METHODS

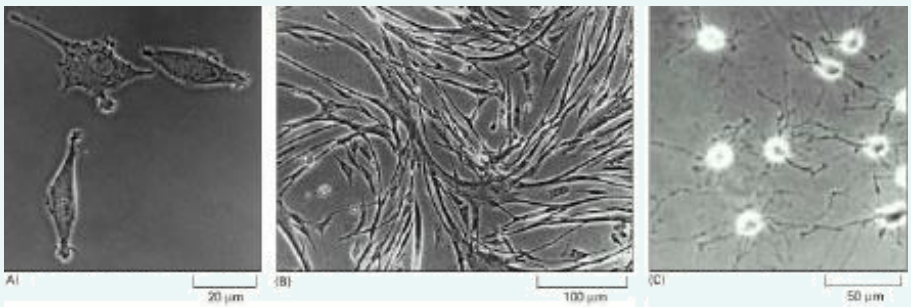
ISOLATING CELLS AND GROWING THEM IN CULTURE

FRACTIONATING CELLS

VISUALIZING CELL STRUCTURES AND LOCALIZING MOLECULES WITHIN CELLS

CULTURE OF ANIMAL CELLS REQUIRES NUTRIENT-RICH MEDIA AND SPECIAL SOLID SURFACES

Growth of vertebrate cells in culture requires rich media containing essential amino acids, fatty acids, and peptide or protein growth factors. Most cultures cells will grow only when attached to a negatively charged substratum that is coated with components of the ECM. Transformed cells grow indefinitely in culture, forming cell lines. Certain cell lines can be induced to undergo differentiation in culture to generate different types of cells and are widely used in cell biological studies.

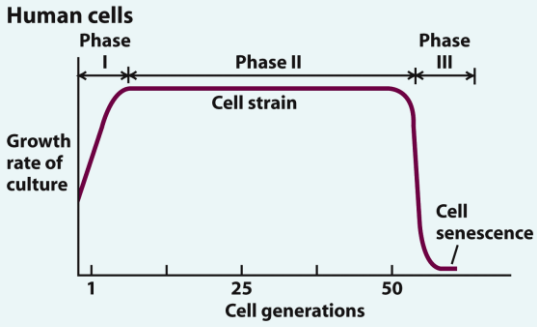


Cells in culture. (A) Phase-contrast micrograph of fibroblasts in culture. (B) Micrograph of myoblasts in culture shows cells fusing to form multinucleate muscle cells. (C) Oligodendrocyte precursor cells in culture. (A, courtesy of Daniel Zicha; B, courtesy of Rosalind Zalin; C, from D.G. Tang et al., *J. Cell Biol*. 148:971 984, 2000)

CELL LINE*	CELL TYPE AND ORIGIN
3T3	fibroblast (mouse)
BHK21	fibroblast (Syrian hamster)
MDCK	epithelial cell (dog)
HeLa	epithelial cell (human)
PtK1	epithelial cell (rat kangaroo)
L6	myoblast (rat)
PC12	chromaffin cell (rat)
SP2	plasma cell (mouse)
COS	kidney (monkey)
293	kidney (human); transformed with adenovirus
CHO	ovary (chinese hamster)
DT40	lymphoma cell for efficient targeted recombination (chick)
R1	embryonic stem cells (mouse)
E14.1	embryonic stem cells (mouse)
H1, H9	embryonic stem cells (human)
S2	macrophage-like cells *(Drosophila)*
BY2	undifferentiated meristematic cells (tobacco)

PRIMARY CELL CULTURES AND CELL STRAINS HAVE A FINITE LIFE SPAN

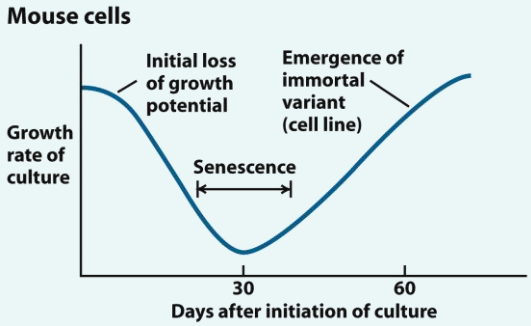
Normal animal tissues (e.g., skin, kidney, liver) or whole embryos are commonly used to establish *primary cell cultures.* When cells removed from an embryo or an adult animal are cultured, most of the adherent ones will divide a finite number of times and then cease growing (***cell senescence***). Thus, even though its lifetime is limited, a single culture, if carefully maintained, can be studied through many generations. Such a lineage of cells originating from one initial primary culture is called a ***cell strain.***

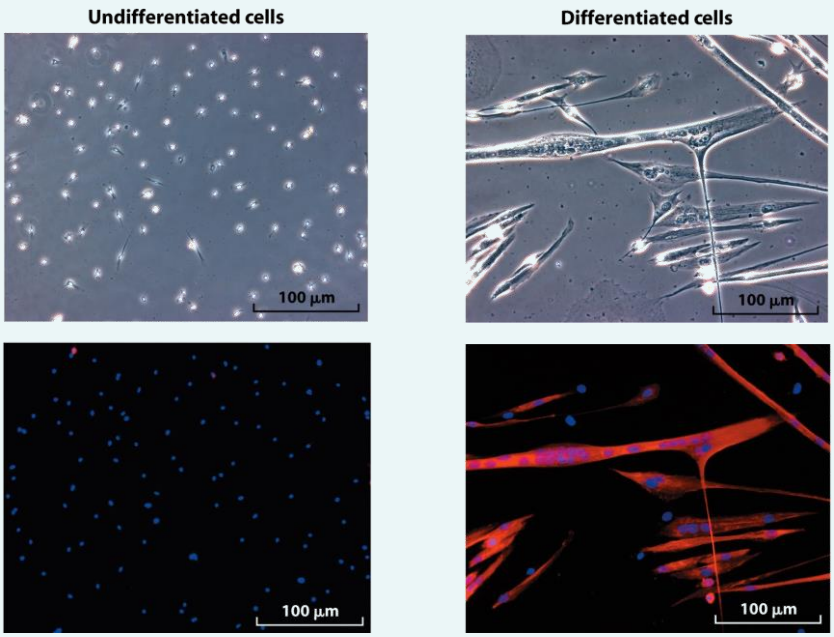


TRANSFORMED CELLS CAN GROW INDEFINITELY IN CULTURE

A culture of cells with an indefinite life span is considered immortal and is called a ***cell line*****.** The HeLa cell line, the first human cell line, was originally obtained in 1952 from a malignant tumor of the uterine cervix.

Although primary cell cultures of normal human cells rarely undergo transformation into a cell line, rodent cells commonly do.





Equipo Básico de un laboratorio de Cultivo Celular

1. Campana de flujo laminar
2. Baño termostatizado
3. Incubador CO_2
4. Autoclave

5. Heladera – Freezer (-20°, -80°)
6. Microscopio Invertido
7. Micro centrifuga
8. Depósito de Nitrógeno líquido

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Campana de Flujo Laminar

Esterilidad

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Microscopio Invertido

Seguimiento del cultivo: viabilidad, multiplicación, contaminación.

Control sobre cambios morfológicos (efectos de hormonas, de tratamientos con medicamentos, de iones, interacciones, etc.)

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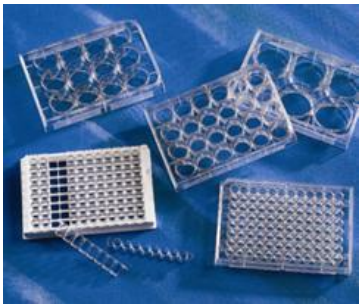
Botellas y Placas de Cultivo







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Medios de Cultivo: Medios Base

Medios base: Eagle's Basal Medium, Dulbecco's modified Eagle's medium (DMEM), RPMI1640, etc.

Contienen: Hidratos de Carbono, ácidos grasos esenciales y otros lípidos, aminoácidos, sales minerales, oligoelementos.

**Cada línea celular tiene unos requerimientos que hacen que crezca mejor en un determinado tipo de medio*

C. Von Bilderling

Medios de Cultivo: Aditivos

- **Antibióticos:** estreptomicina (Gram +/-), penicilina (Gram +), amfotericina (levaduras y hongos)
- **Tampón pH:** Hepes, bicarbonato, etc.
- **Indicador pH.**
- **Suero fetal:** Bovino, ovino, humano. 10% (2-50%).
- **Aditivos específicos:** Suplemento de piruvato, suplemento de glucosa, insulina, etc.

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Medios de Cultivo

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Preparación del medio

- Filtrado (0.22 µm) para esterilidad.

Previamente a la realización de experimentos los medios deben ser probados por contaminación.

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Conceptos básicos en el mantenimiento de una línea

- Confluencia
- Tiempo de duplicación
- Estado de la línea

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División del cultivo

Objetivos:

- Propagación de la línea celular
- Producción de células para la experimentación

Usualmente se realizan al llegar a c**onfluencia.**

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División del cultivo

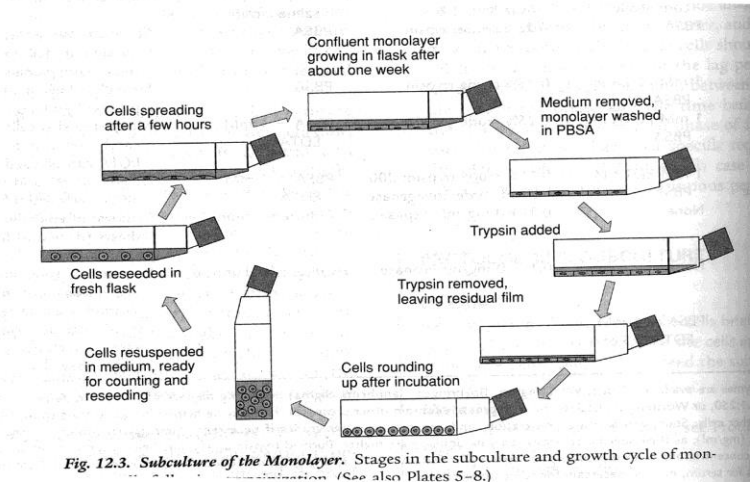

Fig. 12.3. Subculture of the Monolayer. Stages in the subculture and growth cycle of mon-

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Protocolo división de células

Depende de las características de la línea celular con la que se esta trabajando.

1. **Células en suspensión**

2. **Células adheridas al substrato**

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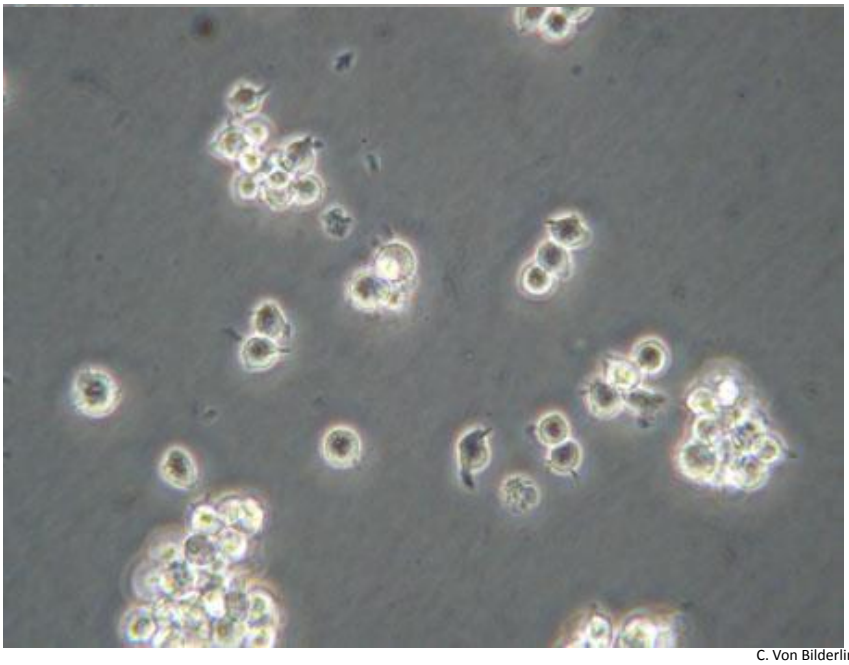

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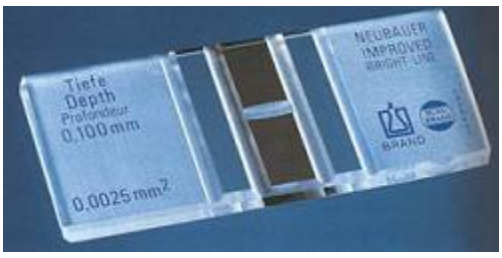
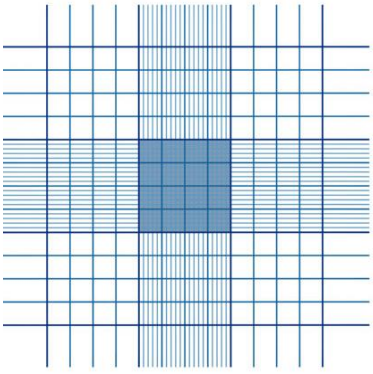
Recuento de células

Cámara de Neubauer





$$\text{células por u. de volumen} = \frac{\text{células contadas}}{\text{superficie contada} \ . \ \text{profundidad de la cámara}}$$

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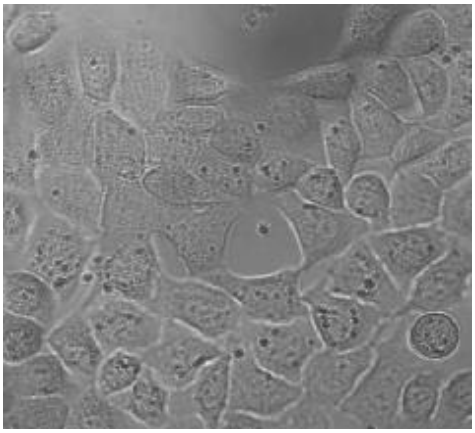
Hasta acá: células



Línea HC11: epitelio mamario ratón

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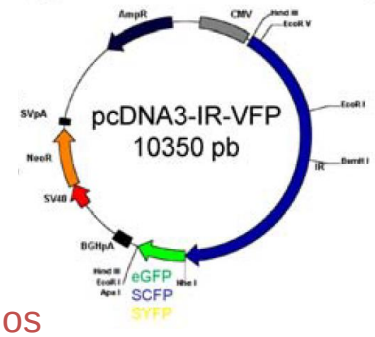
Transfección

Transfección: Introducción de DNA externo con un vector de expresión.

TIPOS: Transitoria o estable.



Plásmidos

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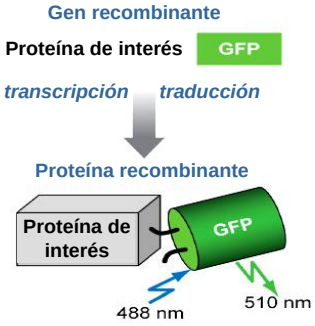
Expresión de proteínas fluorescentes



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Métodos de transfección

- Físicos
  - Microinyección
  - Electroporación
- Químicos
  - Fosfato Cálcico
  - DEAE dextrano
  - Lipofección

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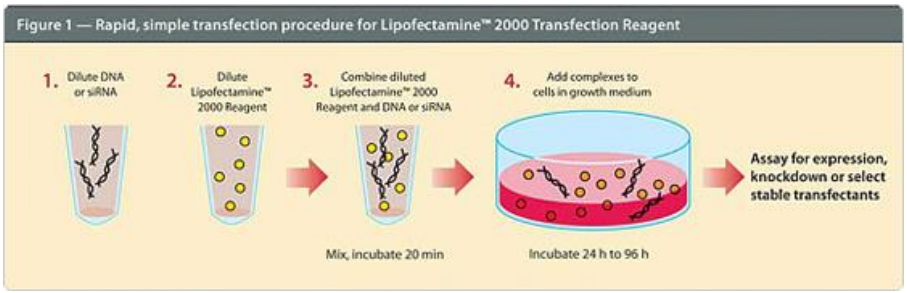
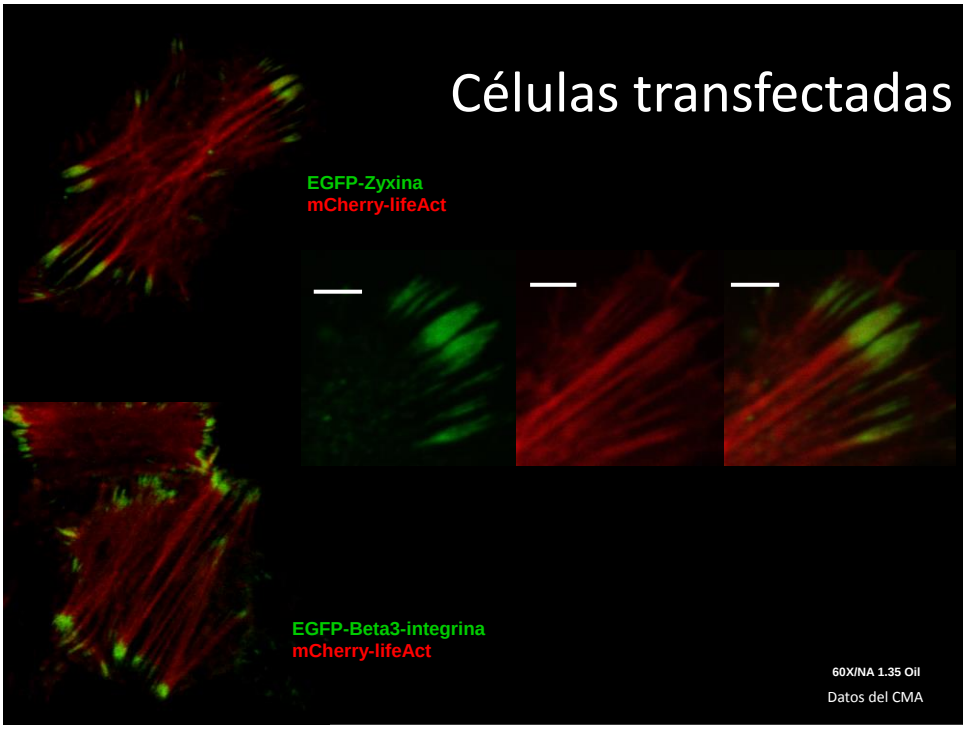
Lipofectamina



C. Von Bilderling



ISOLATING CELLS AND GROWING THEM IN CULTURE

Tissues can be dissociated into their component cells, from which individual cell types can be purified and used for biochemical analysis or for the establishment of cell cultures.

Some cells types differ sufficiently in density that they be separated on the basis of the physical property by equilibrium density-**centrifugation** (leukocytes and red blood cells, for instance).

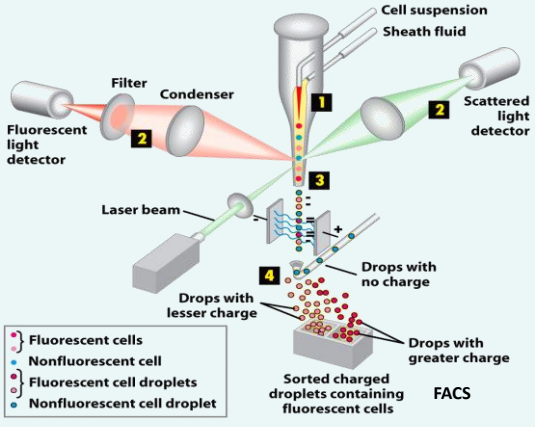
Because most cell types cannot be differentiated so easily, other techniques such as **flow cytometry** must be used to separate them.

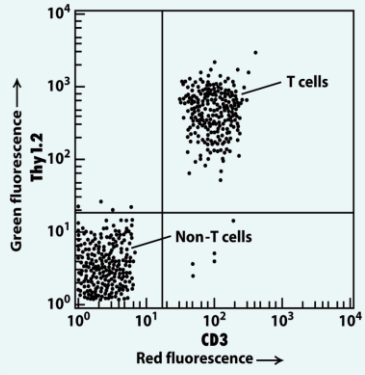
Centrifugation is used for two basic purposes:

1) as a *preparative* technique to ***separate*** one type of material from others
2) as an *analytical* technique to ***measure*** physical properties (e.g., molecular weight, density, shape, and equilibrium binding constants) of cells/macromolecules.

FLOW CYTOMETRY SEPARATES DIFFERENT CELL TYPES

A flow cytometer identifies different cells by measuring the light that they scatter and the fluorescence that they emit as they flow through a laser beam; thus it can sort out cells of a particular type from a mixture.



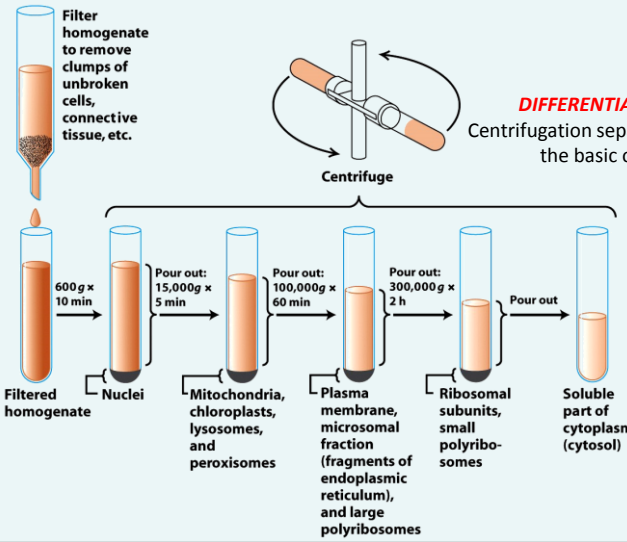


T cells bound to fluorescence-tagged antibodies to two cell-surface proteins are separated from other white blood cells by FACS. Note the logarithmic scale on both axes. [Courtesy of Chengcheng Zhang.]

Fluorescence-activated cell sorter (FACS) separates cells that are labeled differentially with a fluorescent reagent. [Adapted from D. R. Parks and L. A. Herzenberg, 1982, *Meth. Cell Biol.* **26**:283.]

DISRUPTION OF CELLS RELEASES THEIR ORGANELLES AND OTHER CONTENTS

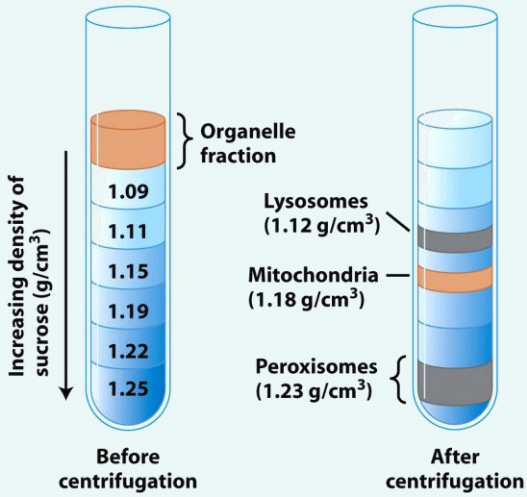
Using gentle mechanical procedures, called homogenization, sonication or other techniques, the plasma membrane of cells can be disrupted so that the cell contents are release.



DIFFERENTIAL CENTRIFUGATION

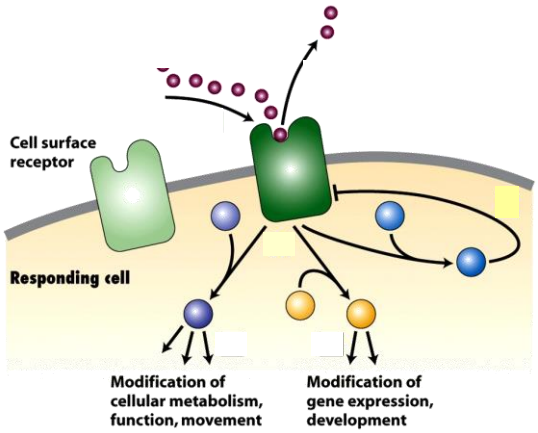
Centrifugation separates cell components on the basic of size and density

An impure organelle fraction obtained by differential centrifugation can be further purified by EQUILIBRIUM DENSITY-GRADIENT CENTRIFUGATION, which separates cellular components according to their density.



Receptores de membrana

Un receptor de membrana es una proteína integral que une específicamente una molécula extracelular (ligando), lo cual induce un cambio conformacional en el receptor, iniciando así una respuesta celular. *El receptor de membrana actúa como un transductor de señales.*



✓ la unión de moléculas secretadas o unidas a membrana, por cambios en la concentración de un metabolito o por un estímulo físico

Receptores de membrana

✓solubilización con detergentes no iónicos

✓ purificación y separación (cromatografía de afinidad)

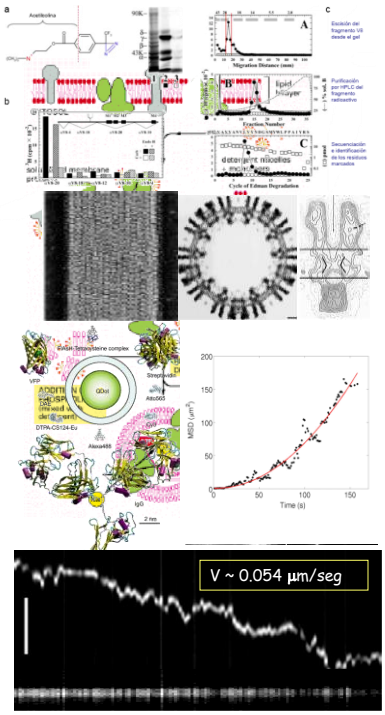
✓detección usando marcaje de afinidad

✓caracterización bioquímica

✓ estudios funcionales

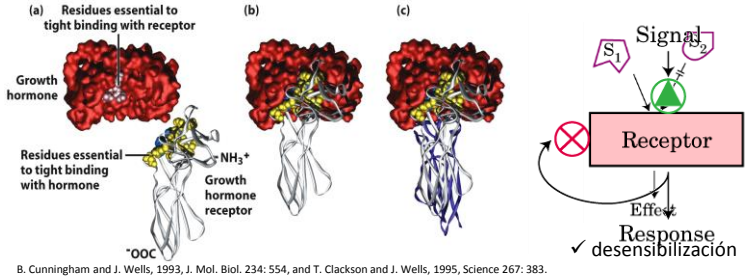
✓ técnicas de DNA recombinante para la identificación y clonado de proteínas receptores

✓localización y seguimiento en células vivas (VFPs, QDs, microscopía, técnicas: FRET, FLIM,)



Los receptores unen ligandos en forma específica y alta afinidad

✓ por las interacciones no covalentes entre un ligando y aminoácidos específicos en la proteína receptor.



B. Cunningham and J. Wells, 1993, J. Mol. Biol. 234: 554, and T. Clackson and J. Wells, 1995, Science 267: 383.

✓ desensibilización

✓ la constante de disociación es una medida de la afinidad de un receptor por su ligando

$$R + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} RL$$

$$K_d = \frac{[R]\,[L]}{[RL]} = 1/\,K_{eq}$$

$K_d \sim 10^{-9}M - 10^{-11}M$

✓ los ensayos de unión permiten detectar los receptores y determinar su afinidad por los ligandos y el número de receptores por célula.

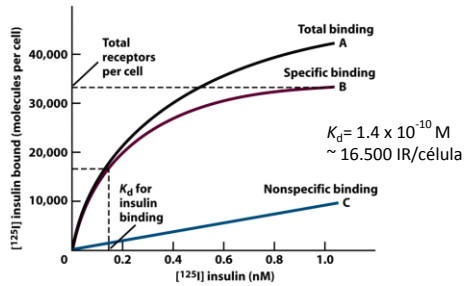
$$R + L \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} RL$$

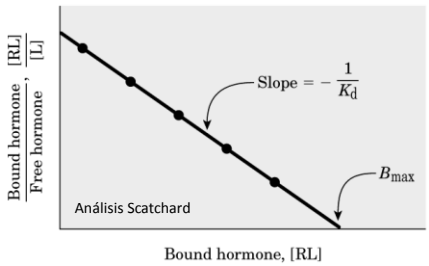
$$K_d = \frac{[R][L]}{[RL]} = 1/\,K_{eq}$$

$$B_{max} = [R] + [RL]$$

$$K_d = \frac{(B_{max} - [RL])[L]}{[RL]} = 1/\,K_{eq}$$

$$\frac{[Bound]}{[Free]} = \frac{[RL]}{[L]} = 1/\,K_d\,(B_{max} - [RL])$$





✓ la máxima respuesta de una célula a un ligando particular generalmente ocurre a las concentraciones de ligando a las cuales la mayoría de los receptores todavía no están ocupados.

METHODS

PROTEIN PURIFICATION, DETECTION AND ANALYSIS

Protein purification and analysis

✓ Purification is an all but mandatory step in studying macromolecules, but it is not necessarily easy.

✓ The first step in the isolation of a protein or other biological molecule is to get it out of the cell and into solution. Many cells require some sort of mechanical disruption to release their content. If the target protein is associated with a lipid membrane, a detergent may be used to solubilize the lipids and recover the protein.

✓ Factors to be controlled at all stages of a purification process: pH, temperature, presence of degradative enzymes, adsorption to surface, long-term storage.

✓ Proteins are purified by fractionation procedures which depend on the protein characteristics are based on:

protein characteristic	purification procedure
solubility	salting out
ionic charge	ion exchange chromatography, electrophoresis, isoelectric focusing
polarity	hydrophobic interaction chromatography
size	gel filtration chromatography, SDS-PAGE, ultracentrifugation
binding specificity	affinity chromatography

Assaying proteins

✓ purifying a substance requires some means for quantitatively detecting it. An assay must be specific for the target protein, highly sensitive, and convenient to use.

✓ Among the most straightforward protein assays are those for enzymes that catalyze reactions with readily detected products. Proteins that are not enzymes can be detected by their ability to specifically bind certain substances or to produce observable biological effects.

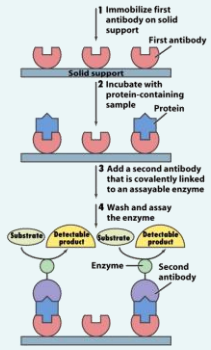
✓ Immunochemical procedures are among the most sensitive of assay techniques.

✓ Radioimmunoassay (RIA), the protein is indirectly detected by determining the degree to which it competes with a radioactively labeled standard for binding the antibody.

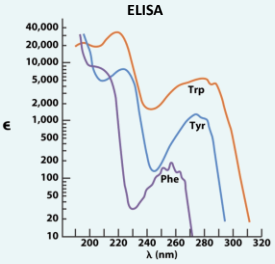
✓ Enzyme-linked immunosorbent assay (ELISA) has many variations, one of which is diagrammed below.

✓ The concentration of a protein in solution can be measured by absorbance spectroscopy (Beer-Lambert's Law; 50 to 100 μg per mL).

✓ The Bradford assay provides a direct measure of the amount of protein present (1 μg of protein per mL).



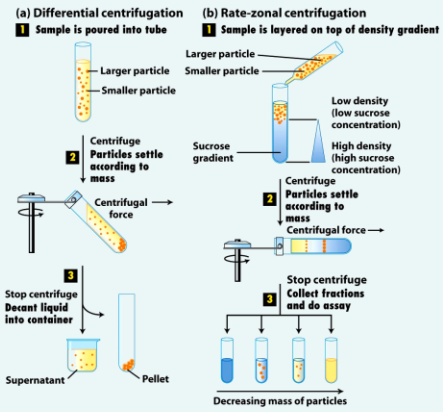
ELISA



UV absorbance spectra

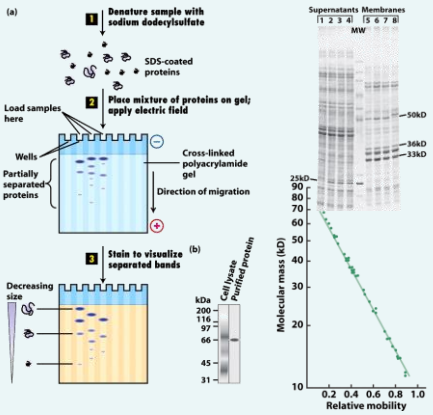
Separation techniques

CENTRIFUGATION



Centrifugation is used for two basic purposes: as a preparative technique to separate one type of material form others and as an analytical technique to measure physical properties of macromolecules (MW, density, shape and K_{eq}).

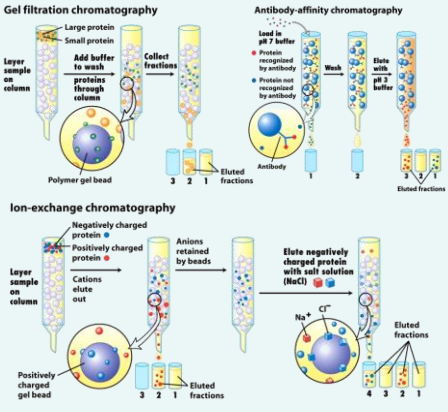
ELECTROPHORESIS



SDS-PAGE separates proteins purely by gel filtration effects, that is, according to molecular mass. The relative mobilities of proteins vary ~ log (molecular mass). The separated bands may be visualized in the gel by an appropriate technique (Abs, radioisotopes).

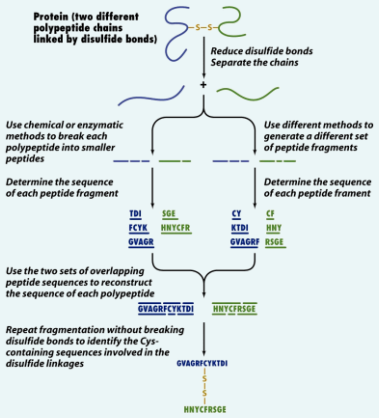
Separation techniques

CHROMATOGRAPHY



Liquid chromatography separates proteins on the basis of their rates of movement through a column packed with spherical beads. Binding and elution of the proteins often depend on the salt concentration and pH.

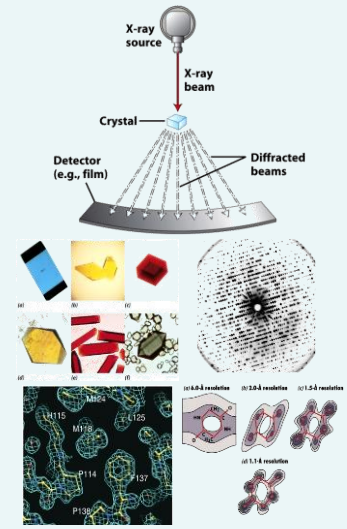
PROTEIN SEQUENCING



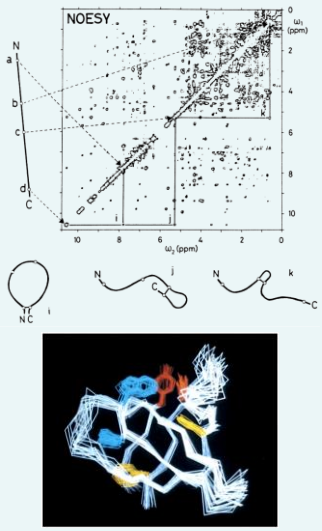
The protein must be broken down into fragments small enough to be individually sequenced, and the primary structure if the intact protein is then reconstructed from the sequences of overlapping fragments (e.g. mass spectroscopy).

Protein conformation is determined by sophisticated physical methods

X-RAY CRYSTALLOGRAPHY

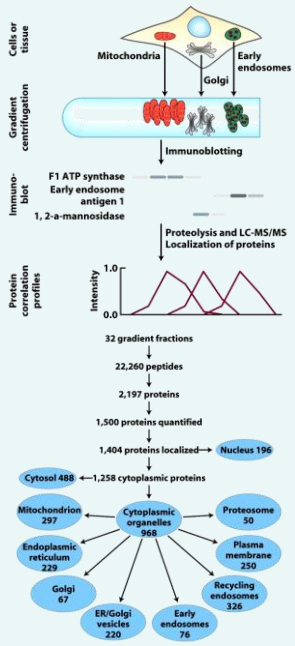


NMR SPECTROSCOPY



X-ray crystallography provides the most detailed structures but requires protein crystallization. Only relatively small proteins are amenable to **NMR analysis**. **Cryoelectron microscopy** is most useful for large protein complexes, which are difficult to crystallize.

PROTEOMICS



✓ Proteomics is the systematic study of the amounts (and changes in the amounts), modifications, interactions, localization, and functions of all or subsets of all proteins in biological systems at the whole-organism, tissue, cellular, and subcellular levels.

✓ Proteomics provides insights into the fundamental organization of proteins within cells and how this organization is influenced by the state of the cell (e.g. differentiation into distinct cell types; response to stress, disease, and drugs)

✓ A wide variety of techniques are used for proteomic analysis, including two-dimensional gel electrophoresis, density gradient centrifugation, and mass spectroscopy.

VISUALIZING CELL STRUCTURES AND LOCALIZING MOLECULES WITHIN CELLS

TP 1: LIGHT MICROSCOPY
TP 2: ATOMIC FORCE MICROSCOPY
TP 3: ELECTRON MICROSCOPY