Cell lines and cloning and Somatic cell-fusion

CELL CLONING :

The traditional microbiological approach to the problem of culture heterogeneity is to isolate pure cell strains by cloning, but, although this technique is relatively easy for continuous cell lines, its success in most primary cultures is limited by poor cloning efficiencies. Cloning of attached cells may be carried out in Petri dishes, multiwell plates, or flasks, and it is relatively easy to discern individual colonies. Micromanipulation is the only conclusive method for determining genuine clonality (i.e., that a colony was derived from one cell), but when symmetrical colonies are derived from a single-cell suspension, particularly if colony formation is monitored at the early stages, then it is probable that the colonies are clones.

Cloning can also be carried out in suspension by seeding cells into a gel, such as agar or agarose, or a viscous solution, such as Methocel, with an agar or agarose underlay. The stability of the gel, or viscosity of the Methocel, ensures that daughter cells do not break away from the colony as it forms. Even in monolayer cloning, some cell lines, such as HeLa-S_3 and CHO, are poorly attached, and cells can detach from colonies as they form and generate daughter colonies, which will give an erroneous plating efficiency. This can be minimized by cloning in Methocel without an underlay and allowing the cells to sediment on to the plastic growth surface. Hematopoietic cells are usually cloned in suspension; depending on the cells and growth factors used, the colony generates undifferentiated cells with high repopulation efficiency, *in vivo* or *in vitro*, or may mature into colonies of differentiated hematopoietic cells with very littlerepopulation efficiency. Cloning then becomes an assay for reproductive potential and stem cell identity.

Continuous cell lines generally have a high plating efficiency in monolayer and in suspension because of their transformed status, whereas normal cells, which may have a moderately high cloning efficiency in monolayer, have a very low cloning efficiency in suspension, because of their need to attach and spread out to enter the cell proliferation cycle. Dilution cloning [Puck & Marcus, 1955] is the technique that is used most widely, based on the observation that cells diluted below a certain density form discrete colonies.

STIMULATION OF PLATING EFFICIENCY :

When cells are plated at low densities, the rate of survival falls in all but a few cell lines. This does not usually present a severe problem with continuous cell lines, for which the plating efficiency seldom drops below 10%, but with primary cultures and finite cell lines, the plating efficiency may be quite low—0.5–5%, or even zero. Numerous attempts have been made to improve plating efficiencies, based on the assumption either that cells require a greater range of nutrients at low den-sities, because of loss by leakage, or that cell-derived diffusible signals or conditioning factors are present in high-density cul-tures and are absent or too dilute at low densities. The intra-cellular metabolic pool of a leaky cell in a dense population will soon reach equilibrium with the surrounding medium, but that of an isolated cell never will. This principle was the basis of the capillary technique of Sanford et al. [1948], by which the L929 clone of L-cells was first produced. The con-fines of the capillary tube allowed the cell to create a locally enriched environment that mimicked a higher cell concentra-tion. In microdrop techniques developed later, the cells were seeded as a microdrop under liquid paraffin, again maintaining a relatively high cell concentration, keeping one colony sep-arate from another, and facilitating subsequent isolation. As media improved, however, plating efficiencies increased, and Puck and Marcus [1955] were able to show that cloning cells by simple dilution

Conditions that Improve Clonal Growth

(1) **Medium.** Choose a rich medium, such as Ham's F12, or a medium that has been optimized for the cell type in use (e.g., MCDB 110 [Ham, 1984] for human fibroblasts, Ham's F12 or MCDB 302 for CHO [Ham, 1963; Hamilton & Ham, 1977])

(2) **Serum.** When serum is required, fetal bovine is generally better than calf or horse. Select a batch for cloning experiments that gives a high plating efficiency during tests

(3) **Hormones.** Insulin, 1×10^{-10} IU/mL, has been found to increase the plating efficiency of several cell types [Hamilton & Ham, 1977]. Dexamethasone, 2.5×10^{-5} M, 10 µg/mL, a soluble synthetic hydrocortisone analog, improves the plating efficiency.

(4) **Carbon Dioxide.** CO_2 is essential for obtaining maximum cloning efficiency for most cells. Although 5% CO_2 is usually used, 2% is sufficient for many cells and may even be slightly better for human glia and fibroblasts. HEPES (20 mM) may be used with 2% CO_2, protecting the cells against pH fluctuations during feeding and in the event of failure of the CO_2 supply. Using 2% CO_2 also cuts down on the consumption of CO_2. At the other extreme, Dulbecco's modification of Eagle's Basal

(5) Medium (DMEM) is normally equilibrated with 10% CO_2 and is frequently used for cloning myeloma hybrids for monoclonal antibody production. The concentration of bicarbonate must be adjusted if the CO_2 tension is altered, so that equilibrium is reached at pH 7.4

(6) **Treatment of Substrate.** Polylysine improves the plating efficiency of human fibroblasts in low serum concentrations

(7) **Trypsin.**

Conditioned Medium ;

Medium that has been used for the growth of other cells acquires metabolites, growth factors, and matrix products from these cells. This conditioned medium can improve the plating efficiency of some cells if it is diluted into the regular growth medium.

Feeder Layers :

The reason that some cells do not clone well is related to their inability to survive at low cell densities. One way to maintain cells at clonogenic densities but, at the same time, to mimic high cell densities, is to clone the cells onto a growth-arrested feeder layer . The feeder cells may provide nutrients, growth factors, and matrix constituents that enable the cloned cells to survive more readily.

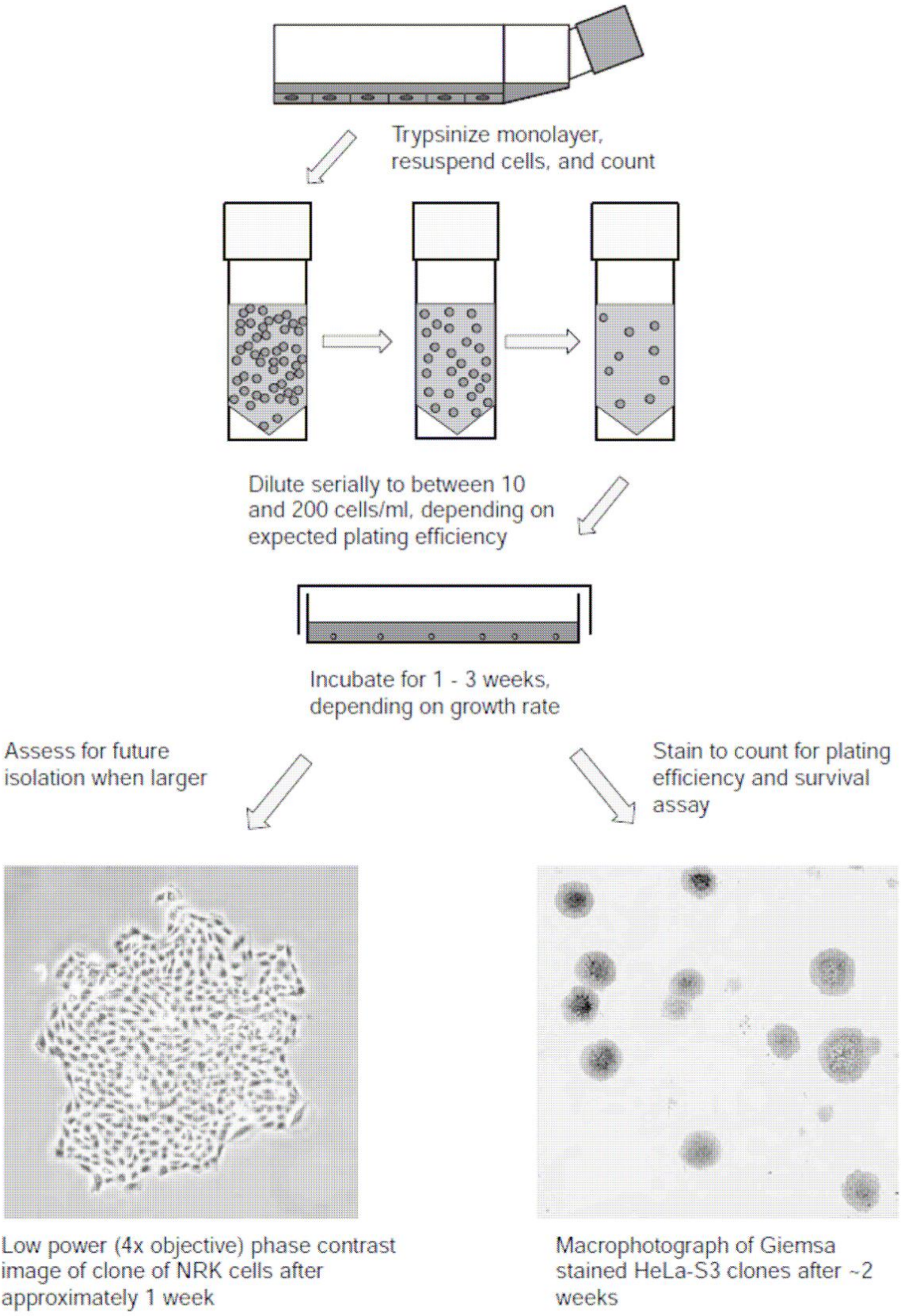

Fig. 14.2. Dilution Cloning. Cells are from a trypsinized monolayer culture that has been counted and diluted sufficiently to generate isolated colonies. When clones form, they may be isolated (*see* Figs. 14.7; 14.8). If isolation is not required and the cloning is being performed for quantitative assay (*see* Protocols 21.10, 22.3), the colonies are fixed, stained, and counted. (*See also* Plate 6a,e).

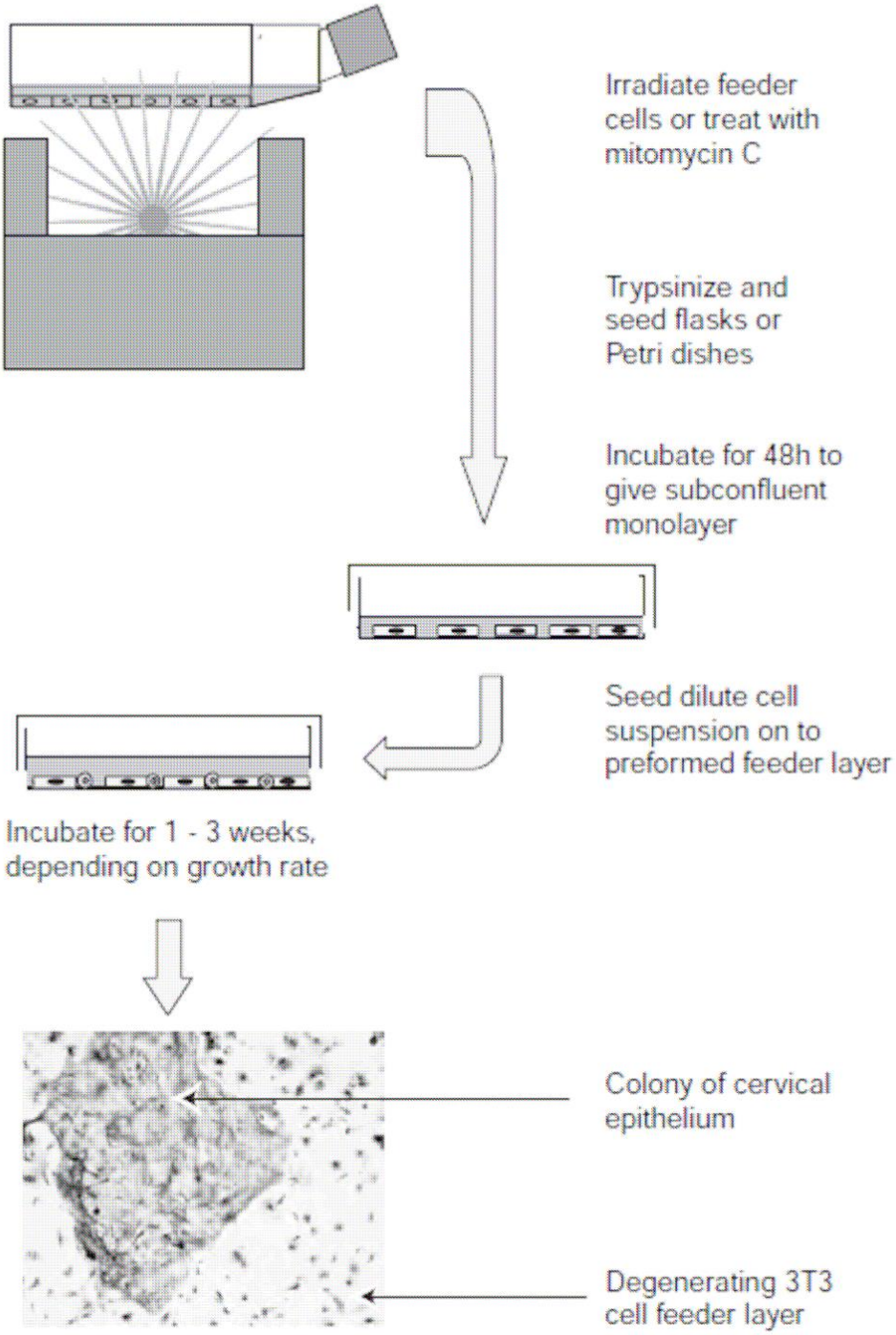

Fig. 14.4. Feeder Layers. Cells are irradiated and trypsinized (or may be trypsinized first and then irradiated in suspension, or treated with mitomycin C) and seeded at a low density to enhance cloning efficiency. (Photo courtesy of M. G. Freshney.)

SUSPENSION CLONING

Some cells, particularly hematopoietic stem cells and virally transformed fibroblasts, clone readily in suspension. To hold the colony together and prevent mixing, the cells are

suspended in agar or Methocel and plated on an agar underlay or into dishes that need not be treated for tissue culture.

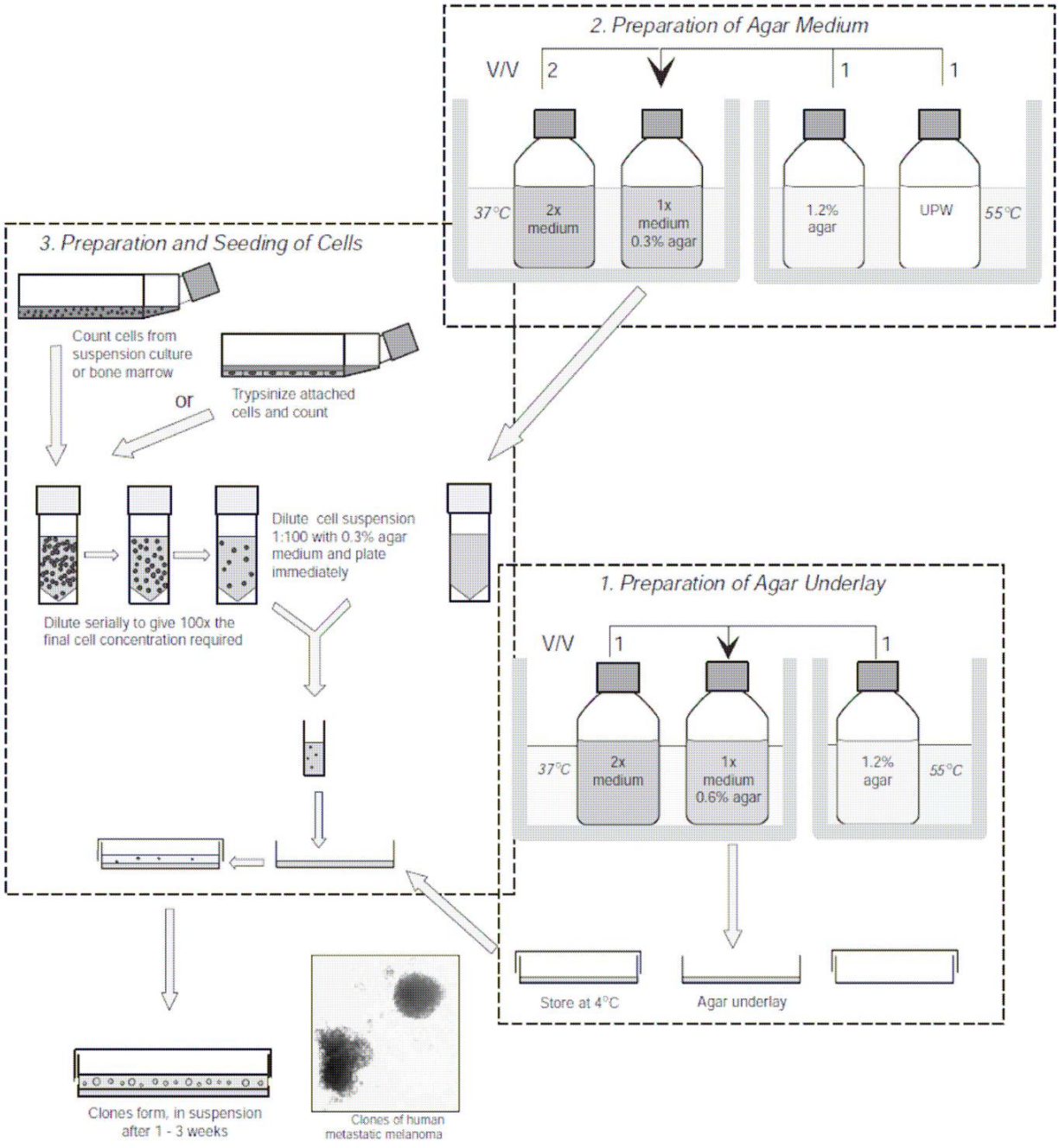

Fig. 14.5. Cloning in Suspension. Cultured cells or primary suspensions from bone marrow or tumors, suspended in agar or low-melting-temperature agarose, which is then allowed to gel, form colonies in suspension. Use of an underlay prevents attachment to the base of the dish. 1. Preparation of agar underlay: Agar, 1.2%, at 55°C is mixed with 2× medium at 37°C and dispensed immediately into dishes, where it is allowed to gel at room temperature or 4°C. 2. Preparation of agar medium: Agar, 1.2%, and UPW are maintained at 55°C and mixed with 2× medium to give 0.3% agar for cloning. The use of low-melting-point agarose allows all solutions to be maintained at 37°C, but this agarose can be more difficult to gel. 3. Cells grown in suspension, derived from bone marrow, or trypsinized from an attached monolayer are counted and diluted serially, and the final product is diluted with agar or agarose and seeded onto an agar underlay.

ISOLATION OF CLONES :

If monolayer cells are cloned directly into multiwell plates (,then colonies may be isolated by trypsinizing individual wells. If cloning is performed in Petri dishes, there is no physical separation between colonies. This separation must be created by removing the medium and placing a stainless steel or ceramic ring around the colony to be isolated.

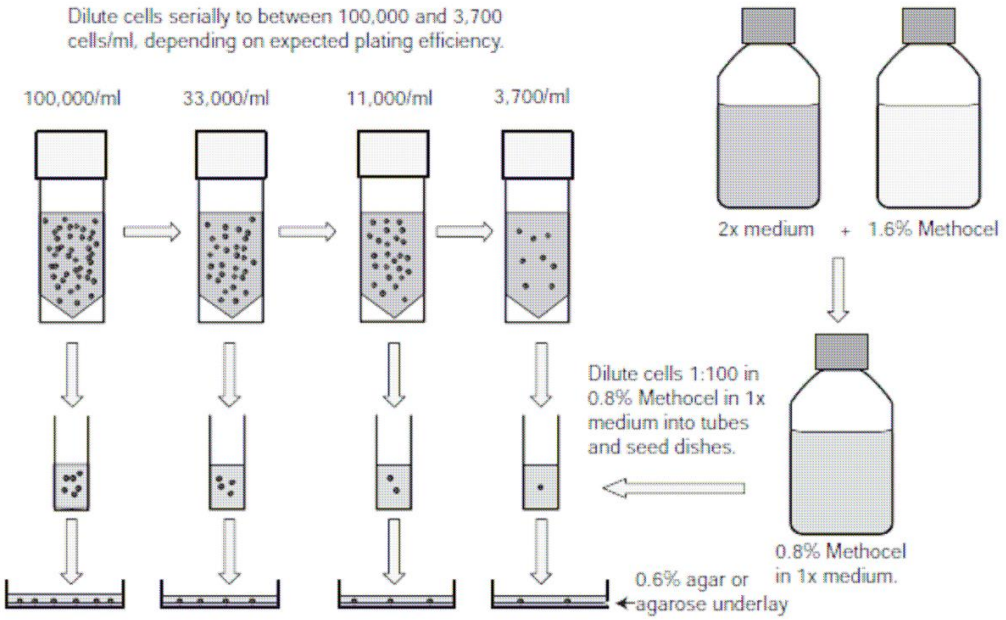

Fig. 14.6. Cloning in Methocel. A series of cell dilutions is prepared as for agar cloning, diluted 1:100 in Methocel medium, and plated into non-tissue-culture-grade dishes or dishes with an agar underlay.

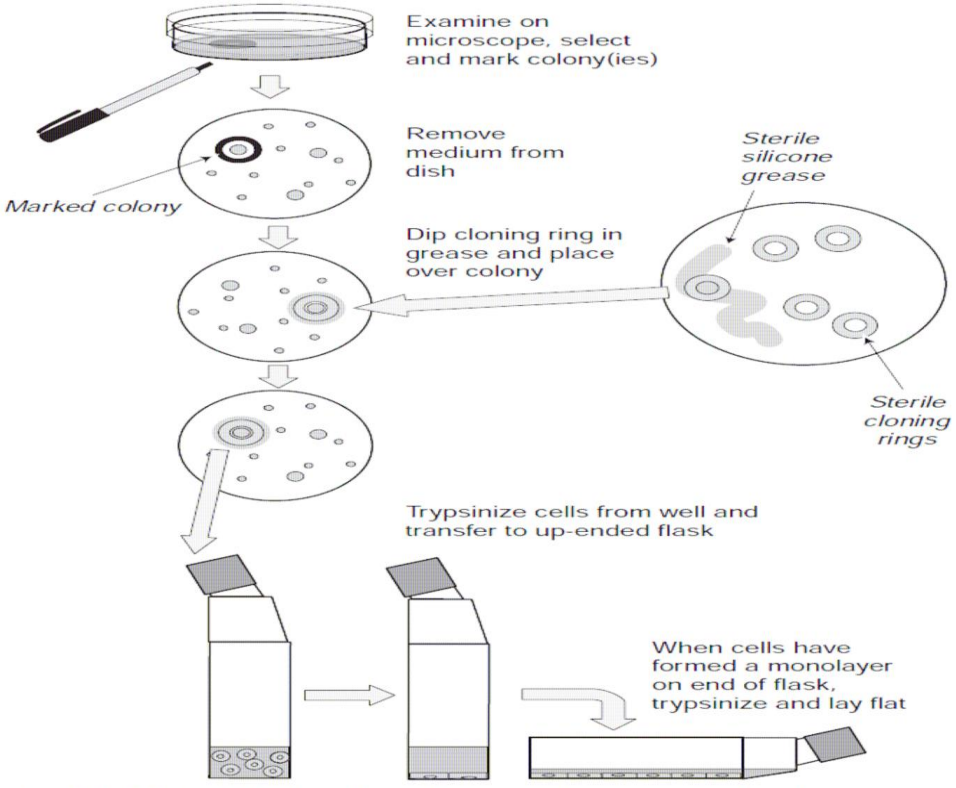

Fig. 14.8. Isolation of Monolayer Clones. The mature colonies are examined on the microscope,

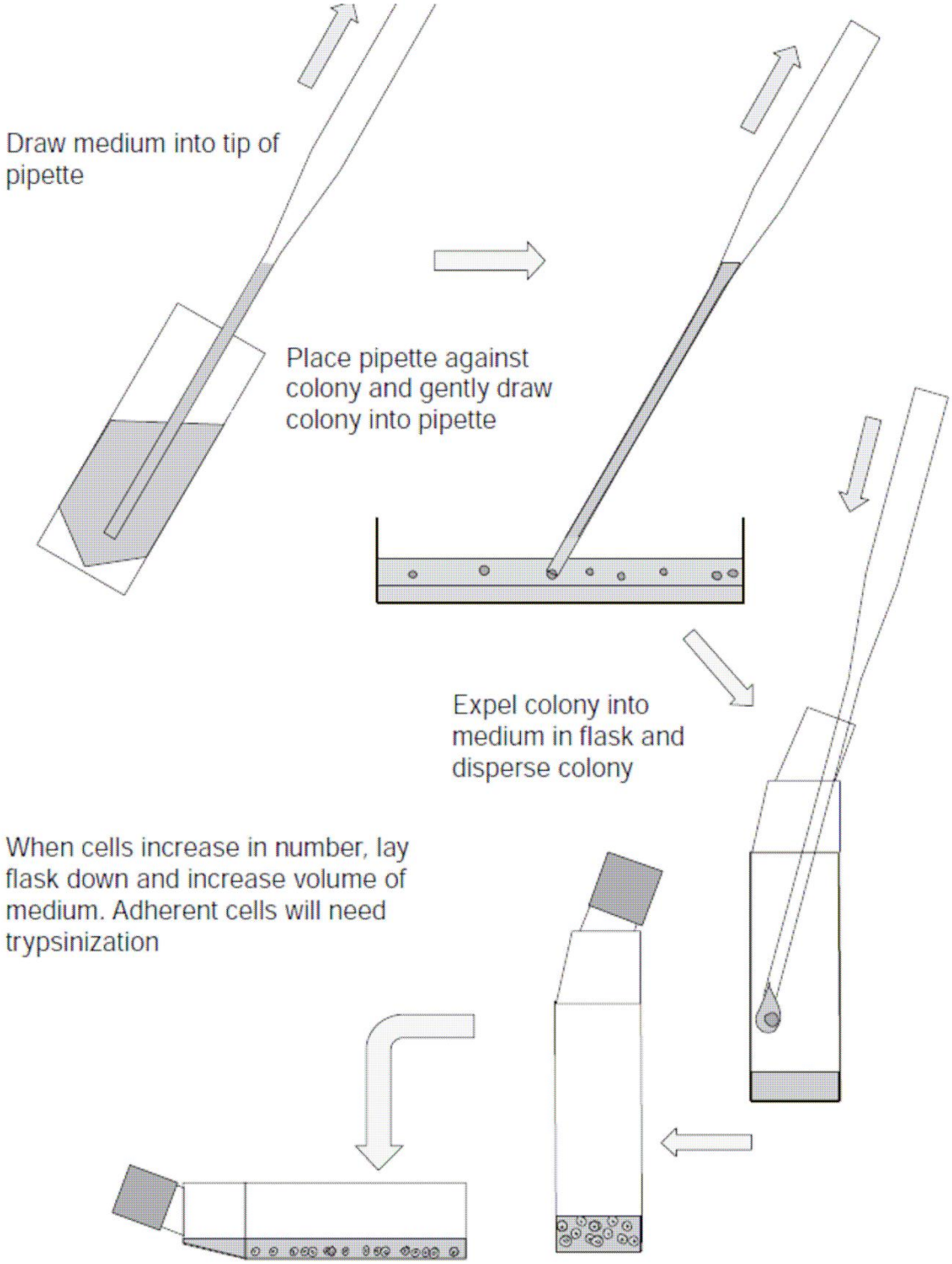

Fig. 14.9. Isolation of Suspension Clones. Mark the colony as for monolayer, then draw the colony into a Pasteur pipette (or pipettor tip). Transfer the colony to a culture flask, disperse it in medium, and incubate it. Make up medium when cells start to grow.

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