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Adherent Cell Culture Protocol

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Materials:

Cells (in this particular example, Human embryonic kidney, or HEK293T, cells)
Cell culture medium (see Ferreira Lab **Making Media/Sterile Technique Protocol**)
Drummond Original Pipet-Aid Pipette Controller (Cat. No. 4-000-220)
Sterile 10 cm Tissue Culture Dishes (CytoOne, Cat. No. CC7682-3394)
Sterile 15 ml Falcon tubes (VWR, Cat. No. 89039-664)
Sterile 5 ml serological pipettes (VWR, Cat. No. 89130-896)
Sterile 10 ml serological pipettes (VWR, Cat. No. 89130-898)
Sterile disposable plastic Pasteur pipette (VWR, Cat No. 10754-264)
Sterile P20 Pipette Tips without filter (Corning, Cat. No. 4121)

Procedures for Culturing Thawed Cells :

1. If cells are frozen, follow Ferreira Lab **Thawing Cells Protocol** and wash the cells
2. Afterwards, take the 15 ml Falcon tube containing the thawed cells in cell culture medium and spin them down at 500 G (or "RCF," depending on the instrument) for 5 mins
    a. Use adapters for the 15 ml Falcon tubes and water balances as needed
    b. While the cells are spinning, label your plate(s) with cell type, dilution (if just thawing, write "thaw"), the date, *passage number, and your initials (*very important to keep track of this number, because some cells, e.g. HEK293T cells, are sensitive to passage number so the more times they are split, the more they tend to lose certain properties, e.g. DNA transfection efficiency)
3. Once the cells are done spinning, aspirate the supernatant. To aspirate:
    a. First check to see how full the vacuum flask is (in the event that it is full and needs to be changed, switch out the flask for a new one - preferably a larger flask so it has to be changed less often - and put 10% bleach on the bottom of the flask to prevent bacteria or mold from growing/sticking to the flask)
    b. Turn on the vacuum by moving the white lever inside the tissue culture (TC) hood from its original vertical position to a horizontal position
    c. Use a glass pipette capped with a sterile plastic P20 pipette tip without a filter to aspirate out the supernatant (aspirate on the opposite side of the pellet)

i. Do not let the glass pipette touch the media, just the plastic tip
ii. If you have to aspirate multiple Falcon tubes, of which contain different cell lines, make sure to use a fresh tip each time
iii. You don't have to be particularly thorough with the aspiration because we're just going to be adding more of the same type of media to the Falcon tube
iv. After aspiration, do not leave your cells without media for too long because they will dry out and die

4. Add 10 ml of media to the wall of the Falcon tube and resuspend the cells
    a. To resuspend:
        i. Pipette up and down roughly 7 times or until you no longer see the pellet/clumps of cells, but do not pipette all the way up and do not pipette all the way down (do not want air to touch the tip of the pipette - your motions should be continuous enough that the tip never comes in contact with air)
        ii. Once the cells are all resuspended, pipette all the way up slowly and gently to avoid bubbles

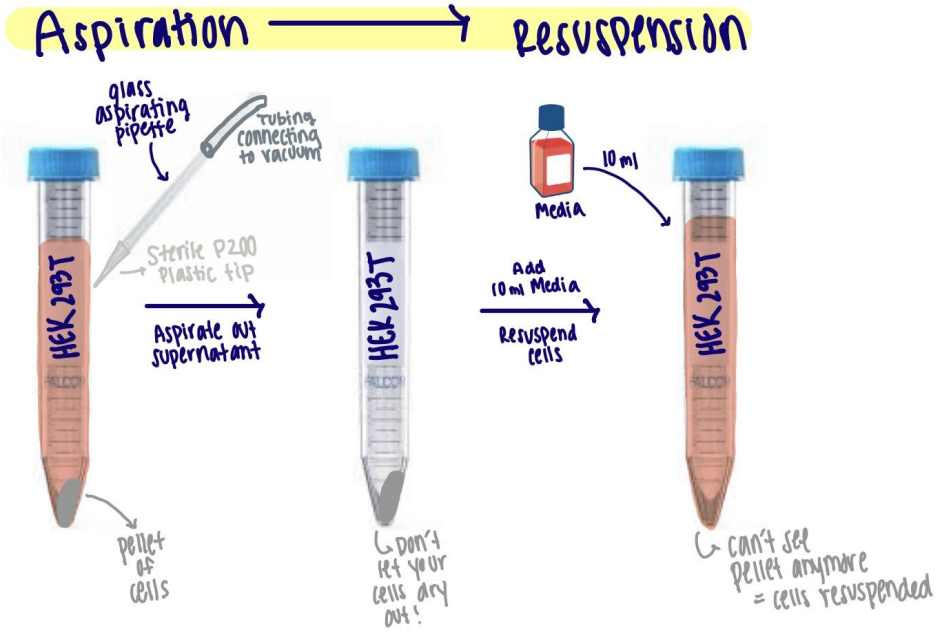

Figure 1. Aspiration and Resuspension of a Pellet of Adherent Cells (HEK293T)

5. Pipette the cells/media into a 10 cm sterile *tissue culture dish (pipette against the wall of the dish)
    a. *The use of sterile tissue culture dishes is recommended (*TC dishes are not the same as Petri dishes, TC dishes are tissue culture-treated)
6. Spread the cells —> "Do the Cross"
    a. Use straight horizontal shaking movements (front↔backwards, left↔right) to spread the cells evenly on the surface of the TC dish

b. Do NOT twirl or spin the dish around, because the cells will all end up in the middle of the dish and we want an equal distribution of the cells on the plate

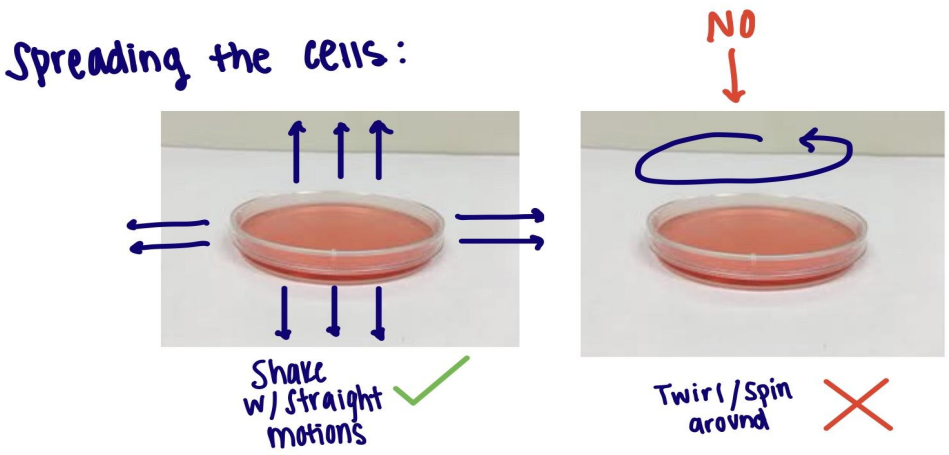

Figure 2. "Do the Cross" (left)

7. Place the plate in the incubator and do the cross once more using the same technique. Leave the cells in the incubator until ready to be split (see below)
8. Close the media and put it back in the fridge
9. Turn off the vacuum by returning the lever to its upright position
10. Wipe down the hood with 70% ethanol (EtOH)

Splitting Adherent Cells

Materials:

Trypsin-EDTA (0.25%), phenol red (ThermoFisher, Cat. No. 25200056)
Media (see Ferreira Lab **Making Media/Sterile Technique Protocol**)
Drummond Original Pipet-Aid Pipette Controller (Cat. No. 4-000-220)
Sterile 10 cm Tissue Culture Dishes (CytoOne, Cat. No. CC7682-3394)
Sterile 15 ml Falcon tubes (VWR, Cat. No. 89039-664)
Sterile 2 ml serological pipettes (VWR, Cat. No. 76093-882)
Sterile 5 ml serological pipettes (VWR, Cat. No. 89130-896)
Sterile 10 ml serological pipettes (VWR, Cat. No. 89130-898)
Sterile P20 Pipette Tips, unfiltered (Corning, Cat. No. 4121)
Sterile Microcentrifuge tubes, 1.5 ml (VWR, Cat. No. 10025-728)
P20 micropipette (SCILOGEX, Cat. No. 713121209999)
Nunclon Delta Surface Sterile 96-well Plate (ThermoFisher, Cat. No. 163320)
DPBS (ThermoFisher, Cat. No. 14190250)
TC20 Auto Cell Counter (Bio-Rad, Cat. No. 1450102)
Cell Counting Slides (Bio-Rad. Cat. No. 1450011)
Trypan Blue Dye (Bio-Rad, Cat. No. 1450013)
Plastic waste container, tri-corner beaker (VWR, Cat. No. BTC0800)

DMi1 Inverted Microscope for Cell Culture (Leica Microsystems, Cat. No. 11526220)
ZOE Fluorescent Cell Imager (Bio-Rad, Cat. No. 1450031)

Experimental Setup:

- Spray the hood down with 70% EtOH
- Several steps of this protocol are time sensitive, thus it is necessary to gather the following materials ahead of time: media, trypsin, DPBS, and the Pipet Aid controller. Spray them down with 70% EtOH before bringing them into the TC hood
    - Set up your materials in the order shown below (**Fig. 3**), left → right
    - Also, gather and order your 2 ml, 5 ml, and 10 ml sterile serological pipettes according to **Fig. 3**. Lastly, set up a plastic waste container to keep directly in the hood
    - This setup facilitates the smooth, efficient transitions you will need to work quickly through this time sensitive protocol

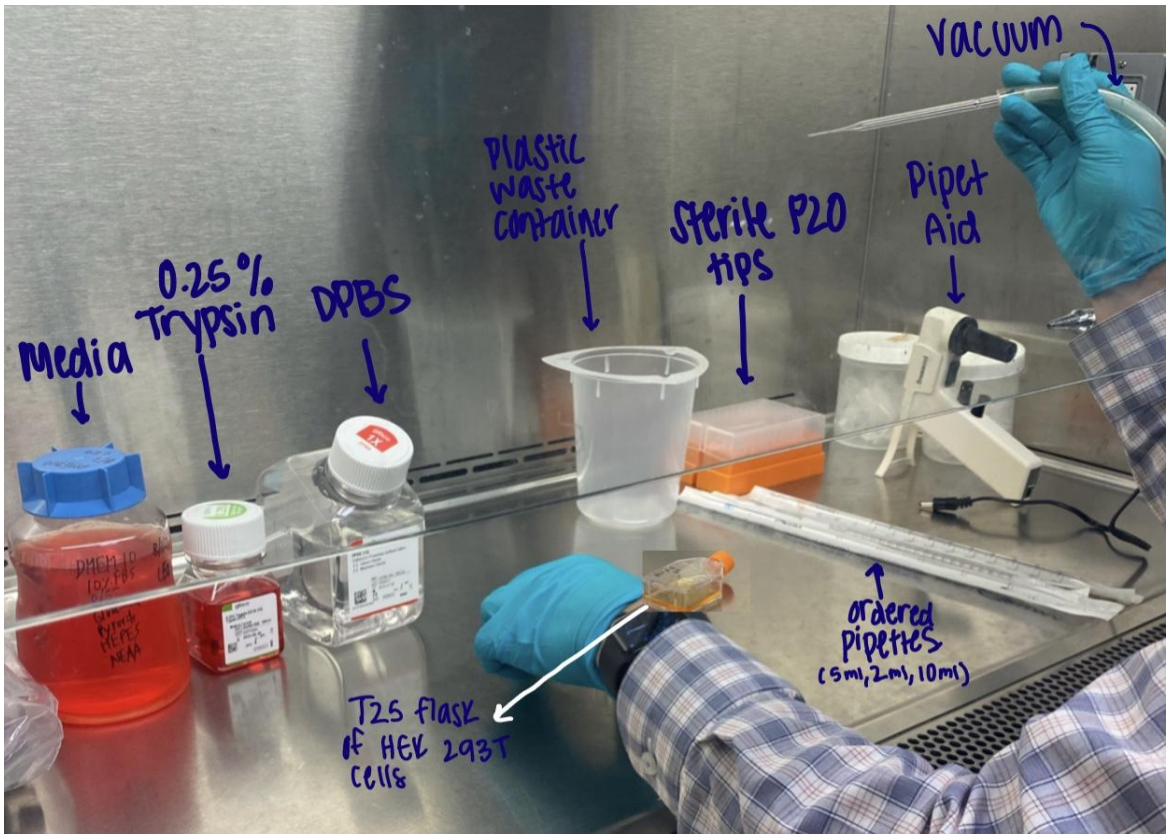

Figure 3. Experimental Setup of Reagents and Other Materials for Splitting Adherent Cells

- **If frozen, make sure the trypsin is taken out of the -20ºC freezer well before starting the experiment to allow time for thawing. Place it in a sterile water bath (autoclaved water) at 37°C to speed up the thawing process. It's very important that the trypsin is thawed and ready to use since adherent cells cannot be harvested without it

- Once the trypsin is completely thawed, as with any other reagent that's been sitting in the water bath, always spray the bottle down with 70% EtOH before bringing it into the hood (since the water bath can be a source of growth/contamination)
- Before starting the protocol, always examine your cells under a tissue culture microscope and photograph the current condition of the cells (can be done by capturing a picture with your phone through one of the microscope lenses or the ZOE Fluorescent Cell Imager)
    - Aim to capture two pictures that are representative of your entire population of cells. For example, if there are areas with lots of clumps, but also areas where the cells are nicely spaced out, be sure to capture photos of both conditions so that you know exactly what you have in that cell population
        - Label your pictures with the date (MM/DD/YY), cell type, magnification, and your initials
    - The first step is to find the cells. If you can't find any cells whatsoever, resuspend them inside the TC hood and try to view them under the microscope again
    - If the cells are clearly there, but not easily visible, try using a phase contrast slider (for our specific microscope, we've found that the cells are best visualized using the 5F0 setting of the phase contrast slider)
        - If your pictures look dark, increase the LED intensity or gain to brighten them
    - In this specific case, HEK293T cells should have a flat shape with a network of projections connecting them to other HEK293T cells
        - If the cells are healthy and exhibiting successful growth, the interconnections these adherent cells form should look like a web/matrix, not like round cells randomly floating/swimming around in the media
        - If the cells appear to be shaking it's because they're not attached to the surface, which means they're probably dead/not doing well. Although, there will likely be some of these dead cells even in largely healthy cell populations

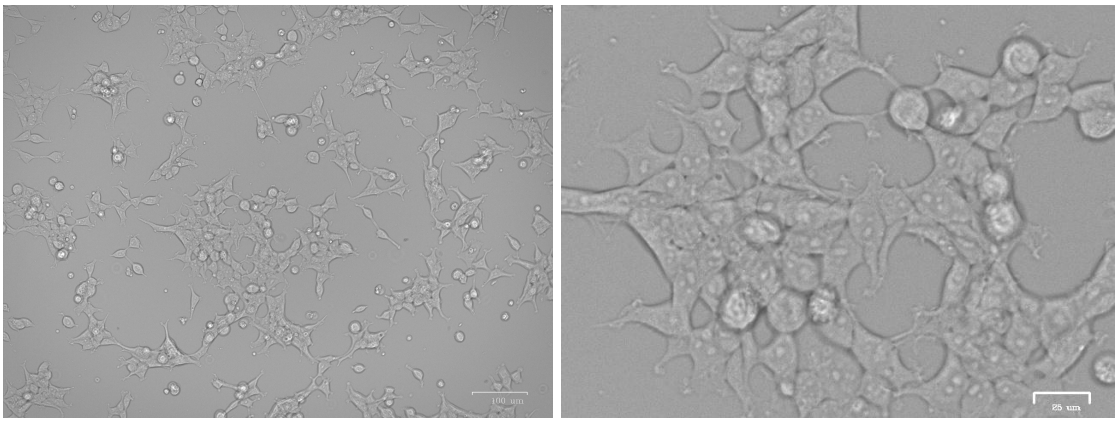

Figure 4. Healthy HEK293T Cell Monolayer (magnification left to right: 4x, 16x)

Procedures:

1. If your cells are frozen, first see Ferreira Lab **Thawing Cells Protocol** and continue on through the beginning of this protocol (see above). If the cells are already in culture, and ready to be split, continue on to **Step 2**. In either case, be sure to first wipe down the hood/any outside materials with 70% EtOH
2. Set up your materials as depicted above in **Fig. 3** and begin by loosening the caps of your reagents so you are ready to pipette easily from each one of them
3. Make sure your cells are the only thing directly in front of you —> maintain sterile technique
4. Check to see how full the vacuum flask is and turn on the vacuum (if it is full and needs to be changed, mix the contents of the flask with bleach and dump down the sink. Remember to put bleach in the bottom of the new flask you will collect waste in)
5. Cap the glass pipette attached to the vacuum with a sterile unfiltered plastic P20 pipette tip, open your *flask (T25 flask in this case) or cell culture dish, and aspirate the media. Discard the plastic pipette tip into your nearby waste container
    a. *It is also very common that your adherent cells will be cultured in a TC dish rather than a flask so be mindful that a flask could be switched out for a TC dish throughout this protocol
    b. When aspirating fluid, tilt your flask or TC dish at a vertical angle
    c. In between each of these steps, it is good practice to replace the cap to the flask (or alternatively the lid to your TC dish), keeping the cells covered as much as possible to prevent contamination (this applies to each of the reagents you will be adding as well, replacing the cap after obtaining the necessary volume each time)
    d. After you've capped the glass pipette with a sterile plastic pipette tip, be sure to put the top back on the box of sterile tips (in general, do this each time you use a sterile tip since it keeps them as sterile as possible)
    e. Double check that you've loosened all of your caps before opening any of the sterile serological pipettes (minimizes wasted pipettes!)
    f. Be careful not to leave your cells without media for too long since they will dry out and die —> as soon as you aspirate the exhausted media, the clock starts ticking
6. Use your sterile 5 ml serological pipette to wash the cells with 5 ml of PBS - before opening the pipette, turn the scale towards you since this will not be able to be adjusted after the pipette is already open
    a. As you pipette the 5 ml of PBS into the flask, make sure you pipette slowly and against the wall of the flask or the dish, not directly on top of the cells, as that would mechanically detach them
    b. When you're done adding the PBS, replace the cap to the flask, slide the 5 ml serological pipette back into its wrapping, and discard it in your *plastic waste container (*this limits movement in/out of the hood so it's more efficient and it diminishes the risk of contamination)

c. Gently swirl the flask or dish to spread the PBS around, then cap the glass pipette attached to the vacuum with another sterile plastic P20 pipette tip. Lift the cap to the flask and aspirate the PBS. Replace the cap to your flask and discard the used tip in your waste container. The cells are now washed

7. Enzymatically detach the adherent cells from their surface so that they can be harvested
   a. Open a 2 ml sterile serological pipette and slowly add 2 ml of 0.25% **trypsin to the flask - this is enough to cover the bottom of a T25 flask or a 10 cm dish. Place the flask in the incubator at 37ºC for *1-2 mins
      i. **Trypsin works best at 37ºC
      ii. If the flask has a vented cap, you can tighten and close it all the way. If it is not vented, loosen the cap a little before placing it in the incubator to let air in
      iii. There is a 'sweet spot' for trypsinizing cells. If we trypsinize the cells for too long, we kill them. If we don't trypsinize them for long enough, we kill them. For this reason, we leave trypsin on the cells in the incubator for *1-2 mins before adding FBS-containing media to stop the reaction - it can be easy to forget about your cells in the incubator as you're preparing your materials for the next steps, but if trypsinized for too long, you will have no cells left to work with - manage your time very carefully
      iv. During this brief waiting period, grab a sterile 15 ml Falcon tube, Trypan Blue dye, a plastic counting slide, a rack, and a P20 micropipette - we are preparing to count the cells
      v. If you have not finished gathering all of your materials, but you're approaching the 1-2 min mark, immediately stop. Microscopically verify your cells are no longer attached and proceed through **Step 8** of this protocol. You can always finish preparing your materials later, once the cells are back in media and 'happy'
   b. After 1-2 mins take your flask out of the incubator and lightly bang the sides of the flask - these horizontal vibrations help the cells come off more easily
   c. You should always quickly place your cells under the microscope again to verify that they are coming off nicely
      i. At this point, the cells should have all lost their flat shape and should be more round. Remember that they will appear to be floating/shaking if no longer stuck to the surface of the flask
   d. For the next step, it is crucial to remember that the cells are no longer attached to the flask. They are floating in the trypsin - do NOT aspirate this fluid or you will lose all of your cells - instead, we will add media to stop the reaction
   e. In this case, we are only working with one cell line so it was not particularly important that we label the 15 ml Falcon tube before the procedure in **Step 8**. However, if we were, it would be necessary to label the tubes ahead of time to prevent confusion/errors

8. In their detached state, the cells are not getting any 'happier' so it is once again a time sensitive situation - add fresh media as quickly as possible
    a. Turn the flask so that it is vertically oriented. Then, using a 10 ml sterile serological pipette, add 8 ml of fresh media to the flask and pipette up and down to resuspend the cells - do not pipette all the way down or all the way up during this addition because now you are going to *resuspend your cells and wash the surface of the flask to collect any cells left behind
        i. *During this process, you always want the tip of your pipette submerged in liquid and never in contact with air - this prevents introducing bubbles that cause you to lose cells
        ii. Remembering to wash the surface is particularly important when working with adherent cells
        iii. When washing the surface of a TC dish rather than a flask, perform a 'half-moon' motion with the pipette. Hold the TC dish at a vertical angle and slowly follow along the arc of the top of the TC dish with your pipette, washing off any remaining cells to collect at the bottom of the dish (still do not pipette all the way down - keep liquid at the tip of your pipette at all times)

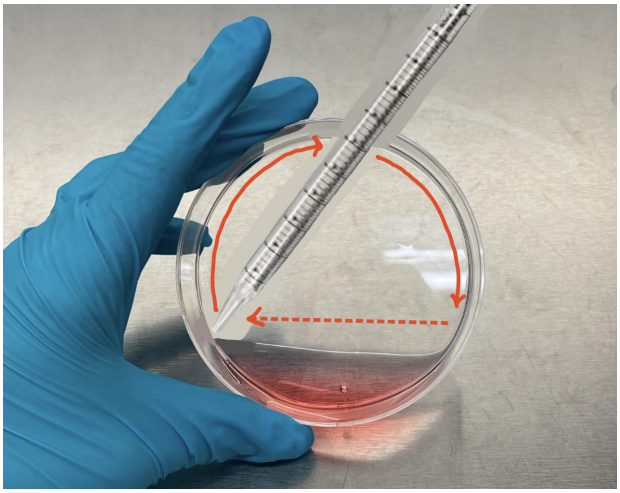

Figure 5. 'Half-moon' Washing Technique

        iv. We are using precise volumes so that it is easier to calculate the dilution later - right now there should be 10 ml total in your flask (2 ml trypsin + 8 ml medium)
    b. After you've resuspended your cells in the flask, pipette all the way up without allowing air into the pipette and transfer the cell suspension to your sterile 15 ml Falcon tube
        i. Add the volume by slowly pipetting against the wall of the tube (since you can't let air touch the tip of your pipette, you will never be able to take up the entire 10 ml from the flask)

c. At this point, if you've successfully resuspended your cells, you should no longer see clumps. If you do, it means the cells are dead because dead cells fuse together and form clumps
d. If you have not already done so, label the 15 ml Falcon tube with the cell line

9. Ideally, whenever you split cells you should count them, since we want to seed the cells at a specific density every time for consistency - especially for replication experiments in order to decrease variability. To count your cells:
    a. Successfully resuspending the cells in your Falcon tube in **Step 8a** is key, because we want to be able to trust the cell count. If the distribution of the cells is not equal throughout the entire volume of the Falcon tube, the count will not be representative, hence not a useful data point for extrapolation later on
    b. Gather the Trypan Blue dye, a plastic counting slide, a P20 micropipette, sterile P20 pipette tips, and a sterile 1.5 ml microcentrifuge tube *under the hood (alternatively, to reduce waste, use a sterile 96-well plate to create the mixture each time you count your cells - 1 well/count - keep the plate in the TC hood and covered in between uses)
        i. *we keep these materials inside the TC hood at all times
    c. *Invert the Falcon tube a couple of times slowly so as to not introduce bubbles (*necessary because cells begin to deposit in the bottom of the tube the second they are left standing still, but we want to see an equal distribution of the cells throughout the volume of the Falcon tube)
        i. If you leave your cells standing for an extended period of time (more than 5 mins), you will need to fully resuspend them again using a pipette rather than just inverting the tube
    d. Then, make a 1:1 mixture of Trypan Blue dye and an aliquot of your cell suspension
        i. Grab a 1.5 ml sterile microcentrifuge tube, taking care to only touch the tube you will use (or use a 96-well plate)
        ii. Loosen the cap of the Trypan Blue you set aside earlier. Then, using a P20 micropipette with a sterile plastic tip, add 10 µL of Trypan Blue to the microcentrifuge tube/well
            1. Make sure you've re-capped the Trypan Blue vial before adding the 10 µL to the microcentrifuge tube/well to avoid spilling the tube
        iii. Discard that tip into your plastic waste container, replace it with a new one, and pipette a 10 µL aliquot of your well-resuspended cells from the 15 ml Falcon tube into the microcentrifuge tube/well
            1. Before adding the 10 µL aliquot of cells to the microcentrifuge tube/well, make sure you've re-capped the Falcon tube containing the remainder of your cells (remember we want to leave the cells uncovered for as little time as possible to ensure sterility)
        iv. Now, pipette up and down in the microcentrifuge tube/well to mix

e. The cell counting chamber accepts 10 µL of volume
    i. After mixing thoroughly, pipette 10 µL of this 1:1 dilution, touch the tip of your pipette to the loading area of one of the chambers on the plastic counting *slide, and slowly release the volume into the chamber (*slides are good for 2 counts - since there are 2 chambers, discard after 2 uses)
    ii. This process works by capillarity - as you slowly pipette down, the Trypan Blue cell suspension mix will fill the chamber on its own. After the chamber fills, discard your tip

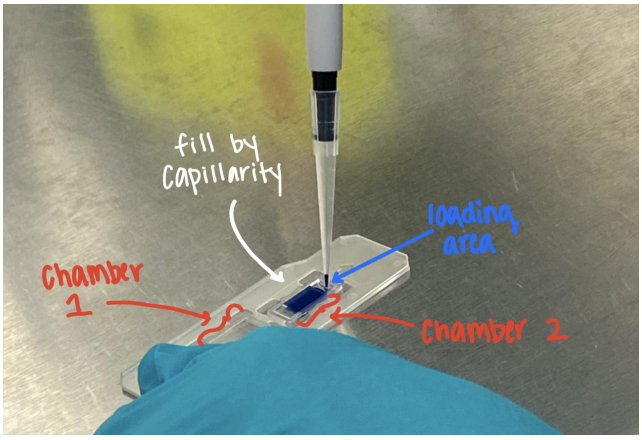

Figure 6. Dual-chamber Cell Counting Slide

    iii. Following the directional arrows on the plastic counting slide, insert the slide into the TC20 Auto Cell Counter (use of alternate cell counter ok, but the following procedures will pertain particularly to the aforementioned model)

f. Data points of particular relevance obtained from the cell counter that should be documented during your procedure include: percentage of live cells, live cell count, and the distribution of cell diameters by histogram depiction
    i. Percentage of live cells - this data point is used as an indicator of how well the cells are growing
        1. Do not use cells for experiments that are less than 90% alive
    ii. Live count
        1. Data point reported in the form of number of cells/ml.
        2. It is not the total amount of live cells you have in the entire volume of your Falcon tube - To extrapolate to that value, multiply the live count by the volume of the entire cell suspension (in this case, it would be # of live cells x 10 ml).
        3. It can be easy to forget to multiply the reported live count by your total volume so be careful not to incorrectly assume your total live count! **This is why resuspension is extremely important - so that you can extrapolate the number of live cells/ml reported in the 10

μL read by the cell counter to the total number of live cells in the entire volume of your cell suspension

iii. Total count - this data point is also reported in the form of number of cells per ml, but it includes both live and dead cells so we don't typically use this data point

iv. Histogram - graph produced by the Bio-Rad TC20 Automatic Cell Counter showing the distribution of cell diameters in your cell population
    1. live cells are displayed in green and dead cells in red
    2. The range feature allows you to change the gate of the graph to limit the portion of cells you are looking at, which will be especially useful in T cell culture (since cell diameter/size can tell us about what percent of the T cells are activated)

v. Dilution calculator - built in tool that is useful if you are not going to be using all of the cells you currently have in suspension (e.g. if you're not making liquid nitrogen frozen stocks of cells)
    1. Using the live count, total volume, and the desired concentration you input, the dilution calculator will tell you how many ml of media/cells to add to produce that desired concentration
    2. The TC20 Automated Cell Counter automatically detects the presence of Trypan Blue in the sample and accounts for the 1:1 Trypan Blue to cells dilution factor. If you ever use a different ratio, make sure to include that in your calculations

vi. It is pertinent that we document these data points because we want to know how many cells we are seeding per square centimeter, that way we can ensure we're seeding the cells at the same density every time. Otherwise, it could bias the results of our experiments, especially if trying to complete replication studies. Counting is important!

10. Now that the total number of live cells you have in your cell suspension is known, determine how many plates of cells you will be able to make based on the desired concentration of cells per plate
    a. In this protocol, our desired concentration will be 3 million cells/plate since that is how we would set up the experiment to carry out DNA transfection
    b. For the sake of simplicity, exact values will be used in the remainder of this protocol. However, these values may vary depending on the number of cells counted in one particular experiment. Be aware that these values are subject to change and should not be used universally
    c. We determined that the total live count in our cell suspension was 27 million cells (2.7 million/ml x 10 ml = 27 million cells). We are aiming for 3 million cells/plate so we will be making 9 plates, each with 3 million cells (27 million/3 million = 9 plates)
    d. Since we essentially performed a single cell suspension in the Falcon tube prior to counting, we can assume that the total live count of 27 million cells is evenly

distributed throughout the entire volume of the Falcon tube. Therefore, 1 ml of the total volume has an equal number of cells as another 1 ml aliquot of the cell suspension. There are 10 ml total in our Falcon tube and we are going to make 9 plates, so the same number of cells can be allotted to all 9 plates by adding ~1 ml of the 10 ml cell suspension to each plate. The remaining 1 ml of the total volume will be spread as evenly as possible between all of the plates since we do not want to waste any cells

11. Once you've determined the number of *plates you are going to make, obtain the necessary amount and label them with the cell type, **passage number, cell number, the dilution, your initials, and the date
    a. The use of sterile TC dishes is recommended (*TC dishes are not the same as Petri dishes, TC dishes are tissue culture -treated)
    b. **passage number is very important in labeling your plates since certain cells - like HEK293T cells - begin to lose some of their properties, e.g. ability to be transfected with DNA, past a certain number of passages
12. Organize your plates into stacked groups of 3 when working with multiple plates. This will streamline the process of adding fresh media to each of your plates
    a. Always add the largest volume to the plates before adding any smaller volumes. Thus, for a 1:10 split, add the 9 ml of medium before adding the 1 ml of cell suspension to each plate
    b. Use a sterile 25 ml serological pipette to pipette 27 ml (up to "-2" on the pipette graduation) of media total (only 1 pipette is necessary since the plates are sterile)
    c. Distribute the 27 ml of media between all 3 plates, adding 9 ml to each plate working from bottom to top
    d. Try to keep the lids off of the plates for as little time as possible, immediately replacing each one after adding the 9 ml of fresh media
    e. Remember to pipette slowly against the wall of the TC dish (no bubbles) with nothing else in front of you
    f. When moving between each of the plates, be mindful of dripping media in the hood or onto the outer surfaces of the plates, as it will stain the hood and allow for growth of fungi/microbes on the plates (will contaminate the incubator)
    g. Shake the plates to spread the media
    h. Repeat this procedure for any remaining groups of three you may have (in this case, the procedure would be followed two more times for the 6 remaining plates)
13. Now that all of the plates have media, grab a sterile 10 ml serological pipette and *resuspend the cells in your 15 ml Falcon tube
    a. *Do not forget to resuspend, since the cells have been standing still for a substantial amount of time and have already begun to collect in the bottom of the tube again. We want an equal distribution of the cells throughout all 9 plates so this is very important to remember to do
    b. While resuspending, make sure the tip of your pipette is fully submerged in liquid so that you do not introduce bubbles. Introducing bubbles may cause you to lose

some of your volume such that you no longer have enough cells to evenly distribute between all of your plates. If you do mistakenly create bubbles, do not pipette the bubbles out - dispense as much volume as possible while still leaving the bubbles contained within the pipette

c. Once you've resuspended, pipette 9 ml of your cell suspension all the way up using your 10 ml serological pipette and replace the cap to the Falcon tube. You will now use this 9 ml to sequentially add 1 ml of your cell suspension to each plate, working through your groups of three, bottom to top (pipette against the wall of the TC dish slowly; immediately replace TC dish lids after adding cells)
d. Distribute the remaining 1 ml of cells in your Falcon tube as evenly as possible between all 9 plates, then discard the empty Falcon tube and used 10 ml serological pipette into your plastic waste container

14. 'Do the Cross' as described in **Fig. 2** to ensure the cells spread over the entirety of the plates instead of forming a large clump in the middle
15. Put all of your plates in the incubator at 37ºC and perform the cross once more
16. Return the media and trypsin to the fridge. If you have not already done so, date and label the PBS as 'sterile' so it indicates to others that it should not be opened outside the TC hood. The PBS can stay in the hood at all times since it can be kept at room temperature and is not light sensitive
17. Turn the vacuum off by returning the lever to its original vertical position
18. Lastly, wipe down the TC hood with 70% EtOH and turn on the UV light for ~30 seconds (do not leave this on for long periods of time)

Supplemental Information:

- HEK293T cells are adherent cells, meaning they grow and attach to a tissue plate, forming a monolayer
    - It's easy to tell if they're alive or not because if they're unhappy, they detach and peel off
    - Also easy to tell if they are overcrowded because they start to grow on top of each other
- HEK293T cells can become confluent within a day - it's very quick, so if it's been a couple of days already and you are not seeing confluence, it probably means the cells are not 'happy' or growing successfully
    - If they're not 'happy,' that presents a major challenge since they will not produce virus under poor growth conditions for example
    - Keeping these cells happy requires keeping passage numbers low and consistent maintenance, including splitting the cells twice a week (never overgrow a plate)
- For adherent cells, we thaw them in a 10 cm tissue culture dish in 10 ml of media and 'do the cross' (see Ferreira Lab **Thawing Cells Protocol** for more information)
- Never overgrow a plate —> will need to split the cells before they get too packed because they are density sensitive so we have to maintain them at the right density

    - This is not the case for some cancer cells, which have lost contact-dependent inhibition, and thus are are less density sensitive - they are ok with being packed together/on top of each other unlike adherent cells
- If your media is beginning to look yellow, that means the cells have grown substantially and most likely need to be split, or at least have their media changed
    - The metabolites cells produce as they grow fill the media and cause it to turn yellow
        - Media contains phenol red, which changes colors with changes in pH. The lactic acid cells produce as they grow lowers the pH such that the phenol red turns yellow
- If the media level is low, it is most likely due to evaporation, so the incubator may need more water (make sure there is a humidity pan filled with water in the bottom of your incubator)
- Trypsin is necessary to harvest adherent cells, since we have to harvest them in their attached state from the surface of the flask/TC dish
- Cells do not like being kept at room temperature, so this is also a reason to be swift with your technique in order to minimize their time outside of the incubator
    - However, when using media from the fridge, it is ok to not warm it in the water bath (more important to minimize possible contaminations from the water bath and maximize time efficiency/compliance with sterile technique when working to swiftly return the cells to the incubator)
- When resuspending, it is helpful to use certain graduations on your serological pipette as guidelines to keep liquid at the tip of your pipette at all times. For example, when using a 10ml serological pipette in cell culture, staying between 1 and 5 ml reduces the risk of creating air bubbles and ensures you never pipette too far up or down
- Trypan Blue distinguishes live from dead cells by only penetrating dead cells, allowing the cell counter to quantify live cell counts/percents
    - However, it is also eventually toxic, so do not incubate cells with it for too long
    - It can stay inside the TC hood since it is kept at room temperature
    - In this protocol, we make a 1:1 mixture of cells and Trypan Blue, but this can be further diluted if there are too many cells to give an accurate count. Although, you should make sure to account for this difference, as the TC20 Automatic Cell Counter automatically assumes a 1:1 cells to Trypan Blue ratio
- The range feature of the histogram produced by the cell counter is useful for T cell culture since T cells change size/shape with activation. Bigger T cells are activated, while smaller T cells are inactive and resting
    - The histogram displays cell counts on the Y axis and cell diameters on the X axis. This means if we can limit the range of cell diameters we're viewing, we can determine the amount of activated cells in our cell suspension
    - The cell counter, however, is not able to tell us much about the morphology of the cells besides the images it provides. These images can be useful to verify that you

can trust your count though since you can see whether you properly resuspended the cells or whether they are still clumped, giving you an inaccurate report
    - Because we resuspended and the cells are no longer attached to the surface, all of the cells will look round (previously, they looked flat since they were stretching out, adhering to the surface/one another to create a matrix)
- Aspirating the exhausted media right before you add PBS when splitting adherent cells is important not only because the FBS in the old media interferes with trypsin, but also because there are dead cells floating around that you need to wash away
- FBS-containing medium impedes the function of trypsin for a couple of reasons:
    - Adding the 8 ml of media dilutes the trypsin such that it becomes 5 times less concentrated so it helps stop the reaction by dilution
    - FBS contains a lot of proteins that compete with trypsin for binding sites. The specific function of trypsin is to cleave proteins so it gets taken up with the addition of FBS. All of the sudden, trypsin gets really busy cleaving added proteins rather than the proteins maintaining the integrity of the extracellular matrix
- If your cells are already starting to detach after only being washed with PBS, it means they are ready to come off and be split since they are starting to overgrow
- If you're going to be producing both liquid and solid waste, have two separate plastic waste containers - one for liquid waste and one for solid waste
- Refrain from conversating/talking when you open the incubator door - minimize contamination
- When preparing HEK293T cells for use in a transfection experiment, it has been suggested that HEPES and Penicillin-Streptomycin (P/S) be left out of the complete cell culture medium since the cell membrane becomes permeable to these molecules during DNA transfection. Intracellular P/S would be toxic, whereas the buffering capacity of HEPES could reduce DNA transfection efficiency using certain techniques, e.g. the calcium phosphate method