Tissue culture and practical Biotechnology

Tissue culture and practical Biotechnology

Progress in **cell purification technology is critical to increase the availability of viable cells for therapeutic,** diagnostic, and research applications.

Technologies for cell purification in

- **science,**
- **medicine,**
- **industrial biotechnology and biomanufacturing**

Bacon K, Lavoie A, Rao BM, Daniele M, Menegatti S. Past, Present, and Future of Affinity-based Cell Separation Technologies. Acta Biomater. 2020 Aug;112:29-51. doi: 10.1016/j.actbio.2020.05.004.

Cell purification

Efficient cell separation is essential in a multitude of fields:

- personalized cell therapy,
- organ recellularization,
- diagnostics and disease monitoring
- drug discovery
- basic cell biology

Bacon K, Lavoie A, Rao BM, Daniele M, Menegatti S. Past, Present, and Future of Affinity-based Cell Separation Technologies. Acta Biomater. 2020 Aug;112:29-51. doi: 10.1016/j.actbio.2020.05.004.

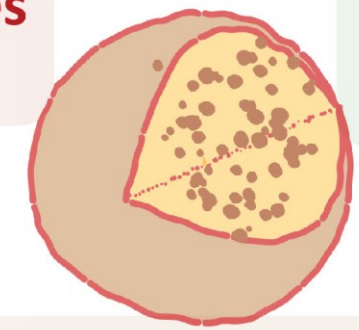
Cell targets and their diagnostic or therapeutic applications.

Examples of clinically relevant cell products

Targets

Erythrocytes
- Estimating erythrocyte aging
- Diagnosing anemia
- Diagnosing vascular diseases
- Diagnosing neurodegenerative diseases

Stem & Progenitor Cells
- Reconstruct decellularized organs
- Tissue and organ engineering
- Treatment of degenerative diseases
- Treatment of Parkinson's disease
- Heart repair after infarction



Lymphocytes
- Assessing immune activation
- HIV infection
- Autoimmune diseases
- Post-operative infections
- Transplant rejection
- Graft-versus-host disease

Mast Cells
- Studying immune and allergic response
- Studying hypersensitivity reactions
- Defense against infections
- Angiogenesis during pregnancy
- Wound healing

Cancerous & Infected Cells
- Cancer diagnostics
- Detection or monitoring of infections
- Reduce patient's viral load

Bacon K, Lavoie A, Rao BM, Daniele M, Menegatti S. Past, Present, and Future of Affinity-based Cell Separation Technologies. Acta Biomater. 2020 Aug;112:29-51. doi: 10.1016/j.actbio.2020.05.004.

Cell purification
Immunoaffinity – MACS – FACS - Microfluidics

Clinical and analytical procedures require highly purified cells

- cell purification method is crucial
- every method offers a different balance between **yield**, **purity**, and **bioactivity** of the cell product.

For most applications, ***the requisite purity is only achievable through affinity methods***, due to the high target specificity that they grant.

Bacon K, Lavoie A, Rao BM, Daniele M, Menegatti S. Past, Present, and Future of Affinity-based Cell Separation Technologies. Acta Biomater. 2020 Aug;112:29-51. doi: 10.1016/j.actbio.2020.05.004.

Cell purification
Immunoaffinity – MACS – FACS - Microfluidics

Cells for therapeutic applications

- **separation technologies** must meet analytical benchmarks and *regulatory compliance*
- **product quality** (cell viability and phenotype purity) is highly controlled to ensure product efficacy and patient safety.
- **presence of *adventitious* agents** is also rigorously monitored, and all processing steps must be compatible with sterility.

Bacon K, Lavoie A, Rao BM, Daniele M, Menegatti S. Past, Present, and Future of Affinity-based Cell Separation Technologies. Acta Biomater. 2020 Aug;112:29-51. doi: 10.1016/j.actbio.2020.05.004.

Tissue culture and practical Biotechnology

Cell therapy, tissue engineering, and gene therapy are called:

“regenerative medicine”

or

“advanced therapies”

or

“advanced therapy medicinal products” (ATMPs, European union legal term)

This represents the most recent branch of the biotechnology revolution in medicine.

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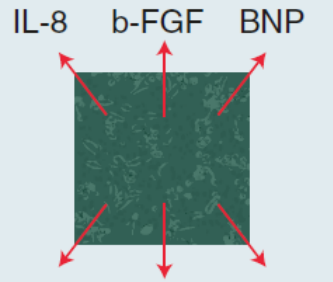
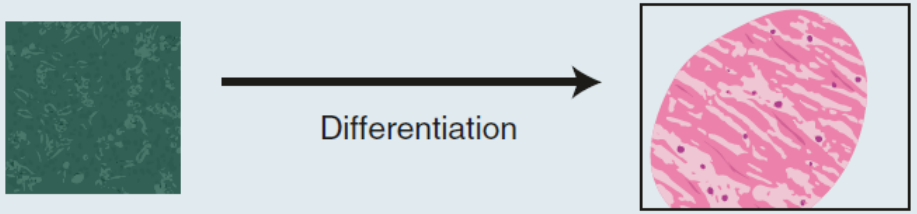
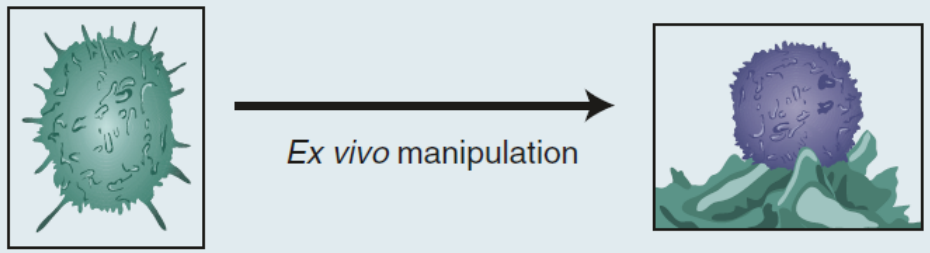
Tissue culture and practical Biotechnology

Pharmacological	 Proliferation Differentiation Survival Function MSCs; HSCs
Engineered tissue (with or without device) Regenerative	 Artificial tissue, e.g., pancreas
Immunological	 Cancer (cytotoxic T lymphocytes); CAR-Ts

Advanced Therapy Possible Mode of Action(s)

in-vivo mode of action of an advanced therapy depends on:

1. the type of cell/tissue;
2. the *ex-vivo* manipulations performed on the cells/tissue in the manufacturing facility (e.g., genetic modification);
3. the route of administration,
4. the *in-vivo* environment the cells/tissue occur.

They are typically subject to regulatory regimes:

- public health legislation
- pharmaceutical legislation

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Tissue culture and practical Biotechnology

Examples of "Stem Cell"

- The fundamental property of a stem cell
  capability to **multiply**
  self-renewal capacity
  ability to go through **numerous cycles of cell division** (through mitosis) while maintaining the **undifferentiated state** and giving rise to a variety of differentiated cells.
- Adult (or somatic)/non-Adult stem cells,
  Mesenchymal stem cells (MSCs) and **induced Pluripotent Stem Cells** (iPSCs) are currently the subjects of intense non-clinical and clinical investigation.
  - iPSCs are capable of unlimited *ex-vivo* (in-culture) growth.
  - MCSs and oligo- and unipotent stem cells cannot be grown in culture indefinitely,
    → i.e., they grow to senescence.

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Tissue culture and practical Biotechnology

Origin, characteristics, and uses of “stem” cells

Type of stem cell	Origin	Characteristic potential	Application
Adult (= somatic) stem cells	Exist in small number in many tissues, often in a well-defined and supportive niche	Multipotent: Give rise to cells of the relevant tissue or local environment	Neural stem cells & limbal stem cells in pre-clinical and clinical development
MSCs (a group of adult stem cells)	A collective term for cells from mesodermal lineage, sourced from stromal or connective tissue (e.g., bone marrow, adipose tissue, and umbilical cord tissue)	Multipotent: A heterogeneous pool of cells. They have a “stem cell-like” character and can differentiate into cells of connective tissues, e.g., chondrocytes, osteoblasts, and adipocytes, but they have also been reported to give rise to many other unrelated cell types	Pre-clinical development & clinical PI-III trials; commercial (Prochymal® and Alofisel®) Treatment-refractory Moderate-to-severe Crohn's Disease
Cord blood-derived MSCs (primitive stem cells, somewhere between ESCs and mature adult stem cells)	A specific source of MSCs. Extracted at birth from umbilical cord blood	Multipotent: Yet to be fully determined. Potentially they could be a source of many cell types for individual patients	Private cell banks are established for cryopreservation of cord blood samples; pre-clinical development and clinical phase I/II trials

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Tissue culture and practical Biotechnology

Origin, characteristics, and uses of “stem” cells

Type of stem cell	Origin	Characteristic potential	Application
ESC (no adult stem cells)	Result from *ex-vivo* culture of the inner cell mass of a blastocyst (embryoblast = 5–9 days old embryo)	Pluripotent	Vital source of differentiated cells for different research applications and clinical first in human (FIH) trials ongoing
iPSC (no adult stem cells	Derived by reprogramming of somatic cells (often skin fibroblasts) taken from an adult biopsy	Pluripotent, although methods for full reprogramming are still in development	From autologous source for disease modelling, drug screening including toxicity testing, and FIH trial; pre-clinical development and plans for human leukocyte antigens (HLA)-matched allogeneic iPSCs for FIH trial; research is ongoing with allogeneic iPSCs eliminating HLA-class I expression using genome editing technologies to generate universal cell lines

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Tissue culture and practical Biotechnology

Categorization of stem cells **on their potency**

Stem cell potency	Explanation and examples
Totipotent (or omnipotent) stem cell	Can differentiate into all embryonic and extraembryonic cell types (i.e., in humans they give rise to the foetus, umbilical cord, and the placenta: morula's cells (0–5 days old embryo)
Pluripotent stem cell	Can **differentiate into all three germ cell types** (endoderm, mesoderm, or ectoderm lineage) but not the placenta and umbilical cord, and subsequently into all embryonic cell types: ESCs, iPSCs
Multipotent stem cell	Can **differentiate into closely related cells**, such as all cells in a particular organ: MSCs, other adult (=somatic) stem cells

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Tissue culture and practical Biotechnology

Categorization of stem cells **on their potency**

Stem cell potency (segue)	Explanation and examples
Oligopotent stem cell	Can **differentiate into a restricted closely related group**, such as a hematopoietic progenitor cell that can produce a subset of blood cell types, such as B and T cells; vascular stem cell that has the capacity to become both endothelial or smooth muscle cells
Unipotent stem cells (or precursor cell)	Have the **property of self-renewal but can only give rise to cells of their own lineage**, such as muscle or skin stem cells. This distinguishes these cells from real stem cells as they do not differentiate into other cell phenotypes

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Tissue culture and practical Biotechnology

Somatic (adult) Cell Technologies

→ Adult stem cells are present in **many tissues**

→ In tissues, they exist in a defined, **organized** environment of supporting cells that define the architecture of the "**stem cell niche**"

→ Adult stem cells are **rare** and they **cannot always be isolated** and grown in culture.

→ Even when they can be grown **in culture, usually they grow to senescence**.

The Hallmark of adult stem cells:

- ability to "self-renew" both *in-vivo* and *ex-vivo*
- undergo asymmetric cell division (give rise to two different cells, one an identical stem cell and the other a partly differentiated progenitor cell)

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Tissue culture and practical Biotechnology

Adult Stem Cells Used as Transplant Product

- Adult stem cells (used since the 1950s) to **treat cancers of blood cells**
  → bone marrow transplants
- HSCs (Hematopoietic stem cells)
- Procedure:
  - whole **body irradiation to kill malignant cells** in multiple myelomas and leukaemia.
  - the patient receives **a bone marrow transplant** containing a few HSCs
  - HSCs home to the **bone marrow stem cell niches** and begin to replenish the blood

- **Rejection and graft-versus-host disease (GvHD) are still threatening complications** of this therapy, but its practice can now be considered routine.
  These products are not medicinal products (transplant products, with a different legislative regime worldwide)

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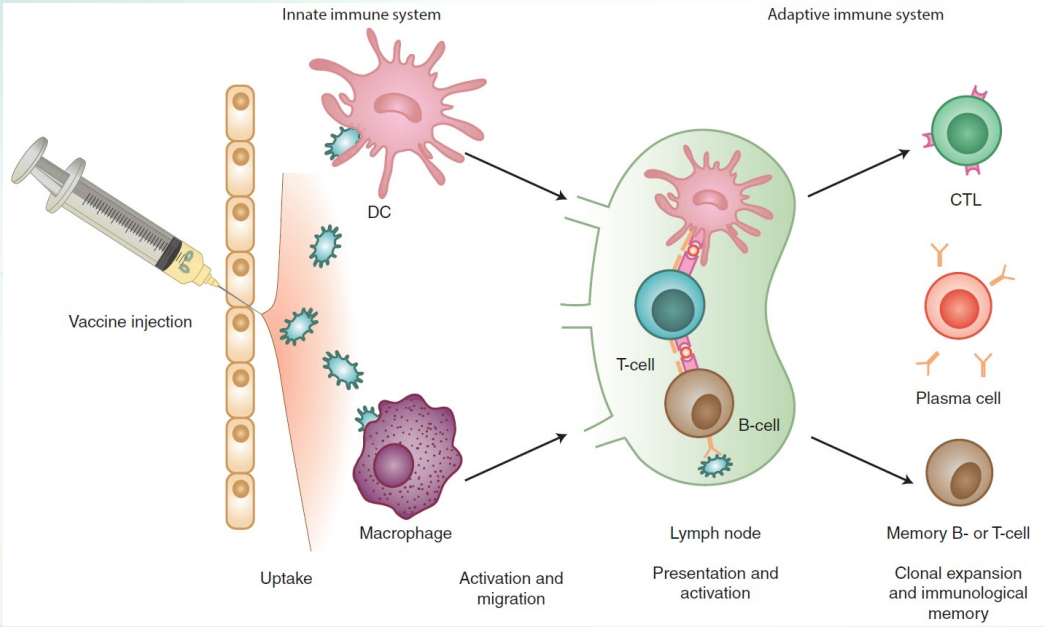
Tissue culture and practical Biotechnology

Adult Stem Cells for Clinical Application: Immune Cells 1/2

Immune cell types currently investigated for their therapeutic value (autologous and allogeneic cells are used as cell sources):

- **DCs (dendritic cells)** and macrophages (Important constituents of the **innate immune system** are APCs like macrophages and dendritic cells (DCs), which reside in tissues)

- **tumor infiltrating lymphocytes (TILs)** (A cellular immunotherapy **to treat cancer relies on interaction of the cellular product** with the patient's immune system for its effect. The in-vivo immunological effect will very likely be different between species.)



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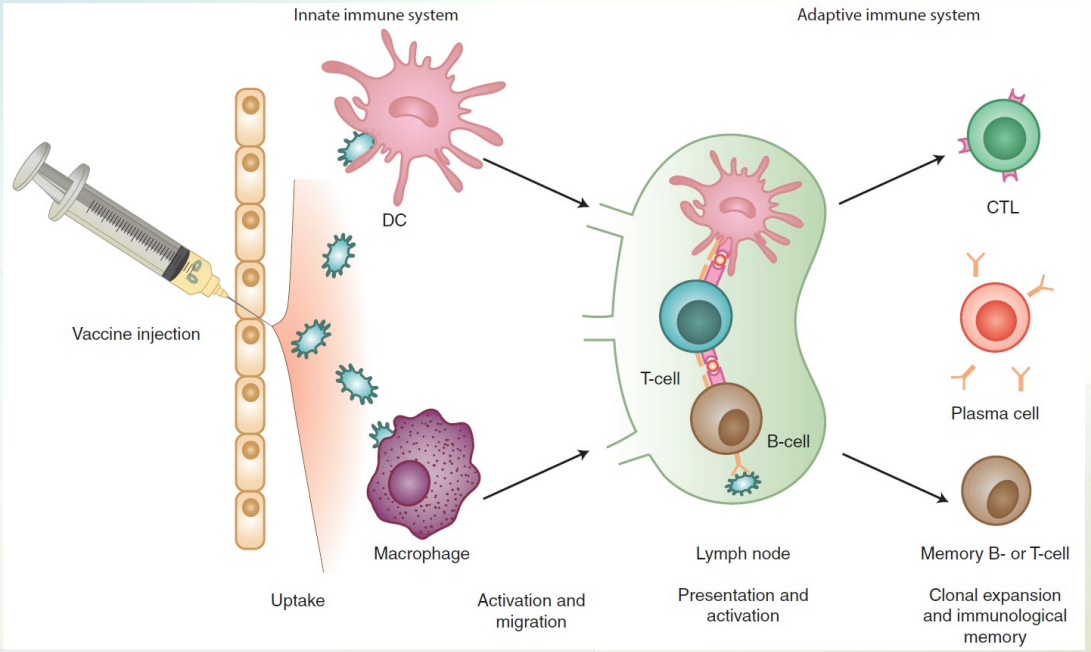
→ Cells do not undergo ADME [Absorption, Distribution, Metabolism, and Excretion) as conventional medicinal products)

Tissue culture and practical Biotechnology

Adult Stem Cells for Clinical Application: Immune Cells 2/2

- **regulatory T cells** (Tregs are subsets of CD4+ T-cells that play an important role in **limiting inflammation through the secretion of anti-inflammatory cytokines, such as IL-10 and TGF-β**.
- → Induction of Tregs may be of interest for vaccines that aim to reduce inflammation in autoimmune diseases).

- **Gamma-delta (γδ) T cells** (a subset of T cells that promote the inflammatory responses of lymphoid and myeloid lineages and are especially vital to the initial inflammatory and immune responses).
  NB: ***the exact mechanisms responsible for γδ T cell proinflammator functions remain poorly understood***



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- **- Viral reconstitution T cells** (protocols to develop **virus-specific T cells (VSTs) were based on *ex vivo* generation and *in vitro* expansion of T cells, leading to a final product comprising polyclonal T cells** (recognizing different immunogenic viral antigens)

A rapid way to obtain VSTs from the donor is the direct selection of specific T cells using **viral peptide multimers conjugated to magnetic beads**, to select highly pure cytotoxic T cells,

(Cells do not undergo ADME as conventional medicinal products)

Tissue culture and practical Biotechnology

Adult Stem Cells for Clinical Application: MSCs

MSCs can be isolated from bone marrow, adipose tissue, and umbilical cord tissue

- several private companies offer personal **cell banking service**s and public cord blood banks supply pooled cord blood samples for clinical use
- Trials indications: **bone/cartilage repair**, heart, lung, liver, gastrointestinal, neurological diseases, and rheumatology, Crohn's, and other autoimmune diseases, GvHD after organ transplantation and kidney Diseases

MSCs products have been **approved** globally:

treatment of Crohn's fistulas → **Alofisel™** in the EU and **Prochymal™** in Canada and New Zealand for the

treatment of pediatric acute GvHD → Temcell HS™ in Japan

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Tissue culture and practical Biotechnology

Adult Stem Cells for Clinical Application: MSCs

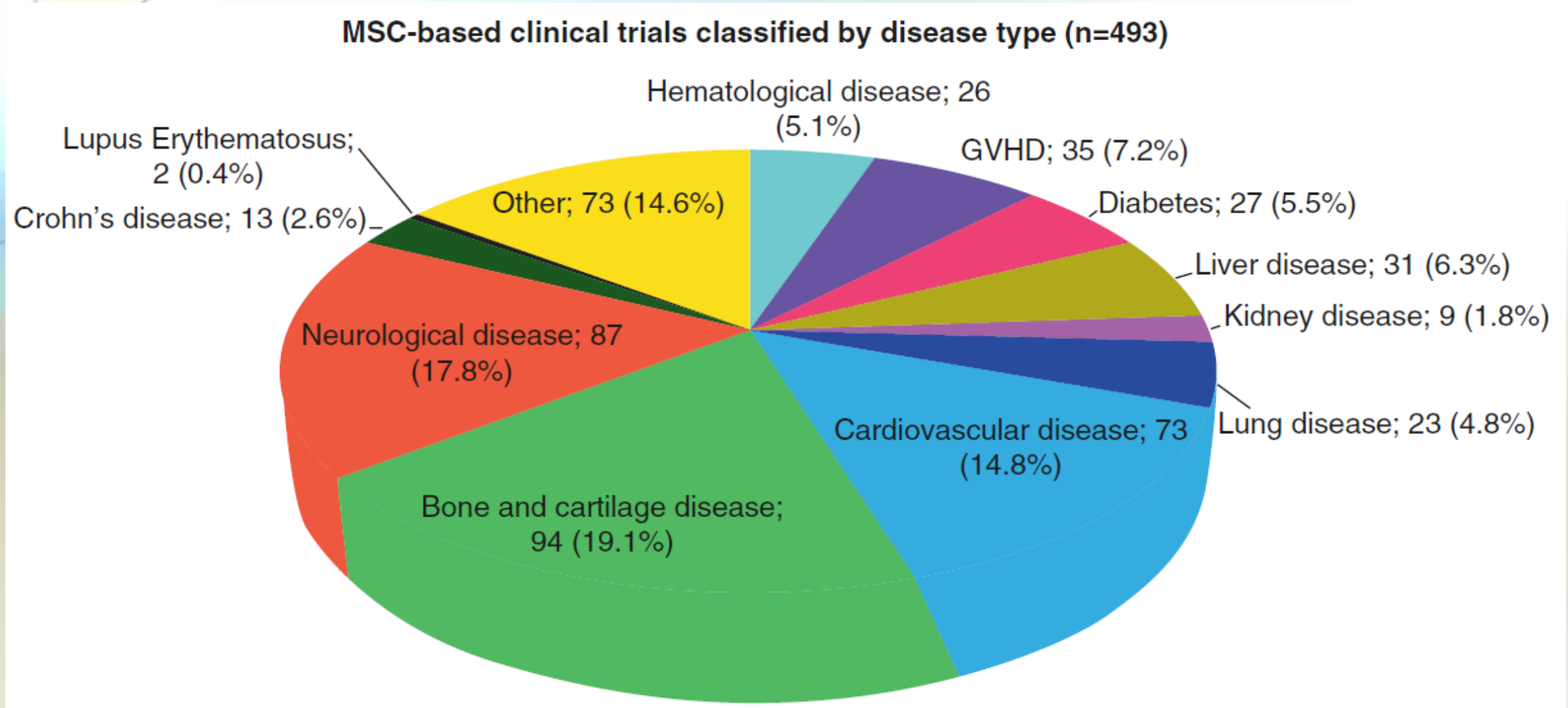

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Table 17.17 Development stage manufacturing and testing challenges for different advanced therapy technologies,

Technologies	Development stage of the field	Current manufacturing technologies	Manufacturing and testing challenges
(a) Somatic cell technologies	Many products in early clinical development phase; few products approved, e.g., Alofisel	Manual process with open handling steps; automated multi-planar flasks and stack systems; micro-carriers in disposable stirred tank systems; hollow fiber growth systems; membrane and contraflow centrifugation systems	Scale-up and control of large scale batches. Recovery of cells from micro-carriers. DSP: Large volume handling, primary container filling at scale using enclosed technologies. Relevant potency assays lacking
(b) Cell immortalization technologies	One product in early clinical development	CompacT SelecT[a] fully automated and programmable scalable cell culture platform consisting of a robot arm that can access T175 flask or multi-well plate incubator. Standard cell culture activities, such as passage or media change, are conducted and controlled with no manual intervention	Similar to protein manufacturing platform technologies

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Table 17.17 Development stage manufacturing and testing challenges for different advanced therapy technologies,

Technologies	Development stage of the field	Current manufacturing technologies	Manufacturing and testing challenges
(c) *Ex-vivo* gene modification of cells using viral vector technologies	Mainly small trials in early and late clinical development phase (gene modified autologous T-cells and HSCs); few products approved, e.g., Strimvelis and Kymriah	Manual processes often not fully enclosed using static bags, gas-permeable pots + lateral movement bioreactors (wave bags) for higher cell yield. Positive or negative cell selection process steps often used. High cell purity becoming possible with sterile cell sorter	Adapting systems to deal with variation in quality and amount of incoming starting material. Lack of product stability pressuring manufacturing and distribution model. Lack of fast QC assays. Low transduction efficiency with non-replicating viral vectors. Enclosed and automated manufacturing systems are becoming available for the entire process (e.g., prodigy)

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Table 17.17 Development stage manufacturing and testing challenges for different advanced therapy technologies,

Technologies	Development stage of the field	Current manufacturing technologies	Manufacturing and testing challenges
(d) Cell plasticity technologies	Mainly pre-clinical phase with few ESC and iPSC-derived FIH trials	Current processes are extremely 'manual' and rely on small scale cell culture and harvest technologies. High risk processes with extensive process and product characterization testing to assess product quality, safety, and efficacy	A two-tier banking strategy (MCB/WCB) scale-up process of pluripotent cells prior to differentiation steps needed. Dynamic cell culture systems to expand PSC numbers. Robotic scale-out of current plate-based iPSC technology is also being explored
(e) 3D-technologies	Mainly pre-clinical phase with few FIH trials	A complex manufacturing interplay between (bio)materials, scaffolds, cells, and biological coatings. Incorporates decellularization/recellularization tissue-based products such as trachea, esophagus, and veins	Enclosed bioreactors to control cell and material interface. Improved stability and delivery systems. Robust product quality to ensure large clinical application

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Cell - practical Biotechnology
Ex-vivo Gene Modification of Cells Using Viral Vector Technologies

Genetic modifications using viral vector technology

- several cell types:
  - **T cells, HSCs, and MSCs**.
- **vector systems** for transfer of genetic information into the cells:
  - adeno associated virus (AAV),
  - herpes virus (HPV),
  - adenovirus (Ad),
  - lentivirus (LV),
  - gamma-retrovirus (γ-RV).

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Table 17.4 EU-ATMP classification definitions according to the EU pharmaceutical legislation

"advanced therapy medicinal products"

ATMP classification	Definition	Examples
Gene therapy medical product (GTMP)	A GTMP is a biological medicinal product (*excluding vaccines*) that: (a) Contains an active substance which contains or consists of a recombinant nucleic acid used in or administered to human beings with a view to regulating, repairing, replacing, adding or deleting a genetic sequence and; (b) Its therapeutic, prophylactic or diagnostic effect relates *directly* to the recombinant nucleic acid sequence it contains, or to the product of genetic expression of this sequence Gene therapy medicinal products shall not include *vaccines against infectious diseases* (see Chap. 14), which have their own set of vaccine specific guidances	Glybera® (see Chap. 16); Kymriah® (autologous CD19+ CAR-T cells)[a]; Strimvelis® (genetically modified autologous CD34+ cells)

aCD19+ (CAR-T cells) = cluster of differentiation (CD) 19 'chimeric antigen receptor T cells', CAR-T cells
bHassan et al. (2013)

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Table 17.4 EU-ATMP classification definitions according to the EU pharmaceutical legislation

"advanced therapy medicinal products"

ATMP classification	Definition	Examples
Somatic cell therapy medicinal product (SCTMP)	A SCTMP is a biological medicinal product which fulfils the following two characteristics: (a) Contains or consists of cells or tissues that have been subject to substantial manipulation so that biological characteristics, physiological functions or structural properties relevant for the intended clinical use have been altered or of cells or tissues that are not intended to be used for the same essential function(s) in the recipient and the donor (b) Is presented as having properties for or is used in or administered to human beings with a view to treating, preventing or diagnosing a disease through the pharmacological, immunological or metabolic action of its cells or tissues	Alofisel® (allogeneic MSCs); irradiated plasmacytoid dendritic cell line (allogeneic) loaded with peptides from tumor antigens

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Table 17.4 EU-ATMP classification definitions according to the EU pharmaceutical legislation

"advanced therapy medicinal products"

ATMP classification	Definition	Examples
Tissue engineered product (TEP)	A TEP is a biological medicinal product that meets the following two characteristics: (a) Contains or consists of engineered cells or tissues, and (b) Is presented as having properties for, or is used in or administered to human beings with a view to regenerating, repairing or replacing a human tissue A TEP may contain cells or tissues of human or animal origin, or both. The cells or tissues may be viable or non-viable. It may also contain additional substances, such as cellular products, bio-molecules, biomaterials, chemical substances, scaffolds or matrices. Products containing or consisting exclusively of non-viable human or animal cells and/or tissues, which do not contain any viable cells or tissues and which do not act principally by pharmacological, immunological or metabolic action, are excluded from this definition. Cells or tissues shall be considered "engineered" if they fulfill at least one of the following conditions: (a) The cells or tissues have been subject to substantial manipulation, so that biological characteristics, physiological functions or structural properties relevant for the intended regeneration, repair or replacement are achieved (b) The cells or tissues are not intended to be used for the same essential function or functions in the recipient as in the donor	Spherox® (autologous chondrocytes); Holoclar® (autologous corneal epithelial cells, which contain stem cells)

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Table 17.4 EU-ATMP classification definitions according to the EU pharmaceutical legislation

"advanced therapy medicinal products"

ATMP classification	Definition	Examples
Combined ATMP	A combined ATMP fulfills the following conditions: (a) It must incorporate, as an integral part of the product, one or more medical devices or one or more active implantable devices, and (b) Its cellular or tissue part must contain viable cells or tissues, or (c) Its cellular or tissue part containing non-viable cells or tissues must be liable to act upon the human body with action that can be considered primary to that of the devices referred to	Allogenic adipose derived regenerative cells encapsulated in hyaluronic acid (TEP + device)[b]; encapsulated allogeneic cells secreting GM-CSF[c] + irradiated autologous tumor cells (GTMP + device)

cGM-CSF = Granulocyte-macrophage colony-stimulating factor

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Examples
(Ex-vivo Gene Modification of Cells Using Viral Vector Technologies)

ex-vivo gene therapy for Children with ADA-SCID
(*Strimvelis* against ADA-SCID, defects in the housekeeping enzyme adenosine deaminase (ADA), which causes metabolites of adenosine to accumulate to toxic levels.)

- **CD34+ cells** (i.e., HSCs that can make lymphocytes) extracted from bone marrow cells
- correct **copy of the gene for ADA** is inserted into CD34+ cells using a γ-RV vector

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Examples
(Ex-vivo Gene Modification of Cells Using Viral Vector Technologies)

ex-vivo gene therapy for Children with ADA-SCID
(*Strimvelis* against ADA-SCID, defects in the housekeeping enzyme adenosine deaminase (ADA), which causes metabolites of adenosine to accumulate to toxic levels.)

- back to the patient **via intravenous infusion**, *Strimvelis* is transported in the blood circulation to the bone marrow
- **genetically modified CD34+** cells start to grow and **produce healthy B- and T-lymphocytes that can produce ADA**

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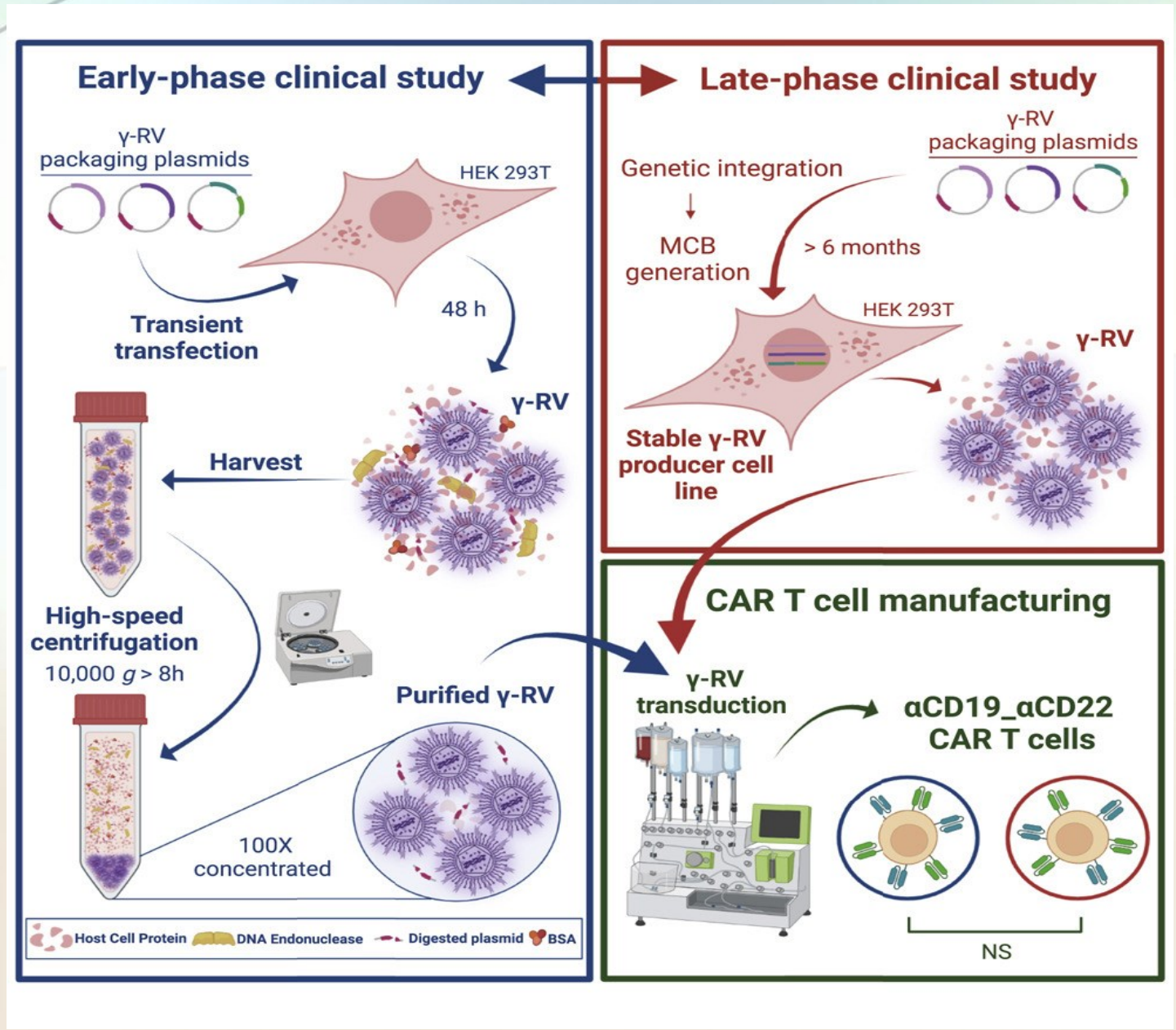

Molecular Therapy - Methods & Clinical Development 2023 28116-128DOI: (10.1016/j.omtm.2022.12.006)

Examples
(Ex-vivo Gene Modification of Cells Using Viral Vector Technologies)

T-cells Ex-vivo Genetically Modified

- Immune surveillance, any molecules identified as non-self are eliminated. (virally infected cells, but also transformed (tumor) cells)
- T-cells play a key role in **cell-mediated immunity**
  genetic modification of the T cells
  - altering the specificity of the T-cell receptor (TCR)
  - introducing **antibody-like recognition in CARs (chimeric antigen receptors)**

Clinical success of response rates to **CAR-T19 cells**:

- B cell acute lymphoblastic leukaemia (B-ALL) --> approved by the FDA (Kymriah®).
- diffuse large B-cell lymphoma (DLBCL) --> Yescarta®

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Tissue culture and practical Biotechnology

Cell purification technology is critical to increasing the availability of viable cells for different applications.

for therapeutic**,** diagnostic, and research applications.

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Cell purification
Immunoaffinity – MACS – FACS - Microfluidics

A variety of techniques are now available for cell separation:

- **Traditional non-affinity and affinity-based purification techniques**, focusing on established ligands and chromatographic formats.

- **Affinity-based pseudo-chromatographic and non-chromatographic technologies**, especially focusing on magnetic-activated cell sorting (MACS) and fluorescence-activated cell sorting (FACS).

- **Emerging trends**, (progress in chemical, material) and **microfluidic sciences** opened new exciting avenues towards high-throughput and high-purity cell isolation processes.

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Cell purification

Different techniques have emerged and classified by:

(i) **physical characteristic** (i.e., cell volume and shape, density, and light scatter properties or fluorescence),

(ii) **surface properties** (i.e., electrical charges, hydrophobicity, etc.) and cell constituents (i.e., such nucleic acids, enzymes and other proteins)

(iii) **adherence/affinity** features

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Cell purification techniques

Techniques are classified into separations by:

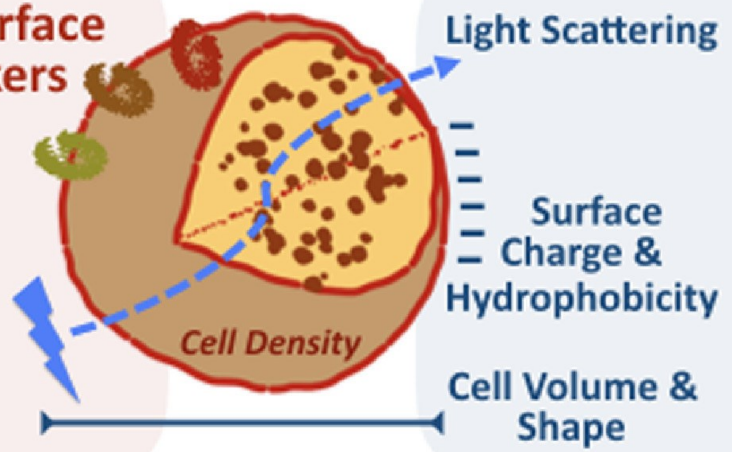
(i) **physical characteristic** (i.e., cell volume and shape, density, and light scatter properties or fluorescence),

(ii) **surface properties** (i.e., electrical charges, hydrophobicity, etc.) and cell constituents (i.e., such nucleic acids, enzymes and other proteins)

(iii) **adherence/affinity** features

Affinity techniques:

- Cell Affinity Chromatography
- (Packed / fluidized beds,
- Cryogels and Membranes)
- Affinity 2-phase Partition
- MACS & FACS



Physical techniques:

- Density gradient centrifugation
- Dielectrophoresis
- Filtration
- Field-flow-fractionation
- Elutriation centrifugation

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Cell purification

A variety of techniques are now available for cell separation:

- **non-affinity methods** such as density gradient, centrifugation, dielectrophoresis, and filtration

- **affinity methods** such as chromatography, two-phase partitioning, and magnetic-/fluorescence-assisted cell sorting.

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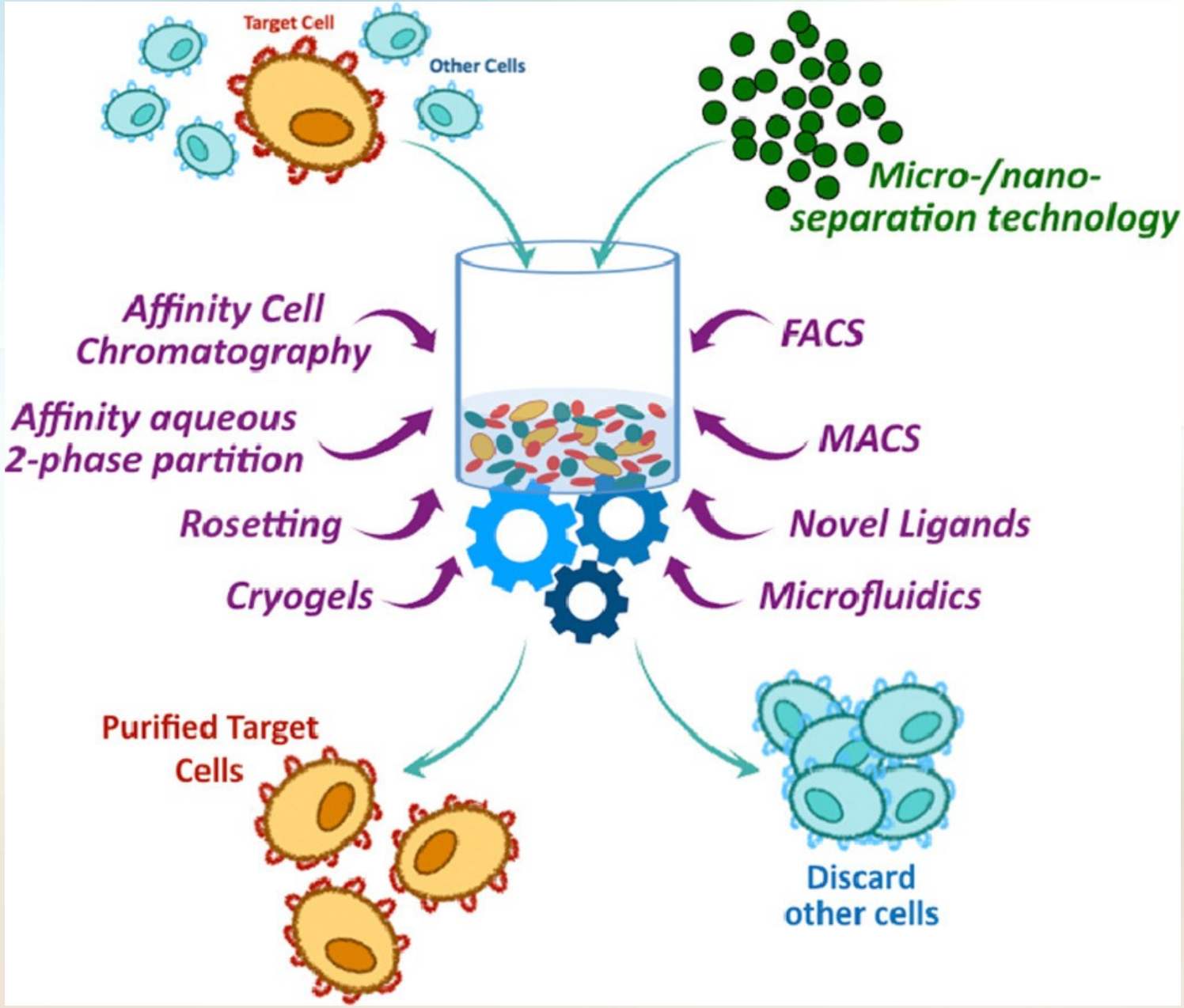
Cell purification Immunoaffinity – MACS – FACS - Microfluidics



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Comparison of physical (non-affinity) and affinity-based cell separation

Non-affinity methods

- density gradient centrifugation
- dielectrophoresis
- field-flow fractionation
- filtration
- elutriation centrifugation

Affinity-based cell separation

- Rosetting
- Chromatography
- Fluidized Beds
- Cryogels

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Physical non-Affinity Methods

Method	Mechanisms	Target Cells
Density gradient centrifugation	Cells migrate through a vertical density gradient (aqueous solutions of biopolymers) during centrifugation and collect in the region where the local density corresponds to their own.	Human mesenchymal stem cells, hematopoietic stem and progenitor cells, blood cells (erythrocyte, platelets, granulocytes, lymphocytes, monocytes), circulating tumor cells, sperm cells, and neurons. Rat pancreatic islets.
Dielectrophoresis	Cells placed in a gradient electric field act as induced dipoles and migrate at different rates based on their size and dielectric properties as well as the dielectric properties of the medium.	Hematopoietic stem and progenitor cells, leukocytes (B and T-lymphocytes, monocytes, and granulocytes), circulating tumor cells, astrocyte and neuron-biased cells, and neural stem and progenitor cells. Isolation of pathogenic bacteria from blood. Fractionation of viable *vs.* non-viable cells (yeast and mammalian cells).
Field flow fractionation	A cell suspension is flown through a channel where a field (*e.g.*, crossflow, sedimentation, and electrical) is applied perpendicular to the direction of flow enabling separation based on mobility differences.	Pathogenic bacteria and yeast cell subpopulations. Human blood cells (erythrocyte, platelets, leukocytes), cancer cells, neurons from cerebral cortices, embryonic stem cells, mesenchymal stem cells, hematopoietic stem cells, electroporated *vs.* non-electroporated cells, and cells undergoing apoptosis.
Filtration	Cells are captured non-specifically on the surface of a material with controlled porosity based on physical properties such as cell diameter (volume) and aspect ratio. Generally used as preparative tool for further purification.	Blood cells (leukocytes, erythrocytes), hematopoietic stem and progenitor cells, adipose-derived stem cells, mesenchymal stem cells, and circulating tumor cells. Bacterial and mammalian (e.g., Chinese Hamster Ovary (CHO) cells) cells for metabolomics preparation.

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Physical non-Affinity Methods

Method	Advantage	Disadvantage
Density gradient centrifugation	Label-free technology, which enables processing large volumes in short process times and concentrating the cell product; high viability of the cell product; reproducible results; facile scale up; commercially available equipment.	Requires knowledge of the density of the different cells in the mixture; low recovery and poor separation of target from non-target cells (low purity); labor intensive.
Dielectrophoresis	Label-free, continuous technology that enables sorting cells based on viability without dilution, thereby reducing sample volumes; short processing time and high sensitivity; can be integrated with microfluidic devices.	Inadequate for highly complex mixtures where differences in dielectric properties may not be sufficient to enable efficient separation of target cells from unwanted cell types; cell viability can also be significantly decreased after exposure to electric field or overheating, which complicates fabrication and can induce cell differentiation.
Field flow fractionation	Label-free, continuous technology that grants high viability and bioactivity of the cell product under short process time; reduced sample volumes; high reproducibility.	Requires dilution of the cell sample prior to separation to avoid cell aggregation and provides limited yield of target cells; unclear criteria for the decision of the field to apply for separation; limited availability of commercial equipment.
Filtration	High-throughput, simple, scalable technology, which can be integrated into a microfluid device platform.	Low-to-medium yield; potential for the filter to clog; likelihood of cell damage caused by the high flow rates and pressures.

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Physical non-Affinity Methods

Method	Mechanisms	Target Cells
Elutriation centrifugation	Cells are separated based on their sedimentation velocity.	Blood cells (granulocytes, lymphocytes, monocytes, platelets), macrophages, Kupffer cells from liver, mast cells, hepatocytes, sperm cells (rats and human), separation into age-related fractions (yeast, erythrocytes), prostate and ovarian cancer cells from tumors, and hematopoietic stem and progenitor cells.

Affinity Based Methods

Method		Mechanisms	Target Cells
Rosetting		Antigen-specific cells are incubated with antigen-coated erythrocytes, with which they aggregate into "rosettes" that can be separated by gradient centrifugation.	Human B and T lymphocytes, and mesenchymal stem cells (negative selection).
Chromatography-based (cell affinity chromatography, CAC)	**Packed beds**	The cell suspension is flown through a packed bed of ligand-functionalized porous particles. Target cells are captured on the surface of the pores, washed, and finally eluted.	Human T lymphocytes ($CD3^+$, $CD4^+$, and $CD8^+$), B lymphocytes ($CD19^+$ cells), monocytes and granulocytes ($CD14^+$ cells), and umbilical vein endothelial cells ($CD31^+$).
	Fluidized beds	A cell suspension is fed upward to agitate/suspend ligand-functionalized porous particles. Target cells are captured on the surface of the beads, washed, and finally eluted.	Yeast cells. Human monocytes and erythrocytes.
	Cryogels	A cell suspension is flown through a continuous, macroporous matrix prepared by gelation-polymerization at sub-freezing temperatures. Target cells can be captured by affinity ligands conjugated to the matrix, and eluted either via traditional methods (buffer with controlled pH) or via thermal/mechanical compression of the matrix.	Bacterial cells and yeast. Human B lymphocytes, hematopoietic stem and progenitor cells, and human acute myeloid leukemia KG-1 cells ($CD34^+$).

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Physical non-Affinity Methods

Method	Advantage	Disadvantage
Elutriation centrifugation	Rapid processing of large volumes of cells featuring a wide range of sizes; applicability at low temperatures to impede cell activation; high recovery and viability of the cell product.	Highly labor intensive (mitigated by impellor); difficult separation of cells featuring similar sedimentation properties; high cost of specialized rotors.

Affinity Based Methods

Method		Advantage	Disadvantage
Rosetting		Commercial products available; high cell bioactivity and purity.	Expensive, complex, slow procedure that requires multiple reagents; when performing positive selection, lysis of the erythrocytes is required to isolate the target cells.
Chromatography-based (cell affinity chromatography, CAC)	Packed beds	Large availability of commercial affinity-based adsorbents; simple operation.	Diffusion limitations: cells are too large to enter pores of beads; difficult elution of captured cells in viable form; shear stress on cells.
	Fluidized beds	Improved mass transfer; large inter-particle volume; and high surface area.	Shear stress on cells; need for large columns; long equilibration times; non-specific capture by the adsorbent matrix; narrow range of appropriate flow velocities; difficult elution; fouling; diffusion limitations.
	Cryogels	Mechanical softness of the substrate, reduced shear stress, and elution by stimuli responsive matrix compression enable high viability of mammalian cell products; pore size appropriate for cells of varying size (1-15 µM); extended life cycle; reduced diffusion limitations and fouling due to convective flow; high storage stability.	Modest cell purity and recovery; the affinity ligands remain bound to cell when eluting via matrix compression.

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Affinity Based Methods

Method		Mechanisms	Terget Cells
Pseudo-chromatographic	**Gel separation**	A cell suspension is flown through a macroporous hydrogel, where target cells are captured by affinity, washed, and later released by dissolving the gel via thermal or enzymatic degradation.	Human spleen cells, and B and T lymphocytes.
	Fibers	A cell suspension is flown through an array of hollow fibers, where target cells are captured by affinity binding, washed, and later released by mechanical, chemical (e.g., controlled pH or reducing agents), or enzymatic treatments.	Human T lymphocytes ($CD4^+$), hematopoietic stem and progenitor cells ($CD34^+$), spleen cells, erythrocyte subpopulations, and cancer cells.
	Membranes	A cell suspension is adsorbed onto a fibermat/membrane, where target cells are captured by affinity binding, washed, and later released via non-specific or competitive elution.	Mouse $CD80^+$ transfected cells. Human **acute myeloid** leukemia cells ($CD34^+$), B lymphocytes, T **lymphocytes** ($CD4^+$), MCF-7 cells ($EpCAM^+$), and PC3 **prostate cancer** cells ($EpCAM^+$).

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Affinity Based Methods

Method		Advantage	Disadvantage
Pseudo-chromatographic	Gel separation	High yield and bioactivity of the target cells owing to gentle elution conditions.	The affinity tag remains bound to the cell; the adsorbent is non-reusable.
	Fibers	The flow rate through the hollow fiber can be used to tune cell binding strength and elution; no diffusional limitations; the matrix is affordable and available at large scale; can be regenerated; possible integration in microfluidic platforms.	Low binding capacity; considerable cell disruption by shear; non-specific binding to fiber.
	Membranes	High throughput (owing to high flow rates and no diffusion limitations); cell elution via flow-/bubble-induced cell detachment; control of flux and fluid velocity enable optimization of adsorption, elution, and fouling; possible thermo-responsive elution; possible integration into microfluidic platforms.	Modest binding capacity due to low specific surface area; high shear stress on cells; modest purity due to non-specific interactions of the cells with membrane surface; current methods of thermo-responsive elution require additional purification to remove the ligand.

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Affinity Based Methods

Method		Mechanisms	Target Cells
Non-chromatographic	**ATPS**	Cells undergo differential migration across the interface between two immiscible polymer solutions based on the properties of the layers and polymer-bound ligands.	Hybridoma cells. Human acute myeloid leukemia cells ($CD34^+$), hematopoietic stem and progenitor cells ($CD34^+$), and $CD133^+$ stem cells.
	MACS	Target cells are captured on the outer surface of magnetic nanoparticles, which are separated from the feedstock using a magnet. Following washing, particle-bound cells are eluted via chemical, enzymatic or competitive mechanism.	Pathogenic bacteria. Human B type acute lymphoblastic leukemia cells ($CD19^+$,$CD45^+$), monoblastic leukemia cells U937 ($CD34^+$), B cell lymphocytes ($CD19^+$), T cell lymphocytes ($CD4^+$, $CD8^+$), circulating tumor cells (EpCAM+), hematopoietic stem and progenitor cells (CD34+), mesenchymal stem cells ($CD90^+$), and megakaryocytic cells ($CD61^+$).
	FACS	Cells tagged with fluorescent ligands are encapsulated into single-cell droplets, which are flown through a detection zone and charged based on their fluorescence. The charged droplets (target cells) fall through electrostatic deflecting plates and are sorted based on their charge.	Neural stem cell subpopulations (CD56, CD133, FORSE-1, SSEA-1, TRA-1-80, etc), undifferentiated embryonic stem cells (SSEA-4+, TRA-1-60+), hematopoietic stem cells ($CD34^+$, $CD59^+$, $Thy1^+$,$CD38^{low/-}$,$c\text{-}Kit^{-/low}$, lin^-), T cell lymphocytes ($CD4^+$, $CD8^+$), antigen-specific B lymphocytes, circulating tumor cells ($EpCAM^+$, $p75NTR^+$), and skeletal muscle stem cells ($VCAM^+CD31^+CD45^+Sca1^+$) .
	Microfluidics	Capture of cells through a combination of affinity-based and field flow-based techniques framed within microfluidic devices.	Mouse adipose- derived stem cells (negative depletion). Human circulating tumor cells (positive or negative enrichment), endothelial progenitor cells ($CD34^+$, $VEGFR\text{-}2^+$, $CD31^+$, $CD146^+$), neutrophils (CD64+, $CD66b^+$), hematopoietic stem and progenitor cells (CD34+), and T cell lymphocytes ($CD4^+$, $CD8^+$).

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Affinity Based Methods

Method		Advantage	Disadvantage
Non-chromatographic	ATPS	Provides high viability of the cell product by minimizing mechanical shear; potential incorporation of stimuli-responsiveness into polymers to promote gentle elution, biocompatibility, short process time, and recycling of the affinity ligands.	Modest recovery and purity; need of separating the target cells from phase-forming polymer after extraction; may require repetitive extractions to achieve sufficient selectivity; difficult to predict how cells will partition based on their physical properties; the interface may be instable.
	MACS	High recovery and purity of target cells; rapid; commercially available immunomagnetic beads; facile automation and integration with other separation techniques.	Harsh elution conditions; low binding capacity; sorting limited to a single surface marker; cannot sort based on expression density; non-specific binding to magnetic bead surface; can trigger cell differentiation.
	FACS	Highly pure cell product; enables cell counting; rapid separation; allows single cell analysis; can sort using multiple surface markers; can sort cells into high-/low-expression populations.	Low throughput; affinity labels remain adsorbed on the surface of the cells; requires low cell concentration; cannot ensure cell sterility; expensive equipment that requires trained operators and long process time.
	Microfluidics	Highly precise and sensitive cell separation resulting in high purity of the cell product; can perform multiple functions and/or combine multiple separation methods in one device; efficient phenotype isolation especially for rare cell types; analysis of small sample volumes and single-cell manipulation; facile automation and realization of point-of-care testing.	Low throughput; potentially expensive fabrication; limited lifespan due to clogging; may require complex sample preparations; high user-to-user variation.

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Cell purification-Summary
Immunoaffinity – MACS – FACS - Microfluidics

- **Affinity-based separations have emerged as the main technology for cell isolation**,
  → for **high yield** and **purity**, **scalability** and **sterile**
- Affinity-based methods for cell purification include
  - traditional chromatographic techniques
  - non-chromatographic or pseudo-chromatographic systems.
- Employing biorecognition agents for capture,
  → traditional protein ligands to synthetic binders

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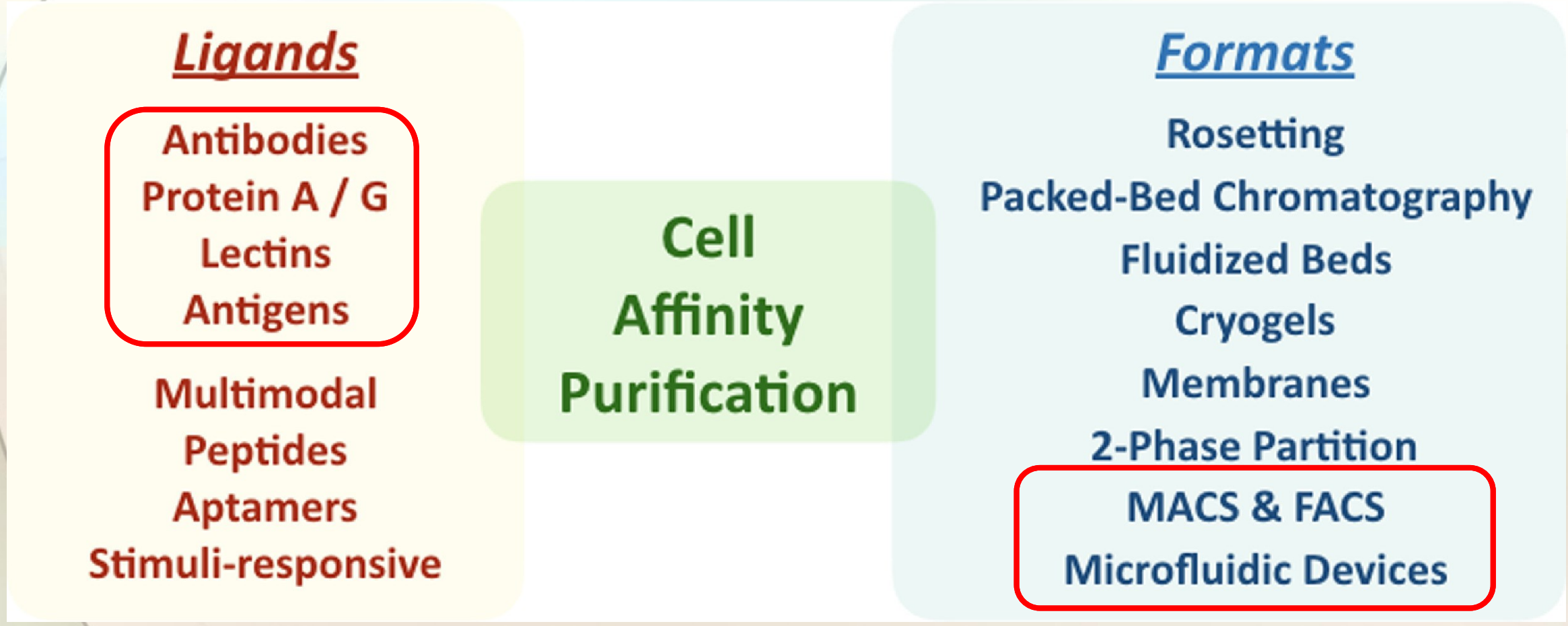
Cell purification
Immunoaffinity – MACS – FACS - Microfluidics



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Affinity-based cell separation

Cell affinity chromatography

- **Difficulty** in identifying **unique biomarkers**
- Cell surface **receptors** may **vary among donors** and **different tissues** isolated from an individual donor → heterogeneity complicates the selection of target cell markers

Examples problem: The case for mast-cells

***purification** by affinity is predominantly **based on CD117 (c-Kit) targeting**,*
(this receptor is not specific to mast cells and is present in many stem cell phenotypes)

NOTE
low receptor expression levels and **low** target **cell abundance**,
→ negative enrichment is the preferred strategy

(microfluidic devices integrating negative selection strategies and physical separation methods (e.g., fluid, electric, or magnetic field) represent the technology of choice

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Affinity-based cell separation

Cell affinity chromatography

- **Selecting the target receptor (disadvantages)**

→biochemical effects that occur upon receptor binding.

→ligand/receptor interactions can trigger undesired events such as **internalization** of the receptor, **metabolic alteration**, and even **differentiation**, in the case of stem cells

Examples problem: The case for mast-cells

- *Metabolic changes caused by affinity binding have been observed on mast cells enriched by targeting **c-kit and FcεRI***
- *These markers, utilized for positive selection, are crucial in IgE activation and are likely to impact cellular metabolism.*

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Affinity-based cell separation

Cell affinity chromatography

- **Ligand selection**

→take into account both kinetic and thermodynamic (K_D) binding parameters

→(K_D) is crucial to ensure product purity,

in the case of positive selection,

High-affinity ligands (low KD), binding target cells specifically, make cell elution difficult,

low-affinity ligands (high KD), allowing for easier elution, may not provide sufficient throughput.

Ideal affinity ligand offers a balance between specific binding and effective elution

→ Cell size, aspect ratio, and receptor density can be used to estimate the number of interactions per cell and to select an appropriate ligand density for a given value of K_D

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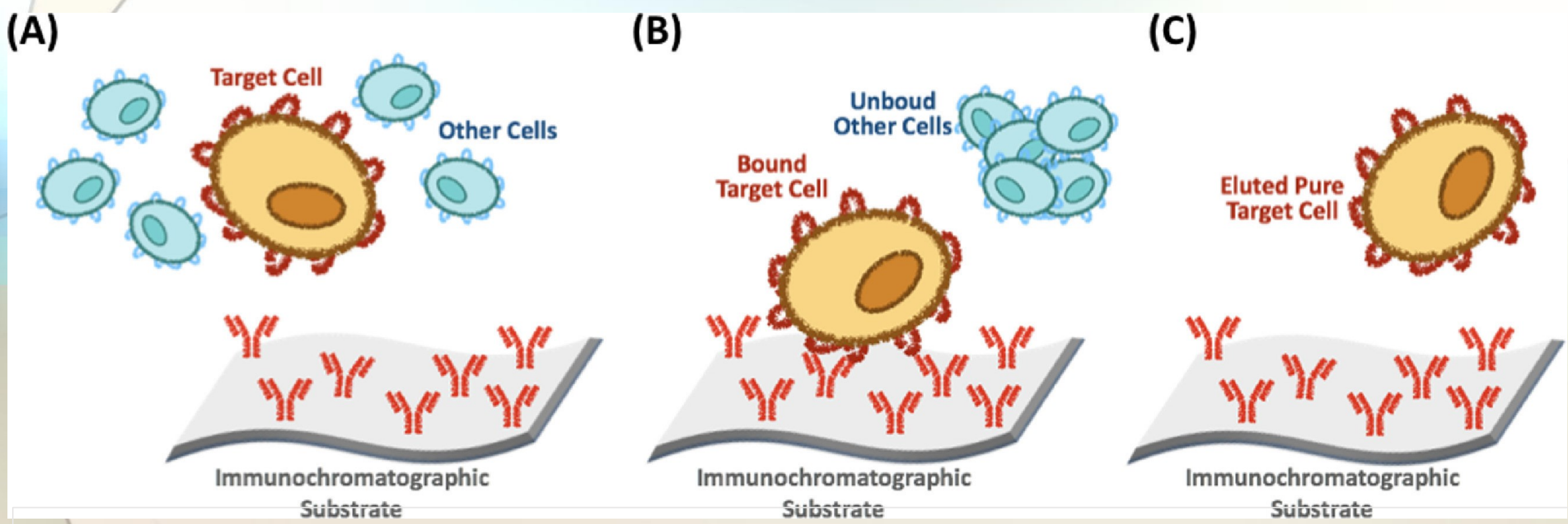
Affinity-based cell separation

Cell immunoaffinity chromatography



The strength of the interaction between the antibody and the target protein….. *A Problem?* → *answer competitive elution and cleavable linkers*

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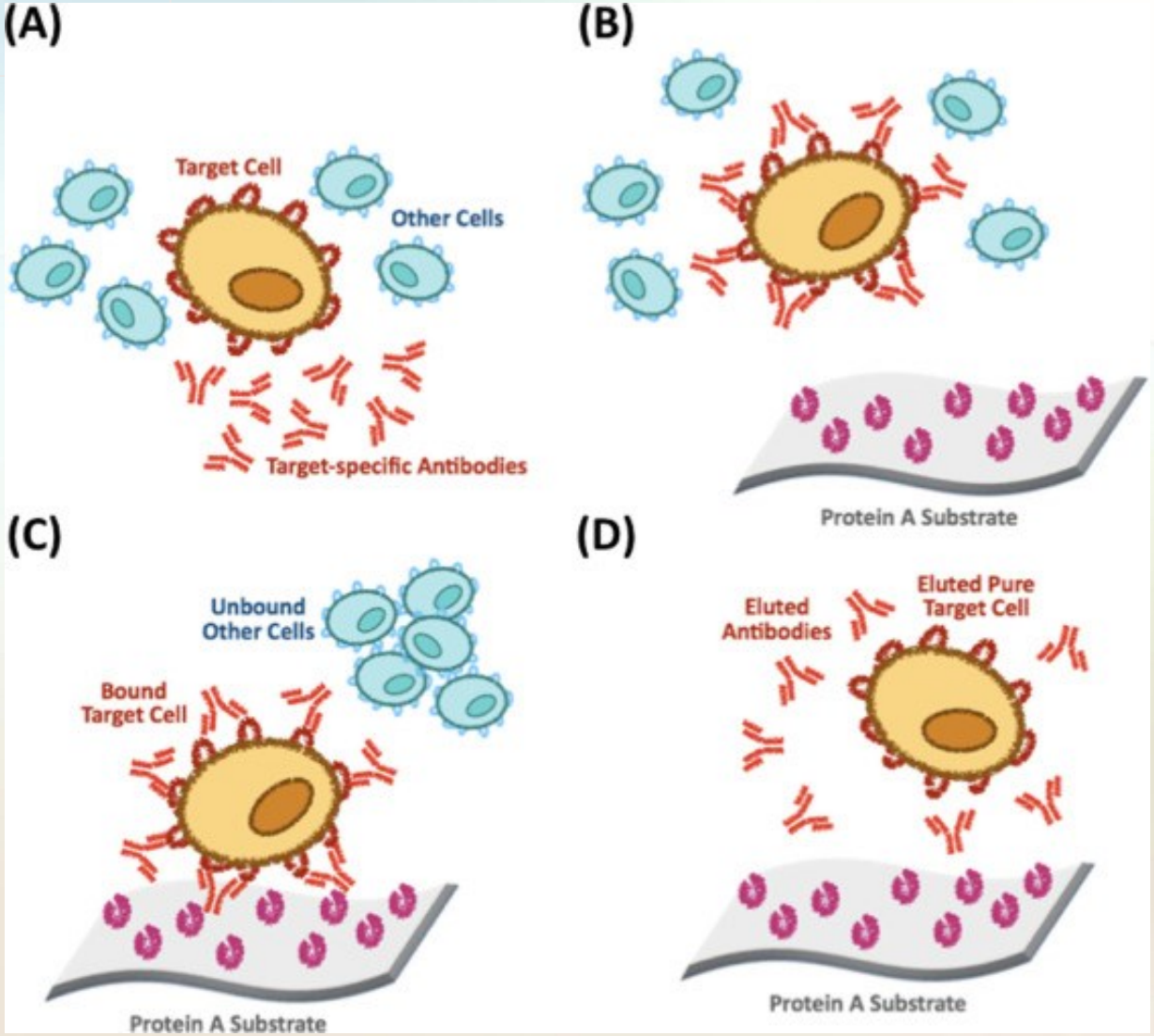
Affinity-based cell separation

Cell immunoaffinity chromatography

Protein A/G-based methods

→ a cell mixture is incubated with a receptor-specific antibody and passed through a Protein A/G-linked adsorbent



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Affinity-based cell separation

Cell immunoaffinity chromatography

- **Synthetic antigens** represent the first use of synthetic ligands for cell purification

Haptens as antigens to stimulate an immune response:
immobilized ligands to isolate white cells with anti-hapten activity

- **Two main difficulties** also encountered in affinity-based capture
  - non-specific binding of non-target cells and
  - detaching cells from the adsorbent without impacting their viability

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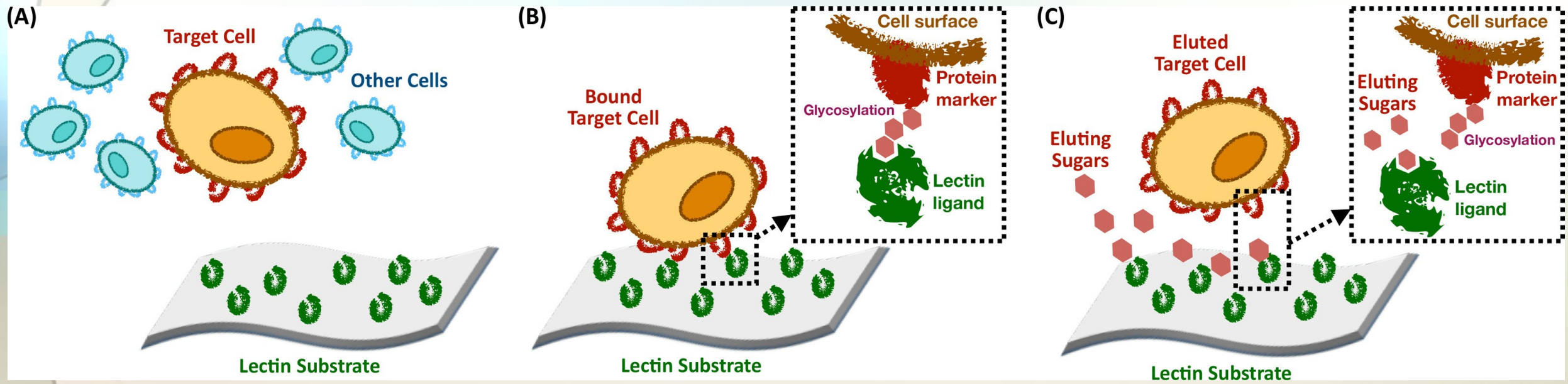
Affinity-based cell separation

Cell affinity chromatography



Advantage of lectins is that cell **elution** can be triggered **by mono- and disaccharides**, which are harmless to cells

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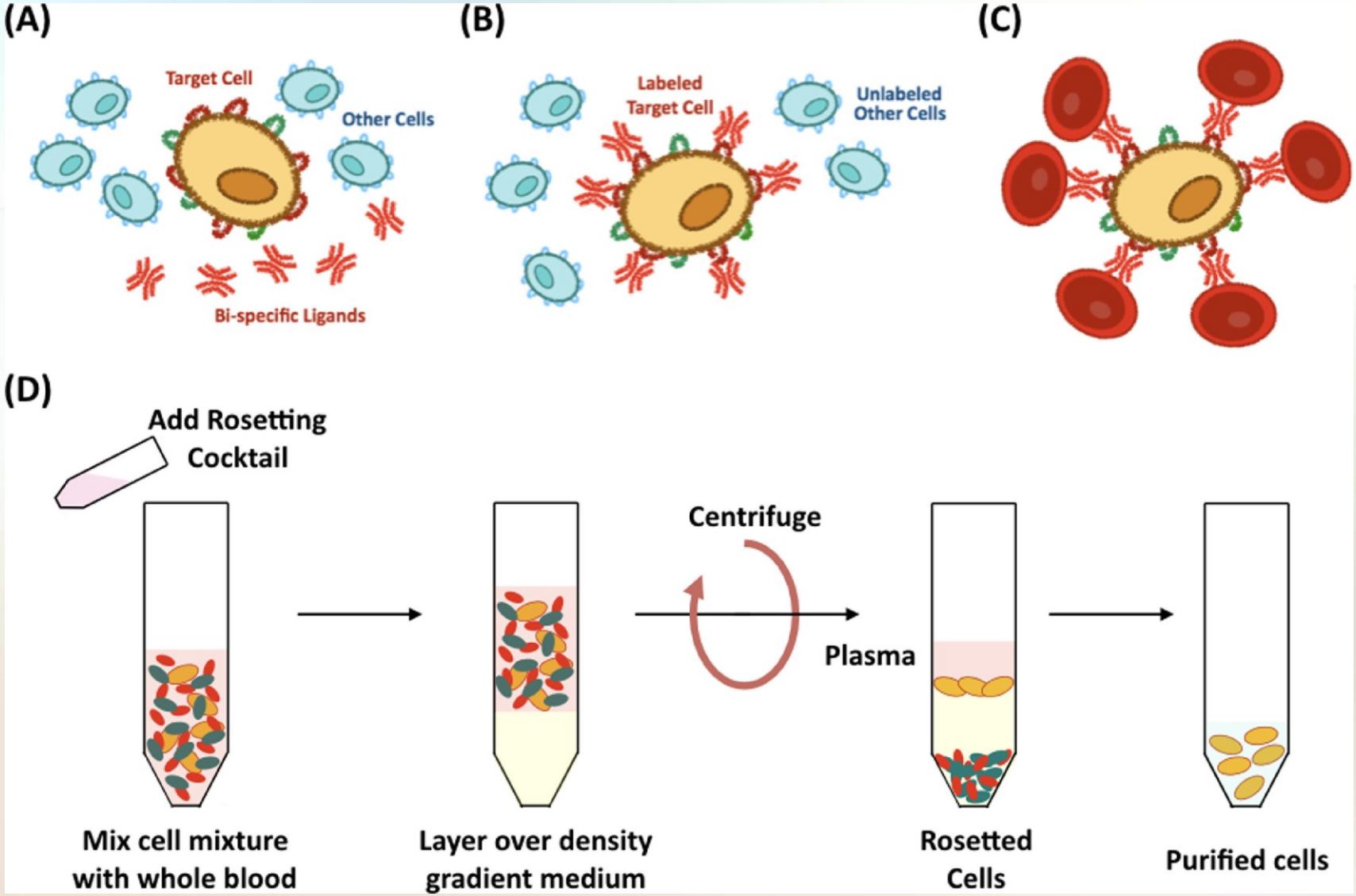
Affinity-based cell separation and density-gradient separation

Rosetting (affinity and traditional density-gradient separation methods)



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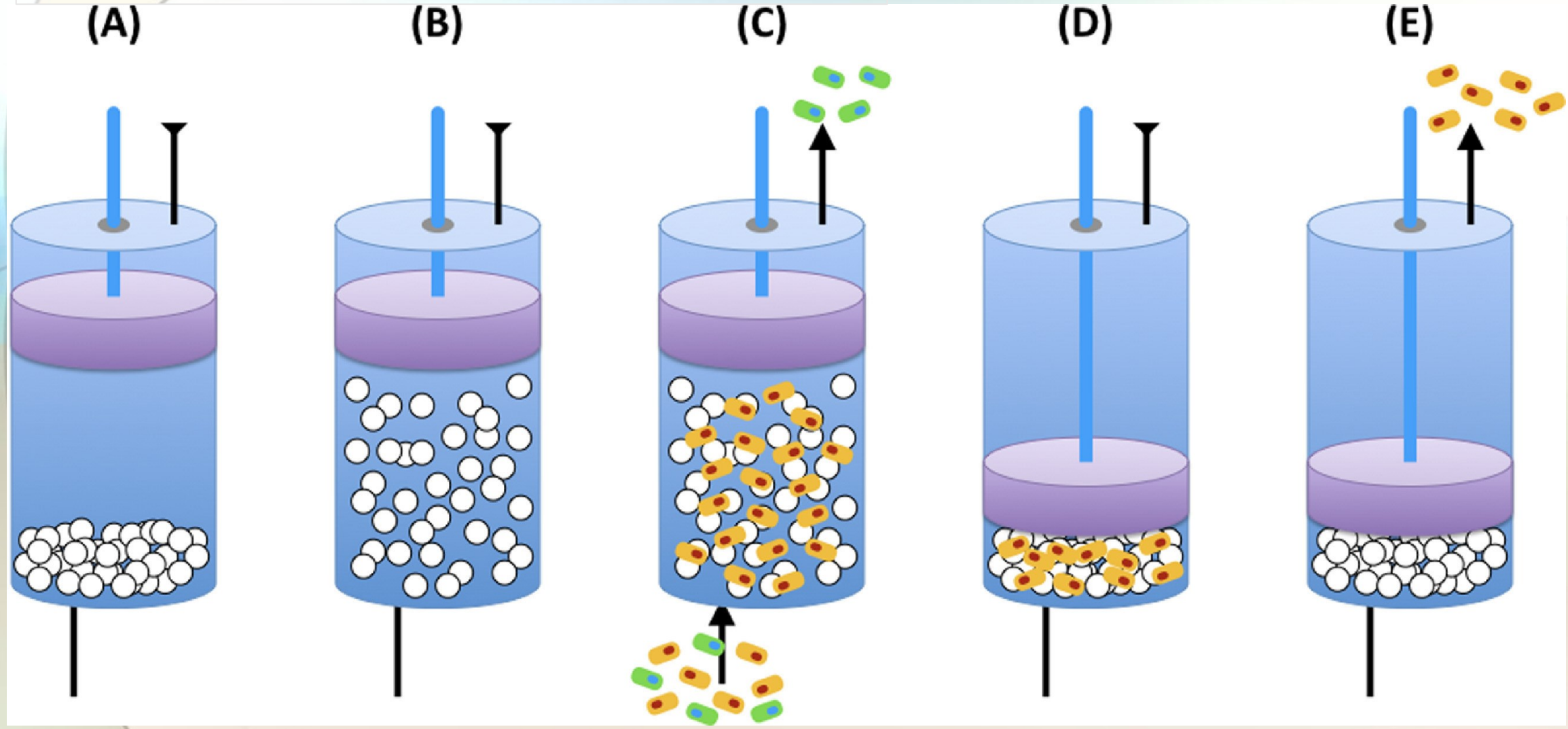
Affinity-based cell separation and Chromatography

Expanded bed chromatography.



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Comparison of affinity-based cell separation

Pseudo-chromatographic systems

- Gel Affinity Separation
- Fiber-based affinity separations
- Affinity Membranes

Non-chromatographic affinity purification

- magnetic-activated cell sorting (MACS)
- fluorescence-activated cell sorting (FACS)

Affinity two-phase partitioning

Aqueous two-phase systems (ATPS) form when two polymers added to a water solution produce two non-miscible liquid layers, across which other components in the solution migrate based on their differential affinity towards the polymers

(Affinity two-phase partitioning is a powerful preparative method for cells, cell membranes and organelles, and viruses)

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Comparison of physical (non-affinity) and affinity-based cell separation

Non-chromatographic affinity purification

- magnetic-activated cell sorting (MACS)
- fluorescence-activated cell sorting (FACS)

- **Elements of MACS and FACS sorting can be combined** in a method known as “ratcheting cytometry” to perform **multicomponent purifications of specific subpopulations**

→ This method is frequently used for continuous and quantitative purification of **T cell subsets for cell therapy manufacturing**.

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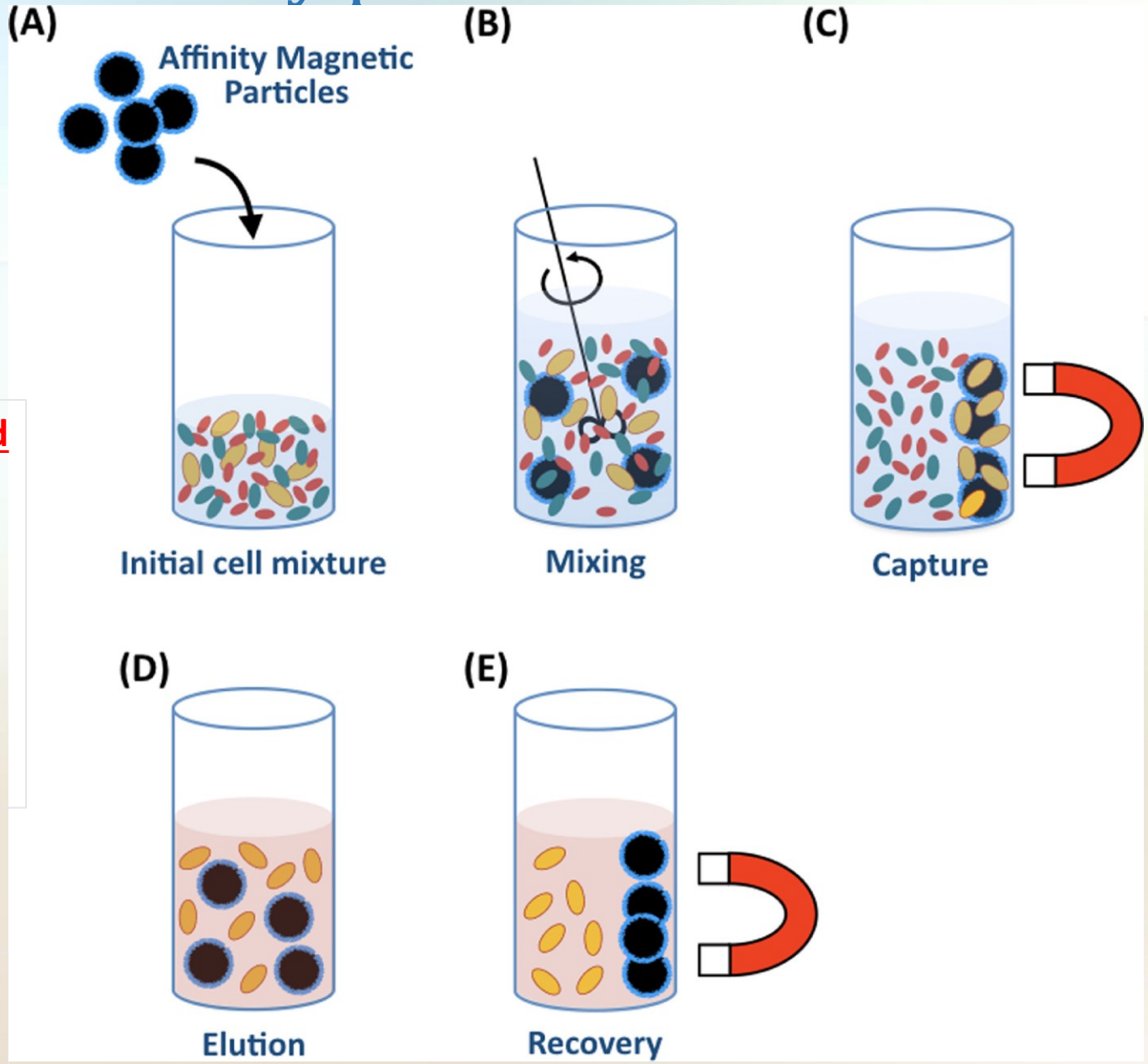
Non-chromatographic affinity purification MACS

Ligand immobilization techniques are highly dependent on the nature of the ligand.

Examples Magnetic nanoparticles coated with bis-Zn-DPA (synthetic ligand that binds Gram-positive and Gram-negative bacteria)
have been utilized for separating *Escherichia coli* from blood with complete bacterial clearance in two separation cycles.



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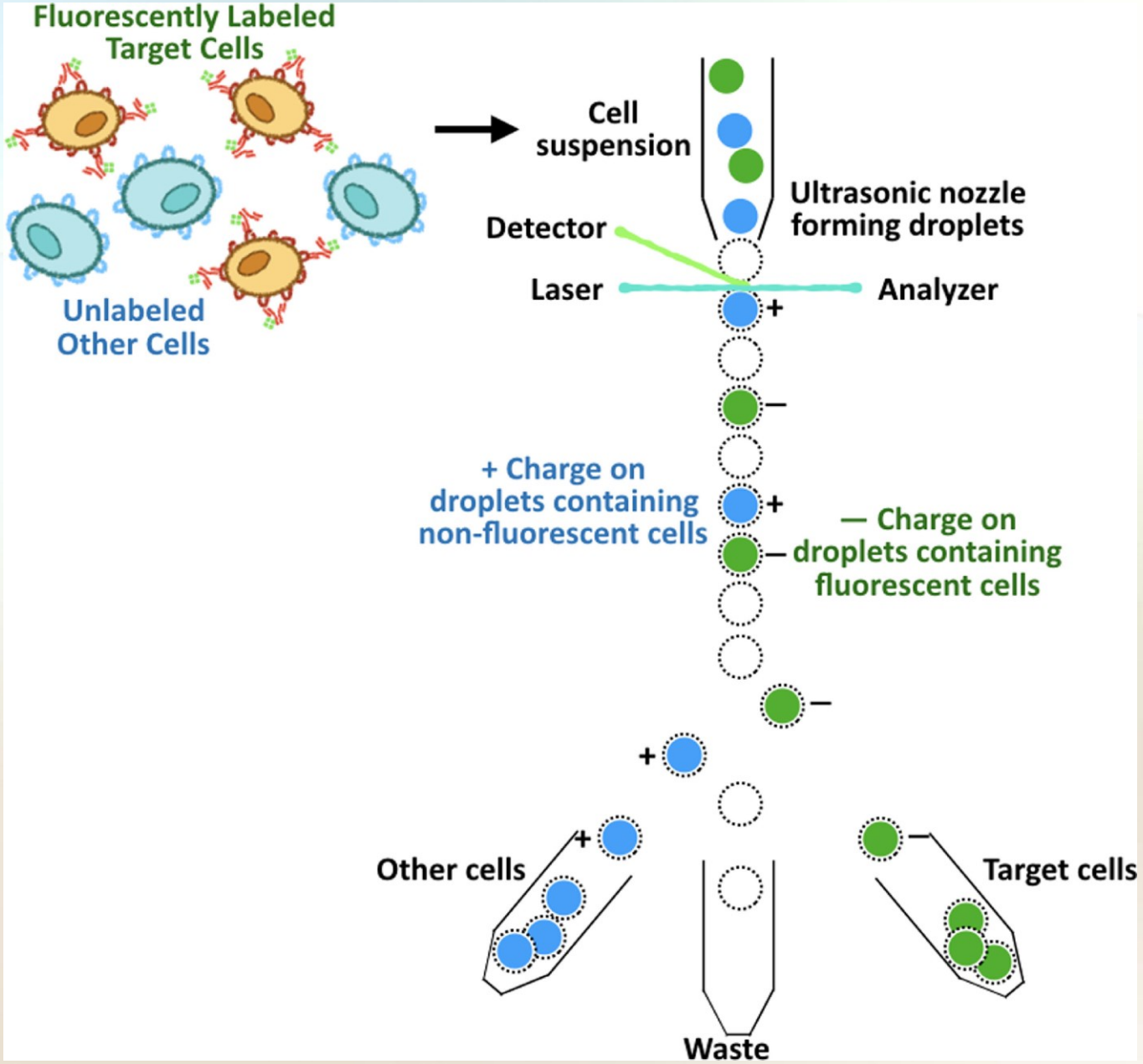
Non-chromatographic affinity purification FACS

FACS
provides highly pure (>95%) cell populations and can sort at the single cell level due to the high sensitivity of fluorescence detection



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Comparison of physical (non-affinity) and affinity-based cell separation

Microfluidic devices for cell separation

The latest frontier of Cell Affinity Chromatography (CAC):

→ microfluidic devices that comprise **sub-millimeter channels coated with affinity ligands (M-CAC)**

The **high surface-area-to-volume ratio of microfluidic channels**, enhanced by micro-fabricated structures with complex geometry, has **enabled the capture of cells at extremely low concentrations by M-CACs**

→ M-CAC systems have been utilized to separate T- and B-lymphocytes at high purity (> 97%) from mixed suspensions.

→To ensure binding specificity, the **channels are often grafted with hydrophilic polymer** brushes (e.g., PEG) or **coated with hydrogels** (e.g., alginate) **functionalized with antibody ligands**

Bacon K, Lavoie A, Rao BM, Daniele M, Menegatti S. Past, Present, and Future of Affinity-based Cell Separation Technologies. Acta Biomater. 2020 Aug;112:29-51. doi: 10.1016/j.actbio.2020.05.004.

Comparison of physical (non-affinity) and affinity-based cell separation

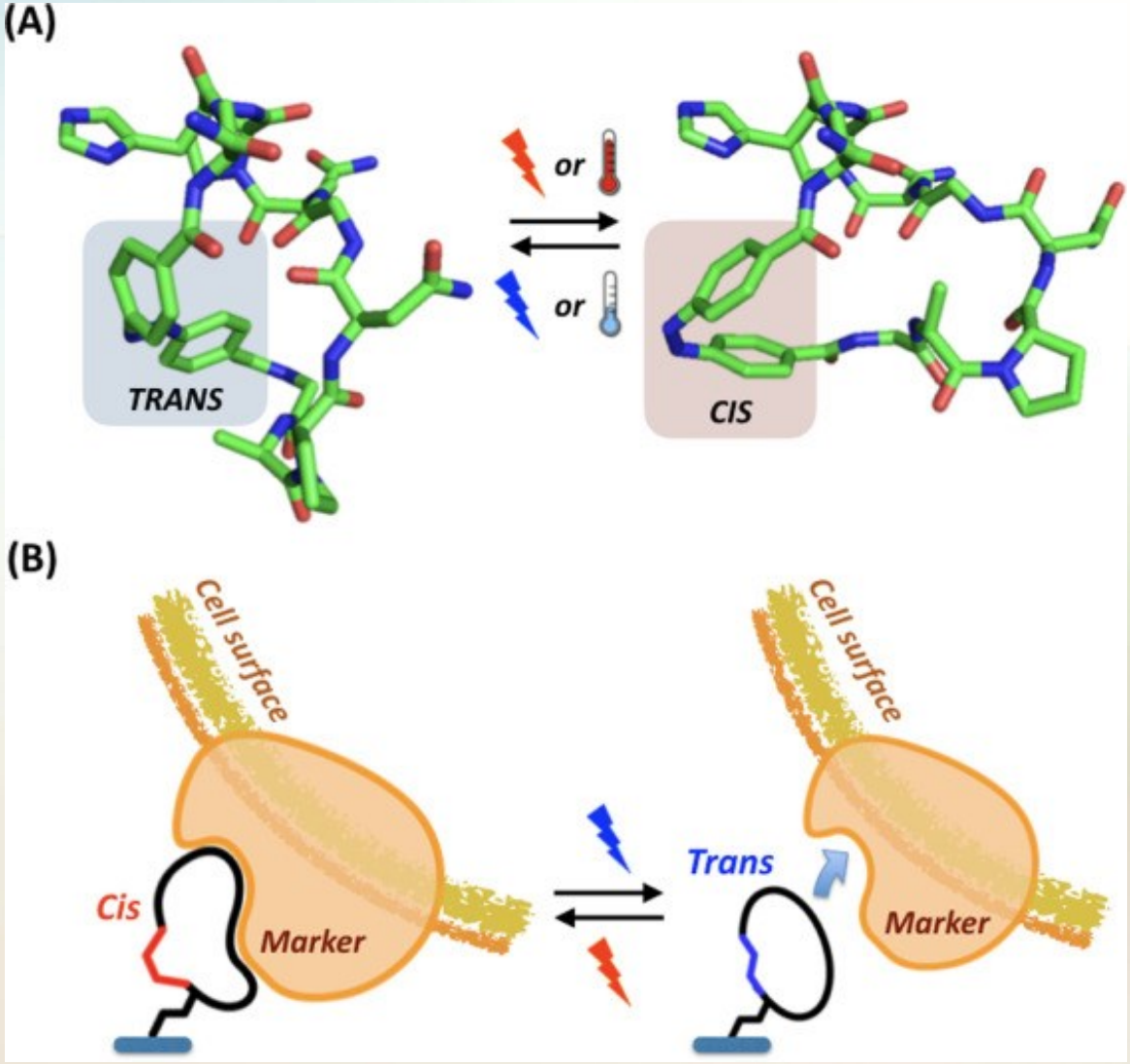
Example of reversible binding of ligand

Peptide ligands can also be engineered to enable the **control of cell binding** and release upon exposure to biocompatible stimuli.

→ stimuli-responsive monomers can be incorporated into the amino acid sequence, allowing the peptide to reversibly

Switch between a binding and a non-binding mode:

- upon cooling
- exposure to light or a magnetic field.



Bacon K, Lavoie A, Rao BM, Daniele M, Menegatti S. Past, Present, and Future of Affinity-based Cell Separation Technologies. Acta Biomater. 2020 Aug;112:29-51. doi: 10.1016/j.actbio.2020.05.004.

Cell purification
Immunoaffinity – MACS – FACS - Microfluidics

Cell separation technologies
for basic research, diagnostic, and therapeutic applications, resulting in cell isolation methods that are more efficient, scalable, and dependable.

- **Affinity-based approaches** are now the most utilized, owing to their ability to achieve high purity.
- **Microfluidic technologies** represent the next frontier of cell manufacturing as they offer the capacity to perform multiple functions (mixing, counting, lysis, single cell analysis, etc.) in a single device.
  - Advances in parallelization and scale-up hold great promise to overcome the low throughput of current devices and enable processing of large sample volumes. Further, the ability to integrate post-sorting molecular, cellular, and functional characterization furthers the appeal of using microfluidic devices for cell separation.

Bacon K, Lavoie A, Rao BM, Daniele M, Menegatti S. Past, Present, and Future of Affinity-based Cell Separation Technologies. Acta Biomater. 2020 Aug;112:29-51. doi: 10.1016/j.actbio.2020.05.004.

Cell purification
Immunoaffinity – MACS – FACS - Microfluidics

Biorecognition front
affinity-based separations are shifting from protein and antibody ligands towards synthetic ligands.

Biological ligands are highly specific but limited by their high cost and exceedingly strong binding.
Synthetic ligands can be synthesized affordably, at a large scale, and with no batch-to-batch variability.

Gentle cell elution conditions → the development of stimuli-responsive ligands, such as **photo-switchable peptides**, whose binding activity can be controlled by exposure to biocompatible stimuli. (further progress in the fields of in vitro and in silico selection methods is needed)

Bacon K, Lavoie A, Rao BM, Daniele M, Menegatti S. Past, Present, and Future of Affinity-based Cell Separation Technologies. Acta Biomater. 2020 Aug;112:29-51. doi: 10.1016/j.actbio.2020.05.004.

Cell purification- Challenge

Currently, the **major challenge for cell therapies and related clinical applications resides**:

An achieving **rapid, efficient, and affordable separation while minimizing costs** and attaining the required **purity, yield, and functionality** of the cellular product.

→***Membrane-based separations*** enable the processing of large-volume cell suspensions at high flow rates, thereby increasing throughput and minimizing processing time, which aids in maintaining the viability, ensure high recovery, purity, and bioactivity of the cell product.

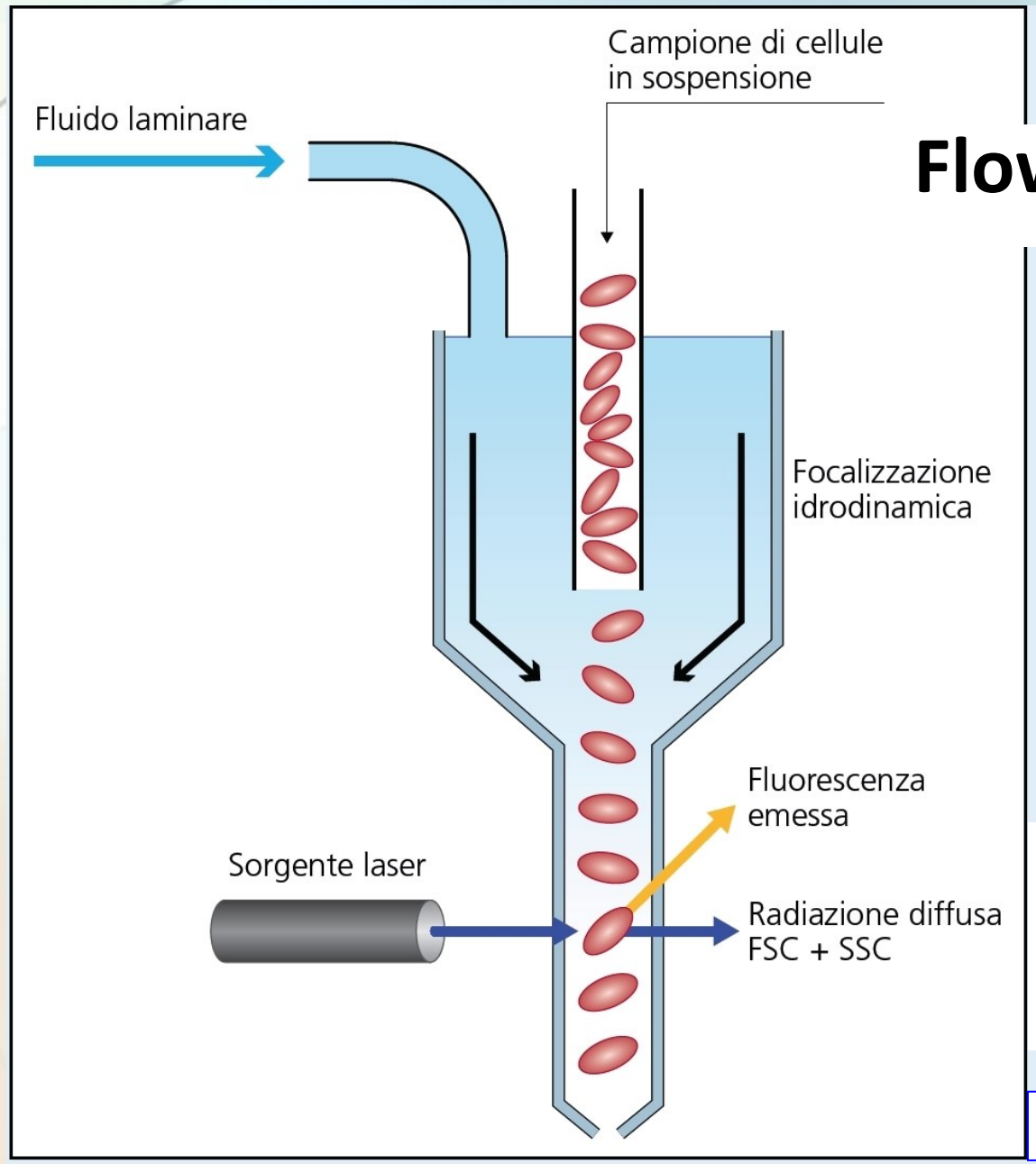
→***Basic cellular research and personalized medicine*** **(**patient-specific cellular therapies)

- identification of highly specific markers defining cell populations advancements

- integrating physical and affinity-based strategies in miniaturized devices

Bacon K, Lavoie A, Rao BM, Daniele M, Menegatti S. Past, Present, and Future of Affinity-based Cell Separation Technologies. Acta Biomater. 2020 Aug;112:29-51. doi: 10.1016/j.actbio.2020.05.004.

Flow cytofluorimetry



FSC: 0° forward scatter (dimensioni)
SSC: 90° side scatter (granulosità)

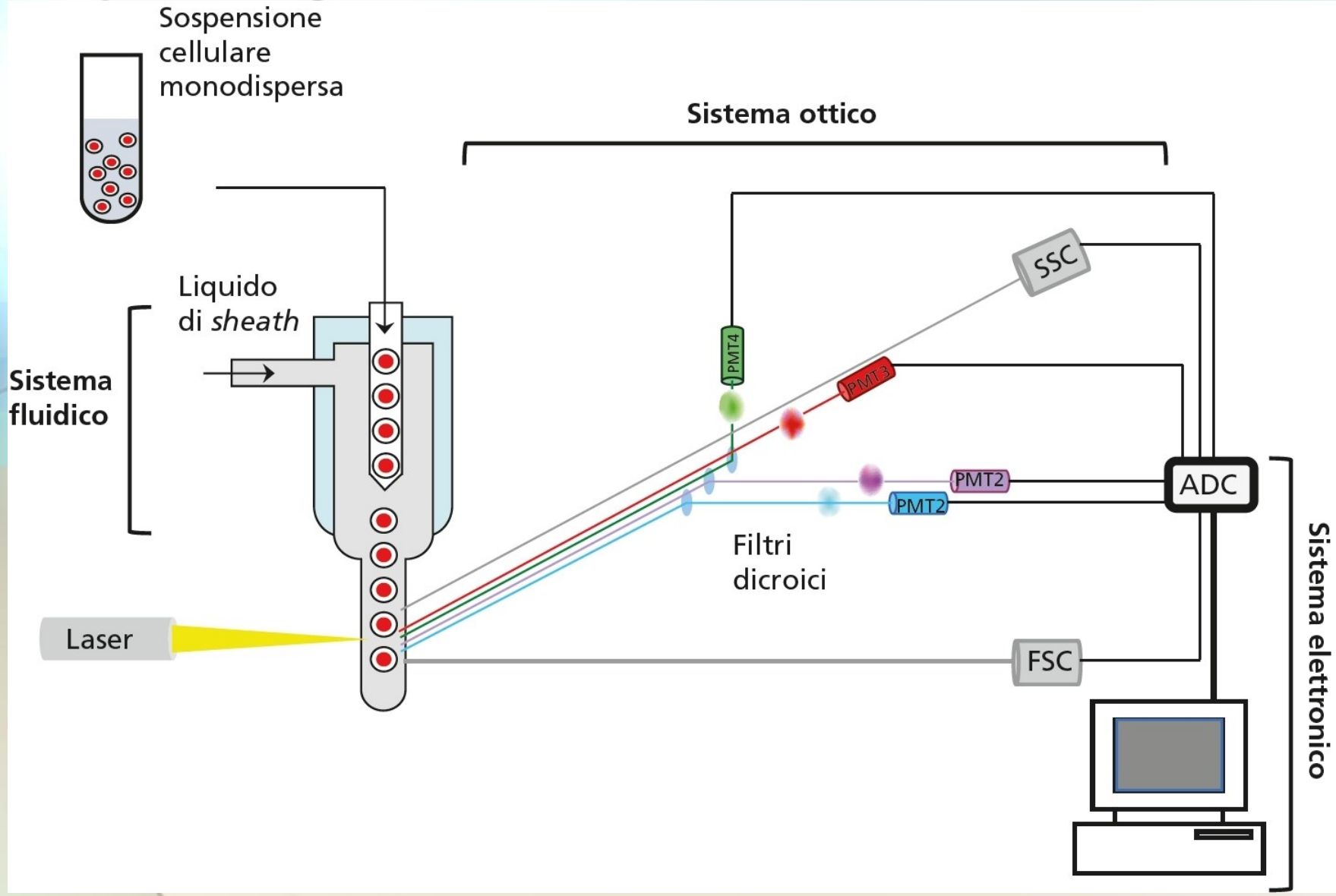
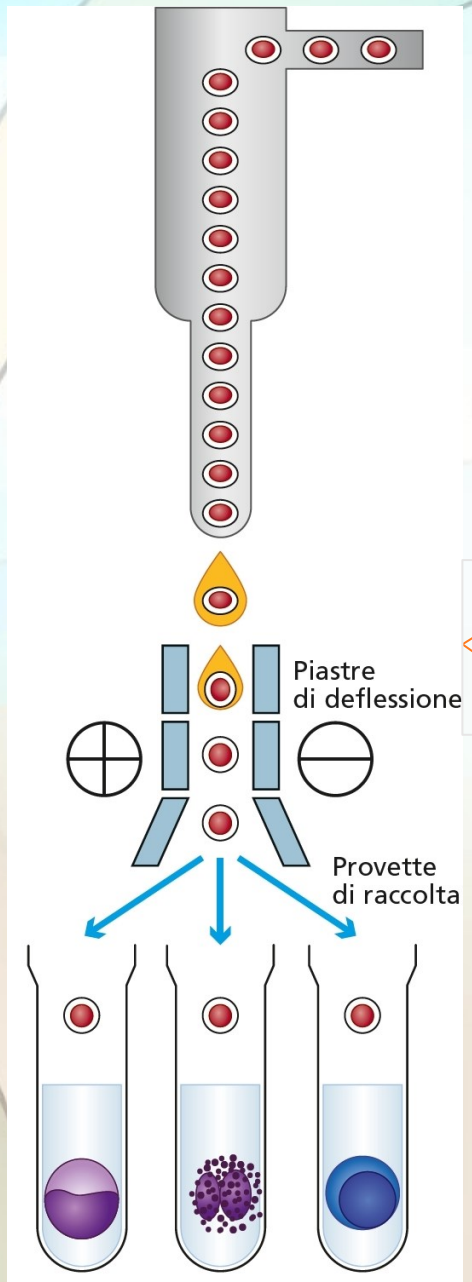
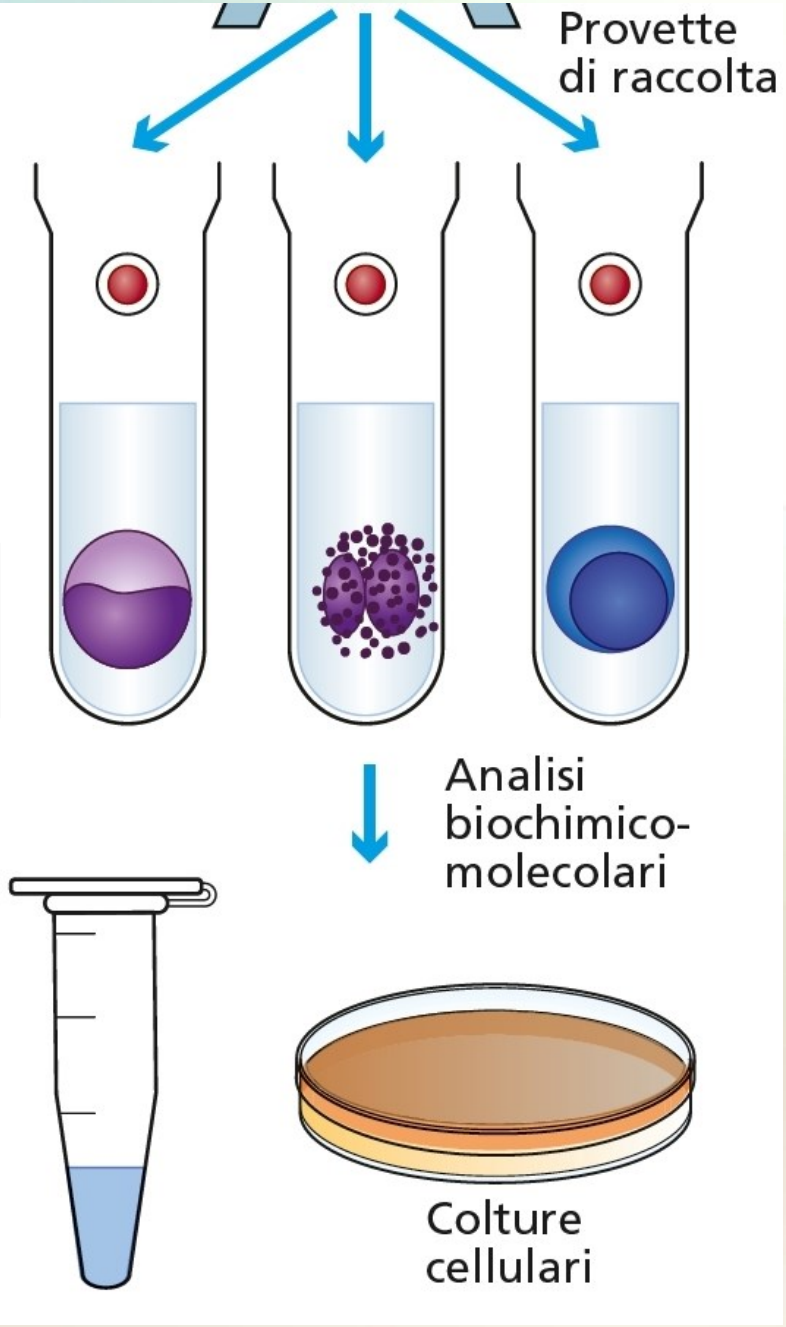


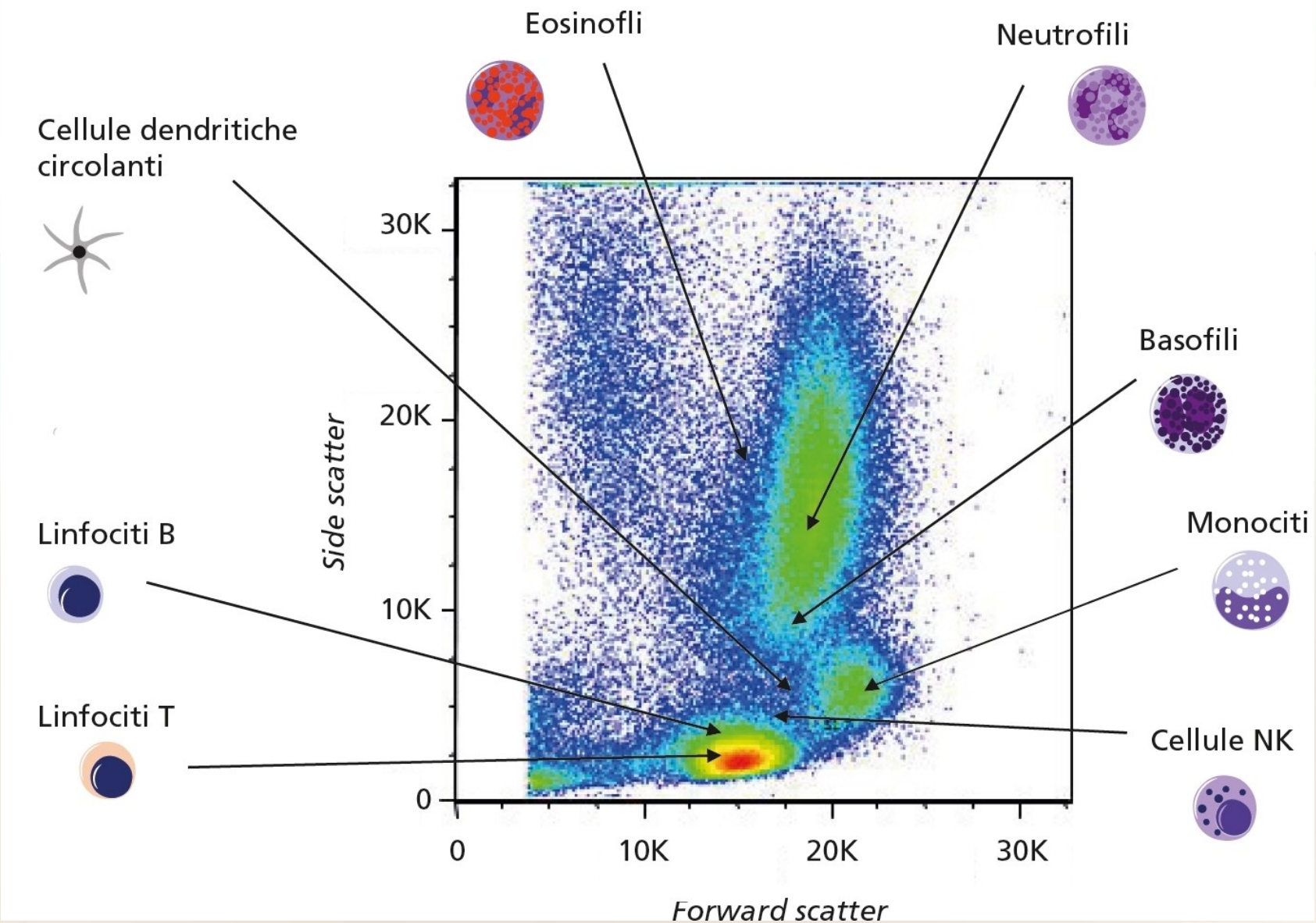
Figura 25.1
Componenti di un citofluorimetro. Una sospensione di cellule marcate con una miscela di fluorocromi passa all'interno del sistema fluidico, in cui le cellule stesse vengono spinte e allineate in maniera ordinata per essere poi colpite da un laser che eccita i fluorocromi. Ciò determina l'emissione di segnali fluorescenti che vengono captati, rilevati e convertiti in segnali elettrici, per essere infine visualizzati al computer.



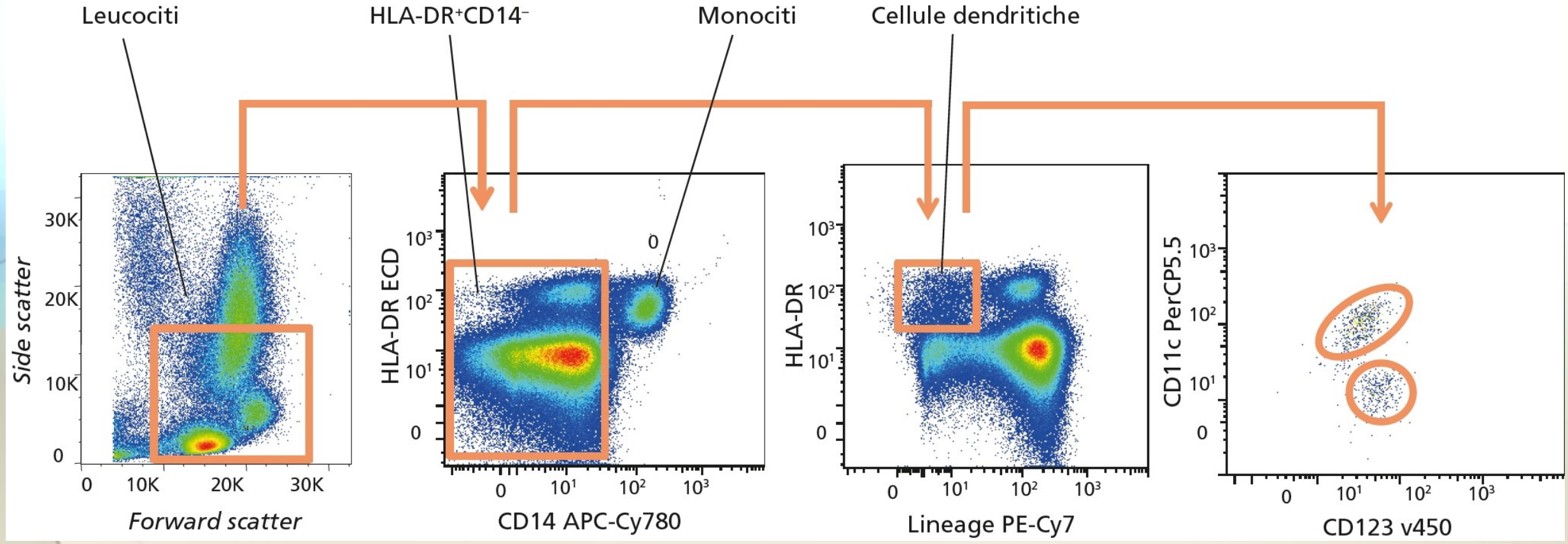
FACS:
Fluorescence Activated Cell Sorting



Flow cytometry (Analysis)

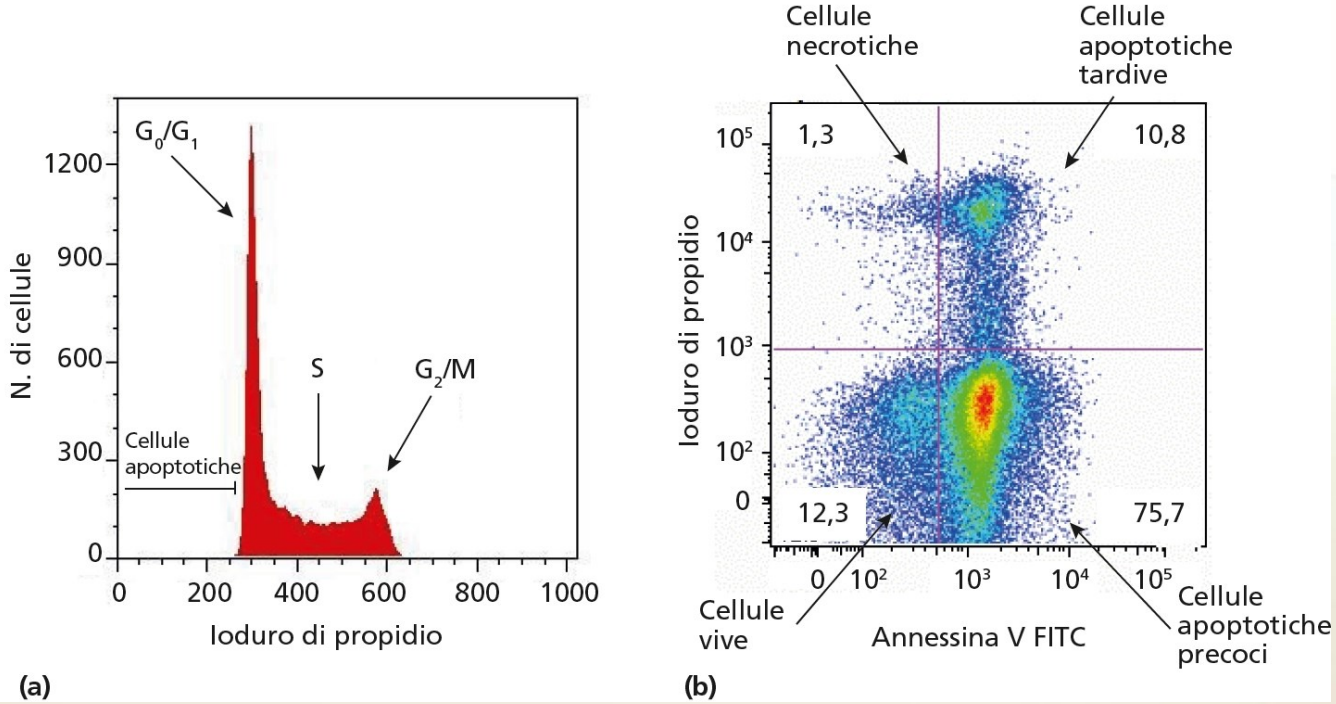


Flow cytometry (Analysis)



Flow cytometry (Analysis)

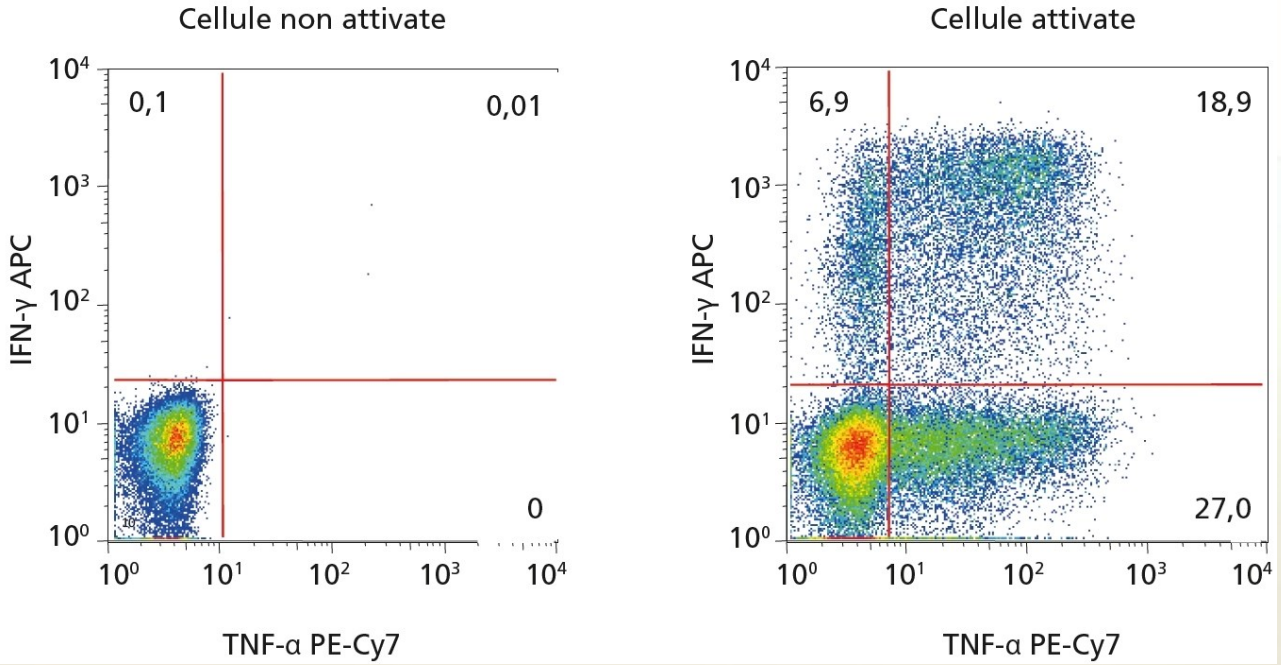
Figura 26.4
Analisi di ciclo e morte cellulare.
(**a**) Tipico istogramma che rappresenta il contenuto di DNA di una sospensione cellulare marcata con lo ioduro di propidio. Tale sonda fluorescente si lega stechiometricamente al DNA e fornisce una fotografia istantanea delle cellule nelle varie fasi del ciclo cellulare (G_0/G_1. S, G_2/M).
(**b**) Grafico bidimensionale che mette in relazione la fluorescenza dell'annessina V (che marca le cellule apoptotiche) con quella dello ioduro di propidio (che a basse concentrazioni marca le cellule necrotiche o nelle fasi tardive del processo apoptotico).



M. Maccarrone, Metodologie Biochimiche e Biomolecolari, Zanichelli 2019

Analisi al citofluorimetro

Figura 26.5
Produzione intracellulare di citochine pro-infiammatorie. Nei grafici bidimensionali è mostrata la produzione di due citochine pro-infiammatorie, il fattore di necrosi tumorale alfa (TNF-α) e l'interferone gamma (IFN-γ), da parte di linfociti T umani non attivati (a sinistra) o attivati (a destra). I numeri all'interno di ciascun quadrante rappresentano le percentuali di produzione delle citochine.



M. Maccarrone, Metodologie Biochimiche e Biomolecolari, Zanichelli 2019

Flow cytometry

https://www.youtube.com/watch?v=7bCZx5xPwt0

https://www.youtube.com/watch?v=EQXPJ7eeesQ

Analysis (software example)
https://www.youtube.com/watch?v=3nsLoitwYYA