Cell Splitting and Counting

Decide whether you will count the cells for a precise seeding measurement or approximate using a dilution.

- **Sterilize** fume hood working area.
- Before the cells are even removed from the incubator, **gather the following tools** and materials in the fume hood:
  - Media container and any intermediate media transfer container
  - Sterile PBS container
  - Trypsin container
  - Electric pipette holder
  - 10 mL pipettes (should already be in fume hood)
  - Ultra-sterilized Pasteur pipettes (should already be in fume hood)
  - P1000 and tips
  - P200 and tips
  - P20 and tips
  - 15 mL centrifuge tube (if counting)
- If counting cells, the following materials should be present on a non-sterilized lab bench:
  - Hemocytometer
  - Cell counter
  - Trypan blue dye (if staining)
  - Eppendorf tube (if staining)
- **Visualize cells under microscope and estimate their confluency.** Plates should have between 60-80% confluency in order to be split. Below 60% will not yield high numbers of cells and above 80%, the cells will have likely senesced and are no longer useful.
  - Check also for signs of contamination: media cloudiness or small moving black dots under microscope (bacterial); long, fibrous growths (fungal).
  - **What to do if contamination strikes:** Infected plates should be aspirated of media (after which the Pasteur pipette should be discarded immediately), sprayed with ethanol, and discarded into the autoclave bin. If using a six well plate, the offending well can be aspirated and (carefully) sprayed with ethanol, provided that the infection hasn’t spread. Check this plate once a day for about 4-5 days to ensure that infection hasn’t spread to the other wells.
- **Aspirate** media from each plate using the Pasteur pipette.
- **Wash** each plate with **5 mL of PBS.** Wait for about 30 seconds before aspirating off PBS. If several plates need to be washed, do the adding and aspiration of PBS sequentially.
- **Add trypsin** to the plate(s) to detach cells; **1 mL for single petri dishes and 0.5 mL for individual wells in a six well plate.** Gently swirl each plate so that the trypsin completely covers the bottom surface.
- **Incubate** the trypsinized plates at 37° C for 2 minutes.
- **Visualize cells** to determine extent of detachment.
  - Cells should not be in large clumps and should move when the plate is gently nudged. If the cells are in clumps or still attached to the plate bottom, hold the plate in one hand and gently tap the palm of your other hand against the side of the plate. **Visualize again to determine the extent of detachment.** If cells are still adhered to the plate, try incubating for another 1-2 minutes. The incubation period of cells mixed with trypsin should not exceed 5 minutes.
- **Add media** to deactivate trypsin. The amount of media added can be in 1:1, 1:2, or 1:3 ratios to the trypsin originally added.
If COUNTING CELLS, go to **A**
If just DILUTING CELLS into new plates, go to **B**

**A**
- Using the 10 mL pipette, **remove the media/trypsin/cells** from each plate and **put it in 15 mL tube(s)**. Take care to think about whether or not you want cells from one plate mixing with another or not. If all the cells are the same and being seeded to the same places anyway, feel free to add all the mixtures to one tube.
- **Place the tube(s) in the centrifuge.** Take care that the placement of tubes in the centrifuge is **balanced**, i.e., a tube with 10 mL should have another tube of the same type with 10 mL directly across from it.
  - There should NEVER be an odd number of tubes in the centrifuge. If another tube is needed to balance, tubes of tap water can be placed. Some of these balance tubes are located to the right of the centrifuge near the back; tap water can be added or removed from them.
- **Centrifuge** the tubes for 5 minutes at level “10.” What the “10” stands for is anyone’s guess, but it is good for centrifuging cells. While the cells are centrifuging, it may be a good idea to prepare the new plates they will be seeded onto.
  - Bags of petri dishes can be found in a drawer opposite of the fume hoods. Since the dishes are sterile in the bag, the entire bag must be sterilized and put into the fume hood before plates can be removed. Take care to tightly seal the bag before removing it from the fume hood and placing it back in the drawer.
- After centrifugation, **aspirate the fluid (supernatant)** above the cell pellets with the Pasteur pipette. Take care not to disturb the cell pellet.
- **Add 1 mL of fresh media** to the tube and resuspend (mix) the cell pellet by gently pipetting up and down with the P1000 until the solution is homogenous (no “cell chunks” visible).

If using Trypan Blue, go to **A2**.
If not staining, continue on to **A1**.

**A1**
- **Remove 10 µL** and insert into hemocytometer.
- **Count four corners of grid**; if amount of cells per corner exceeds 200, dilute the sample in the tube and count again.
- **Use good counting technique!**

![Hemocytometer grid with counts](image)
• Use the following formula to calculate the concentration of cells/mL (see note at end of A2 if staining with Trypan Blue):

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\frac{\text{# of cells counted}}{\text{# of corners counted}} \times 10^4 \text{[the conversion of mm}^2\text{ to mL]} = \#\ of\ cells/mL
\]

• For a specific seeding density:

\[
\frac{\text{seeding density [example, 20,000 cells]}}{\#\ of\ cells/mL} = \text{volume of cells to add}
\]

• Remove the calculated volume from your cell/media tube and add to desired plates/wells.
• Add the amount [10 mL – volume of cell/media just added] of media to each single plate; [2 mL – volume of cell/media just added] to each well in a six-well plate. Gently swill the plate to ensure good distribution.
• Visualize the plates/wells one more time to ensure good density. There should be roughly 5 to 15 cells “per view;” each time you move to look at a new area of the plate under the microscope, 5 to 15 cells should be visible.
• Label your new plates with your initials, the date, the passage number (one more than last time), and HPDLF (for human periodontal ligament fibroblasts). If you have several plates, consider numbering them to help with subsequent observations.

A2
• Pipette 100 µL of Trypan Blue into an Eppendorf tube.
• Pipette 100 µL of cell/media into the same tube.
• Mix by pipetting up and down.

Go to step A1 for counting procedure. Note that you MUST multiply your calculated # of cells/mL by 2 to account for the dilution caused by adding the Trypan Blue.

B
• Decide what dilution factor to approximate. 1:20 is good for most plates; use 1:10 if you’d like the cells to proliferate faster.
• Consider the volume of media/cells/trypsin in your plate. Multiply the total volume currently in your plate by the dilution factor. Add this volume to the new plates.
  o For example, having added 0.5 mL of trypsin and 1 mL of media yields a total volume of 1.5 mL. If the dilution factor is going to be 1/20, then

\[
\frac{1.5\ mL}{20} = 0.075\ mL
\]
• Add the amount [10 mL – volume of cell/media just added] of media to each single plate; [2 mL – volume of cell/media just added] to each well in a six-well plate. Gently swill the plate to ensure good distribution.
• Visualize the plates/wells one more time to ensure good density. There should be roughly 5 to 15 cells “per view;” each time you move to look at a new area of the plate under the microscope, 5 to 15 cells should be visible.
• Label your new plates with your initials, the date, the passage number (one more than last time), and HPDLF (for human periodontal ligament fibroblasts). If you have several plates, consider numbering them to help with subsequent observations.
• Return in 24 hours to change the media (to remove leftover trypsin).
Clean-up

- Ensure that the following have been **put away**:
  - Media bottle (refrigerator)
  - PBS bottle (cabinet above centrifuge)
  - Trypsin tube (refrigerator)
  - Bag of sterile plates (drawer)
  - Pipettes P1000, P200, P20 (rack beside fume hoods)
  - Electric pipette holder (plugged in or returned to rack on center counter)
  - Trypan Blue (cabinet above microscope)
  - Cell counter (drawer to right of microscope)

- Ensure that the following have been **sterilized and disposed of into the autoclave bin and any leftover media/cells or other fluid has been aspirated**:
  - Old plates/wells
  - Centrifuge tube
  - Eppendorf tube

- The following can be disposed of **into the autoclave bin without the need for sterilization**:
  - Pasteur pipettes (into the sharps jar)
  - Pipette tips
  - Glass pipettes

- **Sterilize and clean** the following:
  - Hemocytometer (drawer to right of microscope)
    - Spray actual hemocytometer and coverslide liberally with ethanol; carefully wipe each dry with a Kimwipe before replacing into the box.
  - Aspiration tube
    - Remove and dispose of Pasteur pipette in autoclave bin. With vacuum still on, place end of hose onto the hole of an ethanol squeeze bottle, allowing the ethanol to pump through the tubes connecting to the vacuum. Make sure that the tube under the fume hood is clear of any “pink” fluid before turning off the vacuum.

- **Sterilize** the surface of the fume hood again and organize objects inside so it looks neat.
- Ensure that the following are **turned off**:
  - Vacuum under fume hood
  - Microscope
  - Water bath
  - Fume hood fluorescent light

- **DO NOT close the fume hood window or turn off the blower.**