lab # 6

Tissue Subculture

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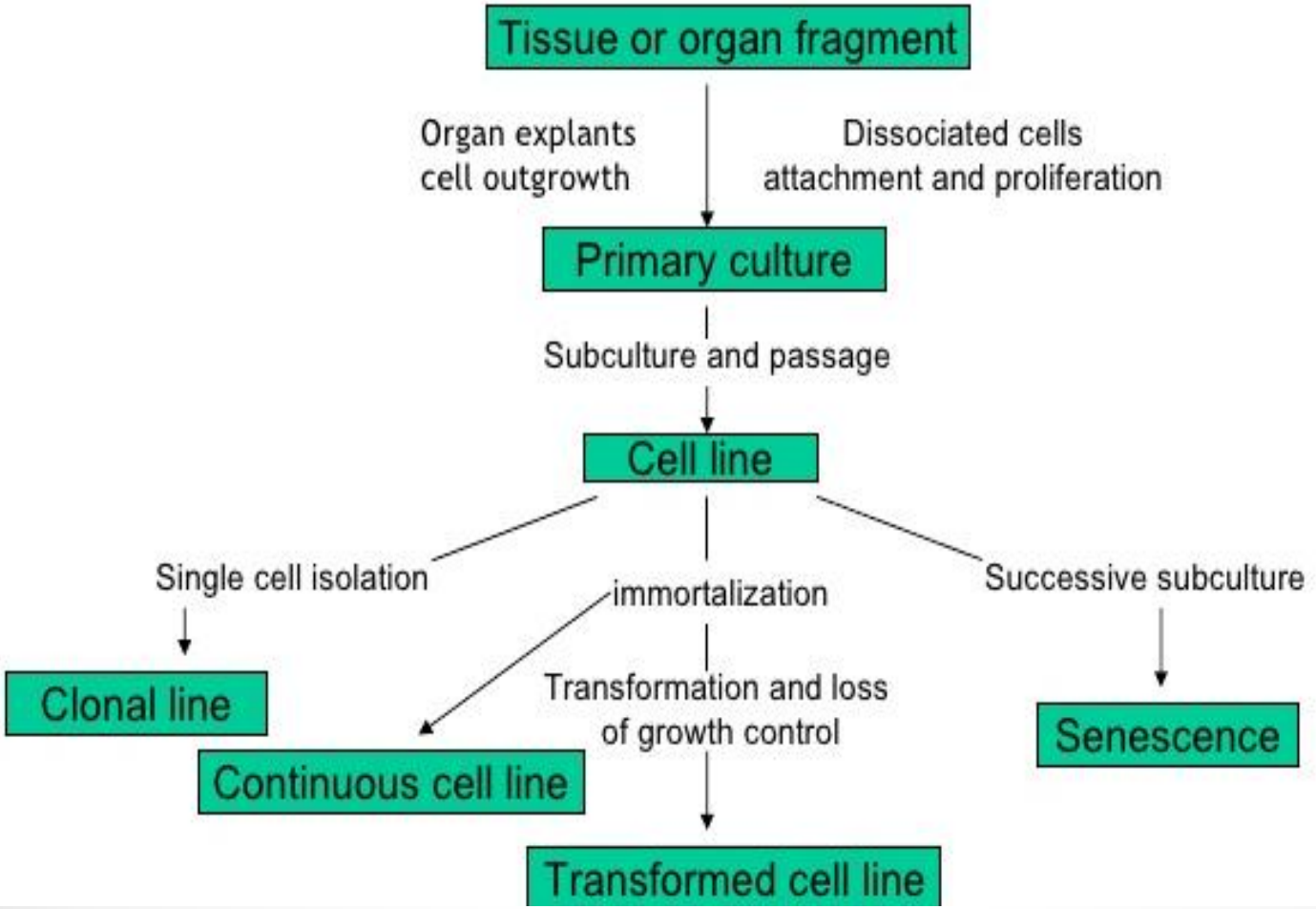
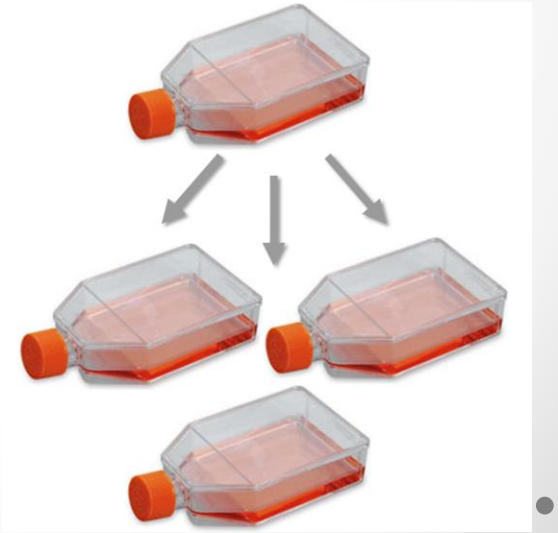

Tissue or organ fragment
Organ explants
cell outgrowth
Dissociated cells
attachment and proliferation
Primary culture
Subculture and passage
Cell line
Single cell isolation
immortalization
Successive subculture
Clonal line
Transformation and loss
of growth control
Senescence
Continuous cell line
Transformed cell line

What is Subculture?

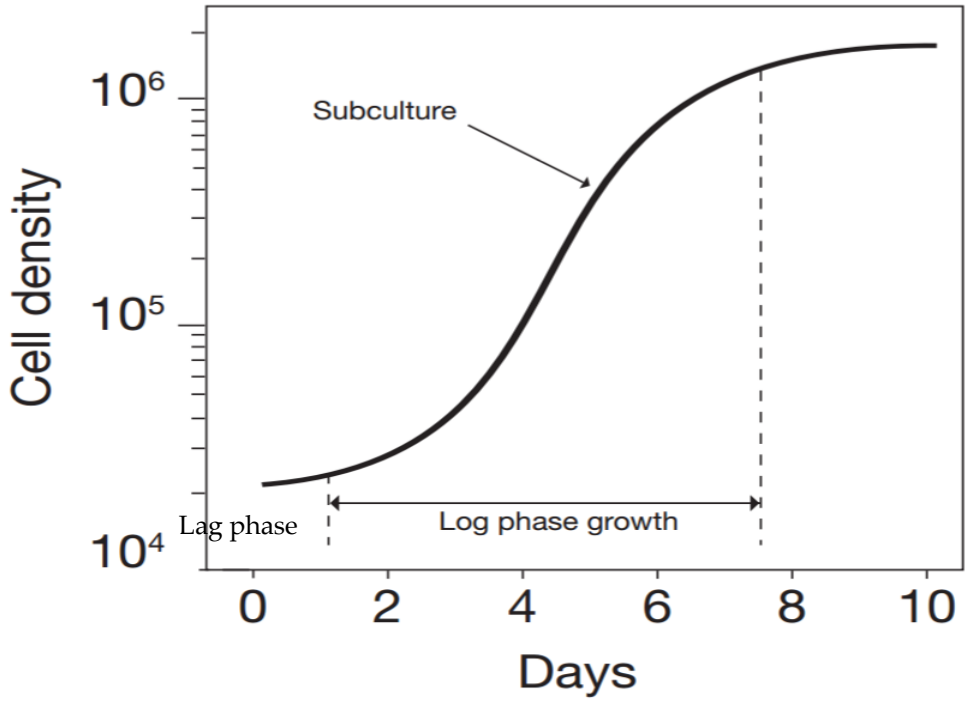
- Sub-culturing, also referred to as **passaging**, is the removal of the medium and transfer of cells from a previous culture into fresh growth medium, a procedure that enables the further propagation (spread) of the cell line or cell strain.



What is Subculture?

- When the cells in adherent cultures occupy all the available substrate and **have no room left for expansion**, or **when the cells in suspension cultures exceed the capacity of the medium to support further growth**, cell proliferation is greatly reduced or ceases entirely.
- To keep them at an optimal density for continued growth and to stimulate further proliferation, the culture has to divided and fresh medium supplied.

Subculture



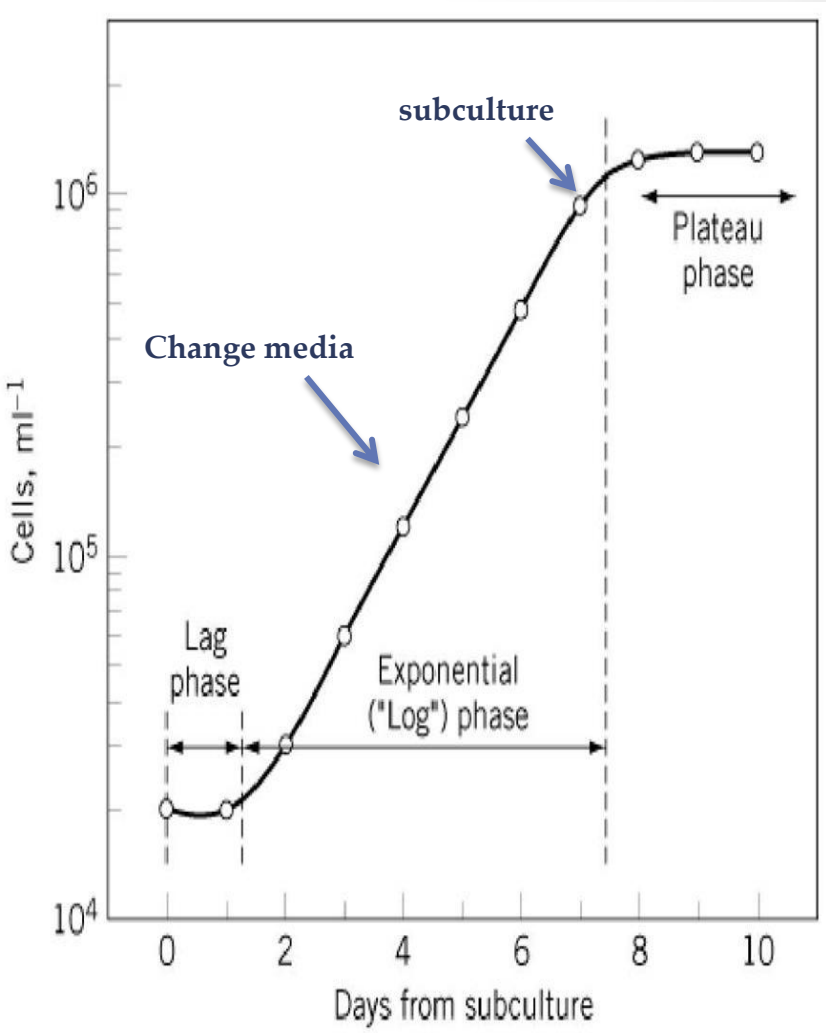
The growth of cells in culture continues from the lag phase following seeding to the log phase, where the cells proliferate exponentially.

Cell Culture Growth phases

- The first phase of growth after the culture is seeded is the lag phase, which is a period of slow growth when the cells are adapting to the culture environment and preparing for fast growth.
- The log phase is a period where the cells proliferate exponentially and consume the nutrients in the growth medium.
- When all the growth medium is spent (one or more of the nutrients is depleted) or when the cells occupy all of the available substrate, the cells enter the stationary phase (plateau phase), where the proliferation is greatly reduced or ceases entirely.

Growth curve for cell subculture

- **Lag phase:** cells adapted to new environment after seeding, minimal increase in cell density.
- **Log phase:** cell population doubles, change media during cell growth, subculture is preferred at confluency 70 – 90%
- **Stationary phase:** Cell proliferation slows and stops.
- **Decline phase:** If the culture medium is not replaced and the cell number is not reduced, the cells lose viability and their number decreases.



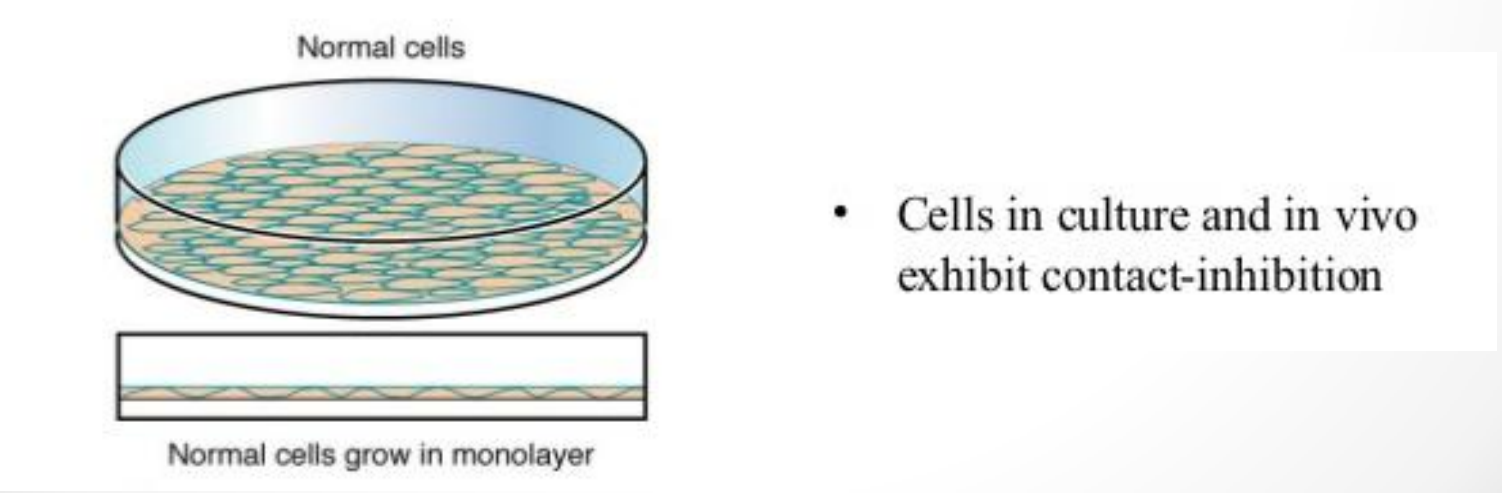
When to Subculture?

- There are three criteria for determining the need for subculture:

1. Cell Density
2. Exhaustion of Medium
3. Subculture Schedule

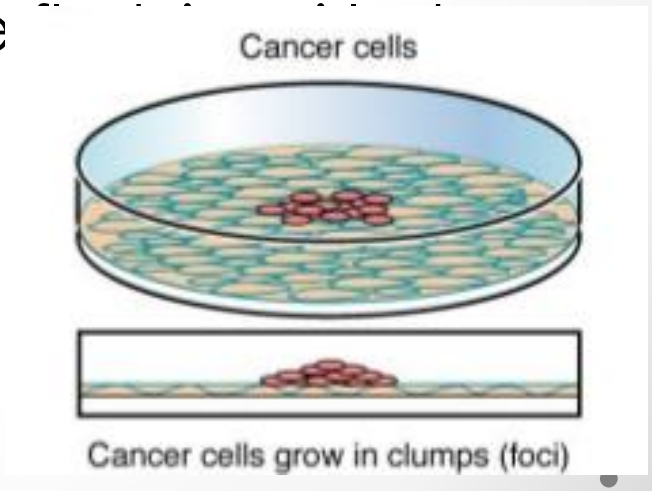
Cell density

- Adherent cultures should be passaged when they are in the log phase, before they reach confluence.
- Normal cells stop growing when they reach confluence (contact inhibition), and it takes them longer to recover when reseeded.



Cell density

- Transformed cells can continue proliferating even after they reach confluence, but they usually deteriorate after about two doublings. Similarly, cells in suspension should be passaged when they are in log-phase growth before they reach confluency. When they reach confluency, cells in suspension clump together and the medium appears turbid when the culture



Exhaustion of Medium

- For most mammalian cells pH 7.2 – 7.5
- A drop in the pH of the growth medium usually indicates a build up of lactic acid, which is a by-product of cellular metabolism.
- Lactic acid can be toxic to the cells, and the decreased pH can be sub-optimal for cell growth.

Exhaustion of Medium

- The rate of change of pH is generally dependent on the cell concentration in that cultures at a high cell concentration exhaust medium faster than cells lower concentrations.
- You should subculture your cells if you observe a rapid drop in pH (> 0.1–0.2 pH units) with an increase in cell concentration.

Subculture Schedule

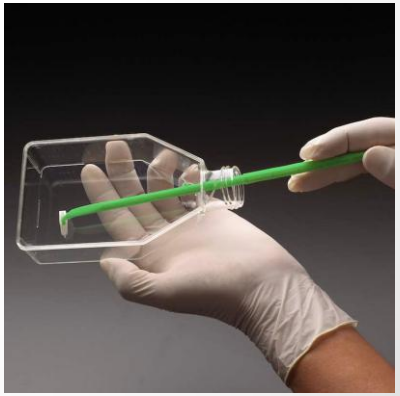
- Passaging your cells according to a strict schedule ensures reproducible behavior and allows you to monitor their health status. Vary the seeding density of your cultures until you achieve consistent growth rate and yield appropriate for your cell type from a given seeding density.
- Deviations from the growth patterns thus established usually indicate that the culture is unhealthy (e.g., deterioration, contamination) or a component of your culture system is not functioning properly (e.g., temperature is not optimal, culture medium too old).

- We strongly recommend that you keep a detailed cell culture log, listing the feeding and subculture schedules, types of media used, the dissociation procedure followed, split ratios, morphological observations, seeding concentrations, yields, and any anti-biotic use.

Dissociating Adherent Cells

- The first step in subculturing adherent cells is to detach them from the surface of the culture vessel by enzymatic or mechanical means.
- The table below lists the various cell dissociation procedures.

Procedure	Dissociation agent
Shake-off	Gentle shaking or vigorous pipetting
Scraping	Cell scraper
Enzymatic	• Trypsin • Trypsin + collagenase • Dipase



Materials Needed

- Culture vessels containing your adherent cells
- Tissue-culture treated flasks, plates or dishes
- Complete growth medium, pre-warmed to 37°C
- Disposable, sterile 15-mL tubes
- 37°C incubator with humidified atmosphere of 5% CO2
- Balanced salt solution such as Dulbecco's Phosphate Buffered Saline (DPBS), containing no calcium, magnesium, or phenol red
- Dissociation reagent such as trypsin or TrypLETM Express, without phenol red

Protocol for Passaging Adherent Cells

- Use proper sterile technique and work in a laminar flow hood.

1. Check the morphology of cells and degree of confluency and the absence of contamination by using inverted microscope.
2. Remove and discard the spent cell culture media from the culture vessel.
3. Wash cells using a balanced salt solution without calcium and magnesium (approximately **2 mL** per 10 cm2 culture surface area). Gently add wash solution to the side of the vessel opposite the attached cell layer to avoid disturbing the cell layer, and rock the vessel back and forth several times.

- **Note:** The wash step removes any traces of serum, calcium, and magnesium that would inhibit the action of the dissociation reagent.

4. discard the wash solution from the culture vessel

5. Add the pre-warmed dissociation reagent such as trypsin or TrypLETM to the side of the flask; use enough reagent to cover the cell layer (approximately 1 – 2 mL per 10 cm2). Gently rock the container to get complete coverage of the cell layer.

6. Incubate the culture vessel at room temperature for approximately 2 minutes. Note that the actual incubation time varies with the cell line used.

7. When ≥ 90% of the cells have detached, aspirate off most trypsin then add the pre-warmed medium 3 mL.

8. Disperse the medium by pipetting over the cell layer surface several times.

9. Inoculate fresh flasks using the recommended split ratio.

10. Incubate under appropriate conditions.

Passaging Suspension Cultures

- Subculturing suspension cells is somewhat less complicated than passaging adherent cells. Because the cells are already suspended in growth medium, there is no need to
treat them enzymatically to detach them from the surface of the culture vessel, and the whole process is faster and less traumatic for the cells.
- The cells are maintained by feeding them every 2 to 3 days until they reach confluency. This can be done by directly diluting the cells in the culture flask and continue expanding them, or by withdrawing a portion of the cells from the culture flask and diluting the remaining cells down to a seeding density appropriate for the cell line.
- Usually, the lag period following the passaging is shorter than that observed with adherent cultures.

Suspension Culture Vessels

- **Spinner flasks** (i.e., stirrer bottles) specifically designed for suspension cell culture allow for superior gas exchange and permit higher volumes of cells to be cultured.

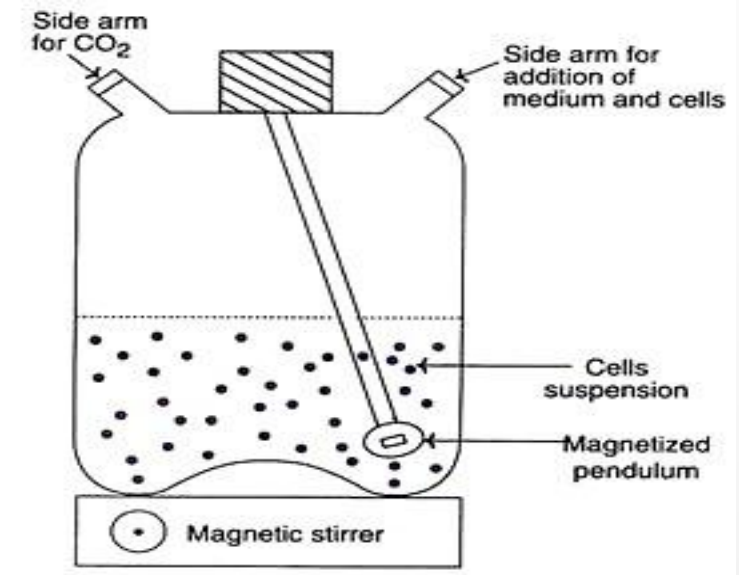


Fig. 37.2 : Diagrammatic representation of a stirrer flask.

Suspension Culture Vessels

- Spinner flasks have two basic designs; the medium is agitated (stirred) by a hanging stir-bar assembly or with a vertical impeller.

- The total culture volume in a spinner flask should not exceed half of the indicated volume of the spinner for proper aeration (airing) (e.g., a 500 mL spinner should never contain more than 250 mL of culture).

Split ratio of cell subculture

- *Suspension cell line* split ratios are done on **volume of culture cell suspension:**

100 mL cell Suspension → Split 1: 4 → 25 ml cell suspension + 75 ml fresh media in 5 separate new flasks
Or
50ml cell suspension + 150 ml Fresh media in 2 larger flasks

.. Remember ..

Sub-culturing should always be done with aseptic technique in sterile conditions

Subculture of Adherent Cells

- https://www.youtube.com/watch?v=mjCZjw4KTYc

Reference

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