 3 minutes. Retrieve (2) new T-25 ventilated flasks.

6.22. Aspirate the supernatant out of your flask using a 1mL pipette once done centrifuging. Be extremely careful to leave your cell pellet undisturbed

6.23. Resuspend your cell pellet using 1mL McCoy's media. Take a 25uL aliquot of your cell suspension and pipette this into a 1.5mL microcentrifuge tube.

6.24. Add 75uL of (1X) DBPS into the same microcentrifuge tube and pipette up and down 3x to ensure single cell suspension. This is crucial for accurate cell count.

6.25. Grab the Millipore Sigma Scepter Cell Counter. Turn on the Scepter cytometer by pressing the black control button on the back of the instrument and wait for on-screen instructions to appear.

6.26. When prompted, attach a sensor to the end of the Scepter unit with the electrode sensing panel facing toward the front of the instrument. Follow the instructions prompted on the screen

6.27. Pipette once to draw 50uL of your sample into the sensor

6.28. Make a note of your cell count on the screen and take a picture. This will be turned in for the MMT.

6.29. Add additional 1mL media into the tube with your cell pellet. Resuspend your pellet by pipetting up and down 1x more.

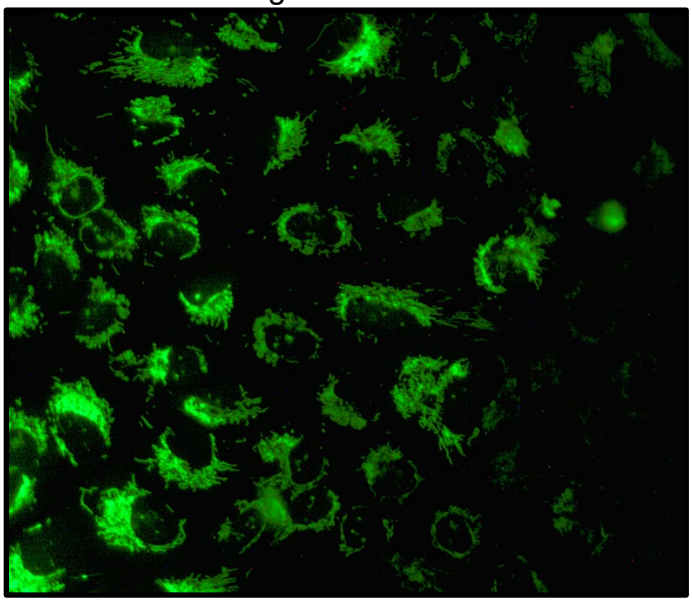
6.30. Pipette 1mL of cell suspension into each of your (2) T-25 ventilated flasks. Remember to label your flasks, keeping in mind the passage number.

6.31. Add an additional 3mL McCoy media to each flask

6.32. Take pictures of both of your newly seeded flasks before placing into the incubator. Make sure you know which picture is which – label this distinctly when submitting images for MMT.

6.33. Leave your cultures incubating over the weekend. Take a brightfield picture of Day 0 (Friday). No pictures need for Day 1 and Day 2. On Day 3 (Monday), image your passaged cultures image using fluorescent **microscope GFP channel in 40X; these are the only photos that need to be in 40X.**

→ How your fluorescent image should look:



6.35. Make sure you discard of all cell cultures, media, etc. properly after experiments and that you have cleaned up after yourself.

2. Troubleshooting

- **Plated cells not growing in 48hrs**

1. If you notice that your cells are struggling to come to confluency within 2 – 3 days, you may have fed your cells the wrong media. The only media that you should be using for your cells is “McCoys 5A + FBS”.
2. Make sure the incubator is at 5% CO2, 37*c, and the water level is full in the bottom circle pan of the incubator (refill with DI water if needed). Let a proctor know if something is off.
3. Make sure you aren’t “power washing” your cells off the bottom of your plate when replacing media; you should always be adding media to the side of the well in which your colonies are on to minimally disturb your adhered cells.
4. If your media is too cold, this will kill your cells. If plating your cells from the vial, you may have caused osmotic shock when thawing your vial. It is crucial that you do not thaw your vial above 37˚C, so make sure your water bath/bead bath is not too hot. You can choose to let it thaw at room temperature as well. It is not recommended to thaw with your fingers.
5. Make sure you are using a VENTILATED flask to allow for oxygen. If you plate your cells in a flask that is not ventilated, they will not grow.

3. References

1. “How to Use ScepterTM 2.0 Cell Counter.” *How to Use ScepterTM 2.0 Cell Counter*, Milipore Sigma, www.sigmaaldrich.com/US/en/technical-documents/protocol/cell-culture-and-cell-culture-analysis/cell-counting-and-health-analysis/how-to-use-scepter-cell-counter?srsltid=AfmBOooBdwnqVm1K2APf6bcFepgk83yruyQsDEDAQGFAhxUHKsfp3J-R. Accessed 18 Sept. 2024.
2. “Introduction to Cell Culture.” *Thermo Fisher Scientific - US*, www.thermofisher.com/us/en/home/references/gibco-cell-culture-basics/introduction-to-cell-culture.html. Accessed 7 June 2024.
3. *U-2 Os - HTB-96 | ATCC*, www.atcc.org/products/htb-96. Accessed 8 June 2024.

4. Module Methods Task (MMT)

Prior to starting experimentation, watch these 2 videos and answer these questions:
(Start at 00:40) Link: https://www.youtube.com/watch?v=nr1tV_LuqJk

a. Why is sterile technique so important?
b. Oh no! You introduced your pipette tip into your media, touched your tip on the inside of your flask, and then reintroduced this into your cell media…. What do you think will happen? What would you have done differently?

(Stop at 04:18) Link: https://www.youtube.com/watch?v=CMRKKl9XSDU

c. We do not do a LIVE/DEAD cell count analysis in this SOP, but why would this be important?
d. What is the purpose of pipetting your cell clump into a cell suspension? Why would this matter?

e. Add all images of your cultures and written observations throughout your days of experimentation in an organized manner below. Make sure to label the day, passage #, etc. chronologically:

f. What did you find your cell count to be? Was this what you were expecting? Insert image below:

g. What was the hardest skill for you to master in terms of cell culture? Why do you think this was?

h. Paste fluorescent microscopy image below. Can you infer why fluorescent tagging cells may be beneficial for research? What organelle of the cell seems tagged and why?

5. Feedback

a. Please provide a brief description of what you thought was the most helpful components of this SOP

b. Briefly describe things you think could be changed to improve this SOP for future students

c. Do the module hour credits appropriately reflect how much time you spent on this skill? If not, did you spend more or less time than the module is worth?

d. Submit your results and module mastery task to Skills Center proctors