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Cell Culture Technologies

Primary Cell Isolation, Growth and Analysis

Edited by Jens Hahne



Cell Culture Technologies - Primary Cell Isolation, Growth and Analysis

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Contributors

Beata Dedičová, Cetin YukseI, Emelie Ivarson, Erika Berenyi, Mohamed Hussein, Niklas Olsson, Oksana A. Leonovich, Per Hofvander, Quinn P. Peterson, Zenith Khashim

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IntechOpen Book Series

Biochemistry

Volume 71

Aims and Scope of the Series

Biochemistry, the study of chemical transformations occurring within living organisms, impacts all of the life sciences, from molecular crystallography and genetics, to ecology, medicine and population biology. Biochemistry studies macromolecules - proteins, nucleic acids, carbohydrates and lipids –their building blocks, structures, functions and interactions. Much of biochemistry is devoted to enzymes, proteins that catalyze chemical reactions, enzyme structures, mechanisms of action and their roles within cells. Biochemistry also studies small signaling molecules, coenzymes, inhibitors, vitamins and hormones, which play roles in the life process. Biochemical experimentation, besides coopting the methods of classical chemistry, e.g., chromatography, adopted new techniques, e.g., X-ray diffraction, electron microscopy, NMR, radioisotopes, and developed sophisticated microbial genetic tools, e.g., auxotroph mutants and their revertants, fermentation, etc. More recently, biochemistry embraced the ‘big data’ omics systems. Initial biochemical studies have been exclusively analytic: dissecting, purifying and examining individual components of a biological system; in exemplary words of Efraim Racker, (1913 –1991) “Don’t waste clean thinking on dirty enzymes.” Today, however, biochemistry is becoming more agglomerative and comprehensive, setting out to integrate and describe fully a particular biological system. The ‘big data’ metabolomics can define the complement of small molecules, e.g., in a soil or biofilm sample; proteomics can distinguish all the proteins comprising e.g., serum; metagenomics can identify all the genes in a complex environment e.g., the bovine rumen. This Biochemistry Series will address both the current research on biomolecules, and the emerging trends with great promise.

Meet the Series Editor



Ana Maria Carmona-Ribeiro has been a full professor of Biochemistry at the University of São Paulo (USP), Brazil, since 2001. She founded the Biocolloids Laboratory at USP in 1993, and since then she has been developing novel important assemblies aimed at drug and vaccine delivery. Her background in Physics, Chemistry, Biology, and Pharmaceuticals has been useful for the development of biomolecular assemblies with potential for novel biomedical applications.

Meet the Volume Editor



Jens C. Hahne received his Ph.D. in biochemistry from the Albert-Ludwigs-Universität Freiburg i. Br. (Germany). During several postdoctoral positions [Department of Molecular Pathology, University of Bonn (Germany), Charité Berlin (Germany), Department of Gynaecology and Obstetrics, University of Würzburg (Germany)], he received broad training and expertise in molecular pathology, oncology, and translational research. Then he worked for 8 years as a Senior Scientist in the Department of Molecular Pathology at the Institute of Cancer Research (ICR, London, UK) before joining the NIHR Biomedical Research Centre in Leicester (UK) as Research Manager. He has published more than 80 papers in reputable journals (with more than 5,500 Citations and an h-index of 32), contributed to several books, and served on the editorial board of reputable journals.

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Preface

The process of growing cells outside their natural environment is called cell culture. Besides established cell lines, the isolation and cultivation of primary cells is of increasing interest and importance. In our days, isolating cells from their native tissue and cultivating them in a controlled, artificial laboratory environment is the basis for many applications and research topics. Cell culture is extensively used in red and green biotechnology, e.g., molecular biology, tissue engineering, regenerative medicine, vaccines, drug discovery, and cellular agriculture.

This book covers various aspects of cell culture technologies and provides an overview of using primary cells in medical, biomedical, and plant fields, while also addressing key points in cryopreservation, recovery, and mycoplasma contamination.

Chapter 1, “Cryopreservation and Recovery of Primary Cells: Best Practices and Pitfalls”, addresses key issues during cryopreservation and provides guidelines for fine-tuning protocols to maintain primary cells’ viability and functionality after thawing. In Chapter 2, “Mycoplasma Contamination of Primary Cell Cultures”, the serious problem of mycoplasma contamination is addressed. Various methods for Mycoplasma detection in primary cultures are discussed, and decontamination methods are summarised. Several updated research findings follow these two general chapters. First, Chapter 3 “Primary Cell Isolation from Solid Tissues and Current Understanding for Their Application in Personalized Medicine and Drug Development” provides a broad overview on how primary cells isolated from tissues can provide more accurate and reliable information about the biology of a disease and how this can direct personalised cancer therapy or can form the base for cell therapies, new drug target identifications and drug developments. Then, Chapter 4 “Generation of Functional β Cells from Pluripotent Stem Cells: Advances and Applications” provides an overview of the differentiation protocols that mimic human embryonic pancreatic β -cell development, highlighting key signalling pathways and transcription factors involved as well as addressing critical challenges in achieving full β -cell maturation, including strategies such as 3D culture, co-culture systems, and environmental conditioning. In addition, methods for isolating and enriching functional stem cell-derived β cells are discussed in detail. Finally, the use of stem cell-derived β -cells in drug screening, disease modelling, and cell replacement therapies for diabetes is discussed. Then, Chapter 5, “Tumor-on-a-Chip: From Biomimetic Tumor-Immune Microenvironments to Therapeutic Insights”, discusses one of the latest developments in cell culture: the use of organoids in combination with microfluidic technology. The focus of this chapter is on recent advancements in the development of tumour-on-a-chip systems and on the integration of the tumour-immune microenvironment, with implications for cancer research and personalized medicine. The challenges and future directions in optimizing these models to enhance their predictive power for clinical outcomes include increasing the complexity of tumour-immune interactions, improving the scalability and

reproducibility of the models, and addressing limitations in long-term culture and physiological relevance for drug testing and personalised medicine.

Last but not least, the use of primary cells in green biotechnology is brought up. In Chapter 6, “Isolation and Regeneration of Wheat Mesophyll Protoplasts: Using a Short Protocol”, specific points in plant primary cell culture are addressed. Given the importance of plant protoplasts as valuable tools for studying the fundamental properties and functions of cells, it is of utmost importance to have a reproducible and concise method for isolating wheat mesophyll protoplasts. This chapter provides a method for isolating wheat mesophyll protoplasts from two spring varieties derived from *in vitro* seedlings. It introduces methods to determine the concentration and viability of freshly isolated wheat protoplasts.

In summary, this collection underscores the importance of primary cells across different areas of research and is a valuable resource for anyone studying or researching primary cell culture applications and techniques, offering valuable insights into emerging trends and techniques.

Jens C. Hahne
NIHR Biomedical Research Centre,
University of Leicester,
Leicester, UK

Cryopreservation and Recovery of Primary Cells: Best Practices and Pitfalls

Mohamed Hussein

Abstract

Preserving primary cells through cryopreservation has become an essential part of modern life sciences, allowing scientists to store these biologically meaningful cells for long periods and use them later without losing their functional value. What sets primary cells apart from immortalized cell lines is that they stay true to their original form, and they keep the same genetic makeup, physical characteristics, and biological behavior as they had in the body. This makes them incredibly useful in areas like disease modeling, regenerative medicine, drug testing, and personalized treatment approaches. But because they have a limited ability to divide, vary from one donor to another, and are more fragile overall, freezing and thawing them without causing damage can be quite challenging. This chapter takes a closer look at how to freeze and store primary cells successfully. It explains why these cells matter, how cryoprotective agents (CPAs) work to shield them during freezing, and what happens inside the cells at the molecular level when they are exposed to ultra-low temperatures. Key issues like ice forming inside cells, changes in water movement, and stress from rapid temperature shifts are all discussed, along with how to fine-tune protocols to keep cells alive and functional after thawing. The chapter also touches on deeper problems, like damage to proteins, enzymes, and tissues, especially when working with complex or lab-grown structures. Overall, it offers both the science and hands-on guidance needed to preserve these sensitive cells effectively and reliably.

Keywords: primary cells, cryopreservation, cryoprotective agents (CPAs), intracellular ice formation (IIF), osmotic shock, vitrification, cell viability, cell-based therapies

1. Introduction

Cryopreservation is a technique used to preserve biological materials like cells, tissues, or even whole organs, by cooling them to extremely low temperatures, typically around -196°C , using liquid nitrogen. At these temperatures, all biological activity comes to a complete stop, effectively putting cells into a suspended state [1, 2]. This allows them to be stored for extended periods without deteriorating, making cryopreservation a vital tool in research labs, fertility clinics, hospitals, and regenerative medicine programs around the world [3].

Although freezing methods are well-developed for immortalized cell lines, those adapted to grow indefinitely in the lab, cryopreserving primary cells is far more complex [4]. Primary cells are directly taken from living tissues and retain the natural characteristics of their original environment [5]. That is exactly what makes them so valuable for studying human biology, disease processes, and personalized treatments. But because they are more fragile and have limited lifespans, they are also much harder to preserve without causing damage [6, 7].

A major concern when freezing primary cells is avoiding cryoinjury, damage that can occur during freezing or thawing [8].

This can happen in several ways: ice crystals may form inside the cell and rupture vital structures [9]; rapid water movement in or out of the cell can lead to osmotic stress or bursting [10, 11]; and the chemicals used to protect the cells during freezing, known as cryoprotective agents (CPAs) like DMSO, can be toxic if not used correctly [12, 13]. Even small mistakes, like freezing too quickly or skipping key post-thaw steps, can dramatically lower cell survival or impair their function [14].

To give primary cells the best chance of surviving and working as intended after thawing, it is crucial to follow well-established best practices [15]. This includes choosing the right CPA, using controlled freezing methods, thawing quickly but carefully, and managing cells gently after they are brought back to room temperature. Just as important is knowing what not to do; there are common pitfalls, such as using the wrong concentrations or skipping wash steps, that can undo all your efforts [16].

This chapter explores how to effectively freeze and recover primary cells, whether you are working in research, diagnostics, or clinical applications. It covers the basic science behind cryopreservation, practical steps for success, and common mistakes to avoid. The goal is to help scientists, lab technicians, and healthcare professionals preserve sensitive cells with confidence and improve the quality and consistency of their results.

2. What are primary cells and why do they matter?

Primary cells are freshly collected from living tissues, whether from humans, animals, or plants, and grown in a lab setting under carefully controlled conditions. Unlike immortalized cell lines that are modified to keep dividing forever, primary cells remain unaltered and maintain their natural state [17, 18]. This means they look and behave almost exactly as they would inside the body, keeping their original shape, gene expression patterns, and metabolic activity. Because of this, they are extremely valuable in research, offering a more accurate picture of how real cells function in living organisms. The trade-off, however, is that primary cells are more fragile and do not survive as long in culture, so they need more attention and careful handling [19].

2.1 Primary cells: Defining features, benefits, and practical constraints

Primary cells are harvested directly from donor tissue with minimal ex-vivo manipulation, so they preserve their native genotype, phenotype, and functional markers, giving them unrivaled physiological relevance for translational and personalized studies. That same provenance imposes several practical constraints: their proliferative window is short (typically ~5–15 passages before senescence), every donor introduces inherent batch-to-batch variability, and strict ethical and biosafety

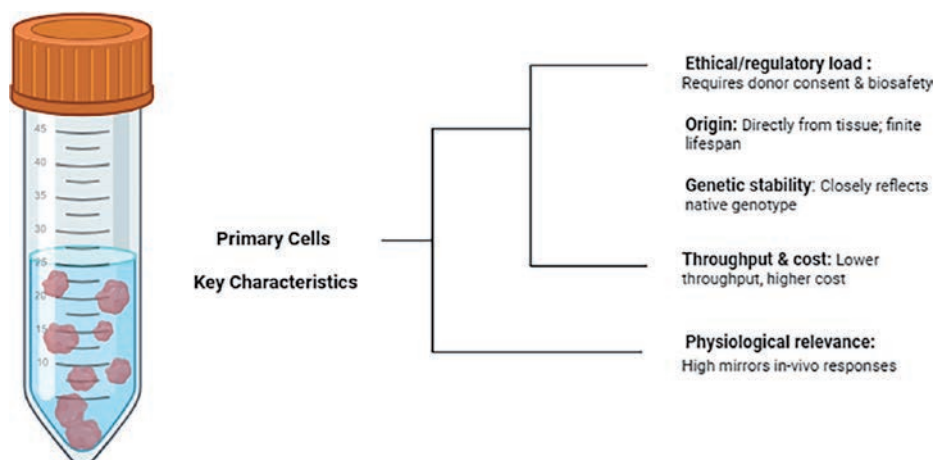


Figure 1.
 Biological profile: cellular, physiological, phenotypic, and intrinsic characteristics.

rules require documented consent and rigorous screening [20]. Because they divide slowly and cannot be expanded indefinitely, experimental throughput is lower and per-sample cost higher than with immortalized lines; yet their limited lifespan also curbs long-term genetic drift, ensuring a genomic profile that mirrors the *in-vivo* state [21]. Taken together, high physiological fidelity, finite lifespan, donor-dependent heterogeneity, ethical oversight, and higher cost, these features define primary cells as the gold standard whenever *in-vivo*-like responses matter more than the convenience of unlimited proliferation (**Figure 1**) [22].

2.2 Cryopreservation: A foundational tool for biobanking, research, and regenerative therapy

Cryopreservation is a cornerstone technique in modern biological and medical sciences, enabling the long-term storage of living cells, tissues, and other biological materials at ultra-low temperatures, typically using liquid nitrogen at -196°C [23]. The fundamental objective of cryopreservation is to suspend all cellular metabolic and enzymatic activity without causing irreversible structural or functional damage [24]. This has become indispensable in various domains such as biobanking, clinical research, regenerative medicine, and personalized therapy. By preserving viable cells and tissues, researchers and clinicians can perform retrospective studies, maintain genetic consistency in longitudinal experiments, and develop new therapies with minimized resource constraints (**Figure 2**) [25].

2.3 The clinical and research utility of cryopreserved biological samples

- i. Cryopreservation plays a central role in biobanking, allowing biological samples like blood, tissue, DNA, RNA, and live cells to be stored for the long term. These collections are crucial for research that translates into clinical practice, large-scale epidemiological studies, and ongoing public health monitoring [26]. Cryopreservation is especially important in rare disease research, where every sample might represent a unique genetic or clinical profile that is nearly impossible to replace [27].

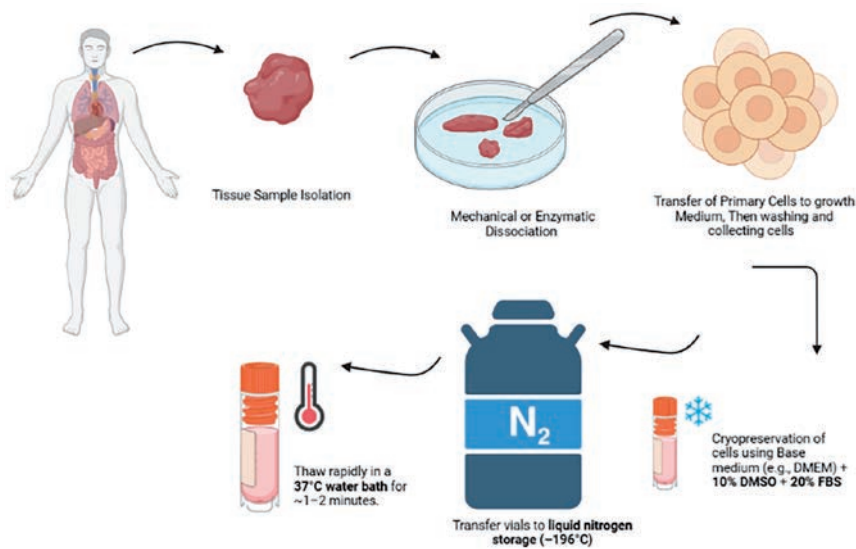


Figure 2.
Tissue-derived primary cell preservation: isolation to liquid nitrogen storage.

- ii. In personalized medicine, cryopreservation allows clinicians and researchers to save patient-specific samples for future use in tailored treatments [28]. For example, tumor biopsies, organoids, and immune cells can be frozen and later analyzed for things like drug response or immune system activity. Induced pluripotent stem cells (iPSCs), which are often used in precision therapies, need to be cryopreserved to ensure consistent results and enable large-scale production. In cancer care, saving immune cells like tumor-infiltrating lymphocytes or CAR-T cells allows flexibility in scheduling treatments and supports centralized manufacturing of cell-based therapies [29].
- iii. In large, multi-site clinical trials, cryopreservation ensures consistency across all participating centers [30]. However, to ensure this level of consistency, all participating centers must adhere to an identical, validated protocol or standard operating procedure (SOP) for every step of the cryopreservation workflow, including sample handling, CPA preparation, freezing rates, and thawing procedures. Any deviation between sites can introduce confounding variability that undermines data integrity, particularly in biomarker analysis or longitudinal studies. Organizations such as the International Society for Biological and Environmental Repositories (ISBER) emphasize the harmonization of SOPs across centers as a fundamental principle for reproducibility in clinical research and biobanking [31].
- iv. Cryopreserving stem cells and engineered tissues is key in regenerative medicine. Whether it is mesenchymal stem cells (MSCs), embryonic stem cells (ESCs), or iPSCs, these cells are stored for use in treatments ranging from cartilage and nerve repair to heart disease therapies [32]. One cutting-edge method called vitrification, which involves freezing cells rapidly enough to avoid forming damaging ice crystals, is proving especially useful for delicate cell types and lab-grown tissues. Although preserving whole organs for transplants is still a major hurdle, advances in cryobiology may 1 day make this a reality [33].

2.4 Thermodynamic and molecular mechanisms of cryopreservation in biological systems

2.4.1 Cryopreservation-induced cellular damage: Ice, osmotic stress, and membrane integrity

2.4.1.1 Intracellular ice formation (IIF)

IIF is widely regarded as the most lethal form of cryoinjury. As temperature drops below freezing, ice begins to form extracellularly, creating a solute-rich environment outside the cell [34]. If cooling is too rapid, water inside the cell does not have sufficient time to move out, leading to the formation of intracellular ice crystals, which mechanically damage organelles, cytoskeletal structures, and membranes. Research shows that IIF is more prevalent in cells with low membrane permeability, such as oocytes and certain primary cells. Ice crystals cause physical rupture of cellular components and irreversible death upon thawing. This is why slow cooling methods are typically preferred for such cells, to allow water to efflux before freezing occurs inside the cytoplasm [35]. See **Table 1** for a concise checklist of universal best-practice principles.

Principle	Why it is essential	Best-practice procedure	Risks of non-compliance
Pre-freeze quality check	Damaged or infected cells accumulate solute stress and ice lesions more readily than healthy cells.	Confirm $\geq 90\%$ viability (Trypan Blue or AO/PI) and mycoplasma-free status before adding CPA.	Lower starting quality cuts post-thaw recovery by 20–40%; contaminated stock seeds future flasks.
Cryoprotectant balance	A penetrating CPA (5–10% DMSO or propylene glycol) protects the cytoplasm, while a non-penetrating CPA (e.g., 2% HSA, 5% PEG, 0.2 M trehalose) controls extracellular ice and osmotic shock.	Prepare fresh, sterile CPA cocktail at 4°C; add dropwise to an equal volume of cell suspension while gently swirling. Equilibrate 5–10 minutes on ice.	Inadequate penetrating CPA $\rightarrow$ intracellular ice; no external additive $\rightarrow$ excessive shrink-swell cycles.
Controlled-rate cooling	Cooling at $\sim 1^\circ\text{C minute}^{-1}$ lets water leave the cell fast enough to prevent intracellular ice but slow enough to avoid lethal solute concentration outside.	From +4°C, cool $-1^\circ\text{C minute}^{-1}$ to -80°C , then transfer to LN_2 ($\leq -135^\circ\text{C}$ vapor or -196°C liquid).	$>2^\circ\text{C minute}^{-1} \rightarrow$ ice spicules inside cells; $<0.5^\circ\text{C minute}^{-1} \rightarrow$ solution effects kill cells.
Rapid thaw and CPA washout	DMSO becomes cytotoxic above $\sim 4^\circ\text{C}$; rapid warming prevents ice recrystallization. Immediate dilution lowers DMSO from 10% to $<1\%$.	Thaw vial in 37°C water bath ≤ 2 minutes. Transfer to $\geq 10\times$ pre-warmed medium/serum, mix gently, centrifuge ≤ 300 g 5 minutes, resuspend.	Slow thaw or small dilution prolongs DMSO exposure, causing membrane rupture and apoptosis.
Post-thaw recovery	Membrane-lipid flip-flops and cytoskeletal repair need time; some lymphocyte functions normalize only after resting.	Incubate cells 1–4 hours at $37^\circ\text{C}/5\% \text{CO}_2$ in complete medium (overnight for PBMCs before functional assays).	Direct use can give false-low viability, poor attachment, or skewed cytokine read-outs.

Table 1.
Universal core principles for cryopreserving any primary cell [36–39].

2.4.1.2 Osmotic shock

As extracellular water freezes, the remaining unfrozen solution becomes hypertonic, causing water to osmotically flow out of the cell [40]. This dehydration can lead to cell shrinkage, membrane distortion, and denaturation of proteins. Conversely, during rapid thawing, influx of water into still hyperosmotic cells can cause them to swell and rupture, termed hypotonic lysis. This dual-phase osmotic stress (dehydration during cooling and swelling during thawing) is a critical factor in designing cryopreservation protocols, particularly in primary cells, stem cells, and embryos [41].

2.4.1.3 Cryoprotectant toxicity

Cryoprotectants like dimethyl sulfoxide (DMSO) and glycerol are essential to mitigate freezing damage, but they are cytotoxic at high concentrations or prolonged exposure [42]. DMSO, for instance, disrupts lipid bilayers and protein structures if left at room temperature or physiological temperature for extended periods. Additionally, certain cell types, such as embryonic stem cells and neuronal progenitors, are particularly sensitive to CPA-induced changes in gene expression, oxidative stress, and differentiation capacity. To mitigate toxicity, CPAs are added and removed gradually and always at low temperatures to reduce chemical reactivity [43].

2.4.2 Balancing protection and toxicity: The role of CPAs in cryopreservation protocols

To protect cells during this process, scientists use (CPAs) such as DMSO, glycerol, ethylene glycol, or trehalose. These substances help prevent ice formation by lowering the freezing point of water inside the cells [44]. Instead of forming sharp, damaging crystals, the water becomes more like a smooth, glass-like solid, a process called vitrification. CPAs also help keep proteins and cell membranes stable by replacing water molecules and preserving important hydrogen bonds, which are essential for keeping cellular structures intact in the cold. However, CPAs are not without risks; they can be toxic if used in high doses or for too long. That is why cryopreservation protocols must be carefully designed [45]. For example, using a slow cooling rate (around $1^{\circ}\text{C minute}^{-1}$) gives water inside the cell time to move out before it can freeze. On the other hand, vitrification, which is often used for more sensitive cells like stem cells or egg cells, needs to happen extremely fast and with high concentrations of CPAs to stop ice from forming altogether [46].

2.4.3 The thawing phase: Minimizing cellular damage post-cryopreservation

Thawing is just as delicate as freezing. If warming happens too slowly, tiny ice crystals that formed during freezing can grow larger, tearing through membranes and causing new damage [47]. Plus, when dehydrated cells suddenly take in water during thawing, they can swell and burst, a problem known as osmotic shock. That is why cells are often warmed quickly, and CPAs are gradually diluted out to reduce stress [48].

2.4.4 Beyond the freeze: Ensuring molecular stability and tissue integrity in cryopreservation

On a molecular level, freezing and thawing can also lead to problems like protein denaturation, enzyme damage, and DNA fragmentation, especially if the freezing process is

not fine-tuned [49]. In larger tissues or lab-grown constructs, there are added challenges like protecting blood vessels, maintaining connections between cells and their surrounding matrix, and dealing with uneven water distribution, which can make freezing more complicated. In short, successful cryopreservation depends on understanding and balancing a wide range of factors, from physical freezing rates to cellular stress responses and biochemical stability. When done right, it lets scientists and doctors safely store cells and tissues for years without losing their function or structure [50, 51].

2.4.5 Ignoring cell-specific needs

One major issue in cryopreservation is using the same protocol for all types of primary cells. Primary cells, such as stem cells, hepatocytes (liver cells), and neurons, are much more delicate than immortalized cell lines, which are designed to be strong and easy to grow [52]. These sensitive cells have unique features, including different membrane structures, water movement, and stress responses. For example, stem cells may die or start to change if freezing or thawing conditions are not carefully controlled. Hepatocytes require oxygen and nutrients shortly after thawing to function correctly, and neurons can be damaged easily by ice due to their fragile structure. Applying a general freezing protocol that works for robust cell lines often leads to poor results with primary cells [53]. Incorrect concentrations of cryoprotectants, inappropriate freezing or thawing speeds, or missing recovery steps can reduce survival and affect how the cells behave. For instance, a method suitable for fibroblasts may cause damage to neuronal cells, affecting their ability to send signals. This can negatively impact research, drug testing, or cell-based therapies. To achieve good outcomes, it is essential to tailor the cryopreservation protocol to each cell type. Preparing the cells properly, using suitable cryoprotectants, and managing the thawing process carefully help maintain cell health and function after storage [54].

2.4.6 Optimizing post-thaw conditions for enhanced primary cell survival and function

Right after thawing is the most delicate time for primary cells [55]. They are under a lot of stress because of the sudden change in temperature, shifts in water movement, and leftover chemicals like DMSO that were used to protect them during freezing. If these chemicals are not quickly and carefully washed away, they can harm the cells by damaging their membranes, proteins, and internal parts [56]. On top of that, rough handling, like shaking the cells too hard, spinning them too fast in a centrifuge, or exposing them to more temperature changes, can physically damage cells that are still trying to recover. If the cells are not returned to the right conditions, such as warm, nutrient-rich culture media, soon after thawing, they may not survive, grow, or function as they should [57]. Even if the freezing process was done perfectly, poor handling after thawing can undo all that effort. To help cells recover and stay healthy, it is important to be gentle, remove harmful chemicals properly, and get them back into good growth conditions as quickly as possible [58, 59]. See **Table 2** for optimized cryopreservation protocols tailored to various primary cell types.

3. Conclusion

Cryopreservation plays a crucial role in modern life sciences by allowing scientists to store primary cells long-term while keeping their natural functions intact. Since

Primary cell type	Cryopreservation medium composition	Freezing protocol	Thawing and recovery protocol	Key considerations	Reference
Human dermal fibroblasts	DMEM + 10% DMSO + 20% FBS	Slow freezing at $-1^{\circ}\text{C minute}^{-1}$ to -80°C , then transfer to LN_2	Thaw rapidly at 37°C in water bath; dilute DMSO gradually with pre-warmed DMEM + 10% FBS	Very resilient, tolerates mild DMSO exposure; minimal cryoinjury if thawed promptly	[60]
Mesenchymal stem cells (MSCs)	α MEM + 10% DMSO + 10% human serum albumin	Controlled-rate freezer preferred; or Mr. Frosty to -80°C overnight	Quick thaw at 37°C ; dilute with culture medium over 5–10 minutes; wash thoroughly	Highly sensitive to DMSO; prolonged exposure post-thaw reduces viability	[61]
Primary human hepatocytes	William's E medium + 10% DMSO + 10% FBS	Isopropanol freezing container (Mr. Frosty) to -80°C , then LN_2	Rapid thaw; immediate transfer to oxygenated culture medium; add antioxidants if needed	Poor attachment post-thaw; requires optimized ECM and oxygen tension	[62]
Neurons (cortical/ iPSC-derived)	Neurobasal + B27 + 10% DMSO + 1% Glutamax	Slow cooling ($-1^{\circ}\text{C minute}^{-1}$); or vitrification for sensitive types	Gentle handling; pre-equilibrate with thaw medium; minimal centrifugation	Extremely fragile membranes, prone to osmotic lysis and differentiation drift	[63]
T cells/PBMCs	RPMI-1640 + 10% DMSO + 10% human AB serum	Slow freezing to -80°C , then transfer to LN_2	Thaw quickly; dilute with RPMI + serum; rest overnight before stimulation	CPA removal is critical to avoid activation artifacts	[64]
Organoids (e.g., intestinal, hepatic)	90% FBS + 10% DMSO (no basal medium)	Directly to -80°C , or snap freeze in LN_2 if small volume	Rapid thaw; embed in Matrigel droplets; refresh medium after 2–4 hours	Prefer serum-only cryopreservation; basal medium can reduce viability	[65]
Adipose-derived stem cells (ADSCs)	DMEM/F12 + 10% DMSO + 10% FBS	Controlled freezing at $1^{\circ}\text{C minute}^{-1}$	Thaw in 37°C bath; wash and seed on fibronectin-coated plates	Retain adipogenic potential post-thaw if washed well	[66]
Induced pluripotent stem cells (iPSCs)	mTeSR1 or E8 + 10% DMSO + ROCK inhibitor (e.g., Y-27632)	Vitrification or rapid freeze + ROCK inhibitor	Thaw in ROCKi-supplemented medium; gentle pipetting only	Require ROCKi to prevent apoptosis post-thaw	[67]

Table 2.
Optimized cryopreservation protocols for selected primary cell types.

these cells closely reflect how they behave in the human body, being able to freeze and later revive them is key for advancing personalized treatments, studying diseases, and developing regenerative therapies. Still, working with primary cells comes with its own set of challenges, they do not divide endlessly, vary between donors, and are more prone to damage from freezing and thawing. This chapter has walked through the science behind freezing cells safely, including how to balance the protective benefits and potential toxicity of cryoprotectants, the effects of freezing and thawing at the cellular level, and the common mistakes that can hurt cell survival. Success depends on getting the details right, like how quickly to freeze, how much cryoprotectant to use, and how to carefully thaw and handle the cells afterward. New approaches, such as rapid freezing techniques (vitrification) and more refined cryoprotectant mixes, are making it easier to preserve even the most sensitive cells. In the end, preserving primary cells is not just a technical skill, it is a vital step for producing consistent, scalable, and reliable results in research and clinical work. When researchers follow best practices and keep up with the latest developments in cryobiology, they set themselves up for success in using these valuable cells across a wide range of applications.

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Conflict of interest

The author declares no conflict of interest.

Acronyms and abbreviations

CPA	cryoprotective agent
DMSO	dimethyl sulfoxide
ESC	embryonic stem cells
IIF	intracellular ice formation
iPSC	induced pluripotent stem cells
ISBER	International Society for Biological and Environmental Repositories
LN2	liquid nitrogen
MSC	mesenchymal stem cells
RNA	ribonucleic acid
DNA	deoxyribonucleic acid
°C	degrees Celsius

Author details

Mohamed Hussein

Biomedical Sciences Department, Dubai Medical College of Girls, Dubai Medical University, Dubai, United Arab Emirates

*Address all correspondence to: dr.m.hussin@dmu.ae

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References

- [1] Dobruskin M, Toner G, Kander R. Optimizing cryopreservation strategies for scalable cell therapies: A comprehensive review with insights from iPSC-derived therapies. *Biotechnology Progress*. 2024;**40**(6):e3504. DOI: 10.1002/btpr.3504. Epub 2024 Sep 13
- [2] Greaves RIN, Davies JD. Separate effects of freezing, thawing and drying living cells. *Annals of the New York Academy of Sciences*. 1965;**125**(2):548-558. DOI: 10.1111/j.1749-6632.1965.tb45413.x
- [3] Meryman H. Modified model for the mechanism of freezing injury in erythrocytes. *Nature*. 1968;**218**:333-336. DOI: 10.1038/218333a0
- [4] Hoyt R, Szer J, Grigg A. Neurological events associated with the infusion of cryopreserved bone marrow and/or peripheral blood progenitor cells. *Bone Marrow Transplantation*. 2000;**25**(12):1285-1287. DOI: 10.1038/sj.bmt.1702443
- [5] Shu Z, Heimfeld S, Gao D. Hematopoietic SCT with cryopreserved grafts: Adverse reactions after transplantation and cryoprotectant removal before infusion. *Bone Marrow Transplantation*. 2014;**49**(4):469-476. DOI: 10.1038/bmt.2013.152. Epub 2013 Sep 30
- [6] Tan Y, Salkhordeh M, Murray ABP, Souza-Moreira L, Stewart DJ, Mei SHJ. Key quality parameter comparison of mesenchymal stem cell product cryopreserved in different cryopreservation solutions for clinical applications. *Frontiers in Bioengineering and Biotechnology*. 2024;**12**:1412811. DOI: 10.3389/fbioe.2024.1412811
- [7] Odanga JJ, Anderson SM, Presnell SC, LeCluyse EL, Chen J, Weaver JR. Impact of post-thaw enrichment of primary human hepatocytes on steatosis, inflammation, and fibrosis in the TruVivo® system. *Pharmaceuticals*. 2024;**17**(12):1624. DOI: 10.3390/ph17121624
- [8] Qi K, Jia D, Zhou S, Zhang K, Guan F, Yao M, et al. Cryopreservation of immune cells: Recent progress and challenges ahead. *Advanced Biology*. 2024;**8**(12):e2400201. DOI: 10.1002/adbi.202400201. Epub 2024 Aug 7
- [9] Ramos TV, Mathew AJ, Thompson ML, Ehrhardt RO. Standardized cryopreservation of human primary cells. *Current Protocols in Cell Biology*. 2014;**64**:A.3I.1-A.3I.8. DOI: 10.1002/0471143030.cba03is64
- [10] Rendal-Vázquez ME, Maneiro-Pampín E, Rodríguez-Cabarcos M, Fernández-Mallo O, López de Ullibarri I, Andiñón-Núñez C, et al. Effect of cryopreservation on human articular chondrocyte viability, proliferation, and collagen expression. *Cryobiology*. 2001;**42**(1):2-10. DOI: 10.1006/cryo.2001.2294
- [11] Xia Z, Murray D, Hulley PA, Triffitt JT, Price AJ. The viability and proliferation of human chondrocytes following cryopreservation. *Journal of Bone and Joint Surgery. British Volume (London)*. 2008;**90**(9):1245-1248. DOI: 10.1302/0301-620X.90B9.20652
- [12] Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ, et al. Cell viability assays. In: Markossian S, Grossman A, Baskir H, Arkin M, Auld D, Austin C, Baell J, Brimacombe K, TDY C, Coussens NP, Dahlin JL, Devanarayan V,

- Foley TL, Glicksman M, Gorshkov K, Grotegut S, Hall MD, Hoare S, Inglese J, Iversen PW, Lal-Nag M, Li Z, Manro JR, McGee J, Norvil A, Pearson M, Riss T, Saradjian P, Sittampalam GS, Tarselli MA, Trask OJ Jr, Weidner JR, Wildey MJ, Wilson K, Xia M, Xu X, editors. *Assay Guidance Manual*. Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences; 2013. p. 2004
- [13] Bhatia SN, Ingber DE. Microfluidic organs-on-chips. *Nature Biotechnology*. 2014;**32**(8):760-772. DOI: 10.1038/nbt.2989
- [14] Pamies D, Bal-Price A, Simeonov A, Tagle D, Allen D, Gerhold D, et al. Good cell culture practice for stem cells and stem-cell-derived models. *ALTEX*. 2017;**34**(1):95-132. DOI: 10.14573/altex.1607121. Epub 2016 Aug 23
- [15] Ferreira JN, Bhummaphan N, Chaisuparat R, Van Phan T, Oo Y, Jaru-Ampornpan P, et al. Unveiling senescence-associated ocular pathogenesis via lacrimal gland organoid magnetic bioassembly platform and HMGB1-Box A gene therapy. *Scientific Reports*. 2024;**14**(1):21784. DOI: 10.1038/s41598-024-73101-8
- [16] Rodboon T, Souza GR, Mutirangura A, Ferreira JN. Magnetic bioassembly platforms for establishing craniofacial exocrine gland organoids as aging in vitro models. *PLoS ONE*. 2022;**17**(8):e0272644. DOI: 10.1371/journal.pone.0272644
- [17] Davidson SM, Andreadou I, Antoniadis C, Bartunek J, Basso C, Brundel BJM, et al. Opportunities and challenges for the use of human samples in translational cardiovascular research: A scientific statement of the ESC Working Group on Cellular Biology of the Heart, the ESC Working Group on Cardiovascular Surgery, the ESC Council on Basic Cardiovascular Science, the ESC Scientists of Tomorrow, the European Association of Percutaneous Cardiovascular Interventions of the ESC, and the Heart Failure Association of the ESC. *Cardiovascular Research*. 2025;**121**(5):702-729. DOI: 10.1093/cvr/cvaf023
- [18] Wang X, Wang E, Zhao G. Advanced cryopreservation engineering strategies: The critical step to utilize stem cell products. *Cell Regeneration*. 2023;**12**(1):28. DOI: 10.1186/s13619-023-00173-8
- [19] Cheng Y, Yu Y, Zhang Y, et al. Cold-responsive nanocapsules enable the sole-cryoprotectant-trehalose cryopreservation of beta cell-laden hydrogels for diabetes treatment. *Small*. 2019;**15**:e1904290. DOI: 10.1002/smll.201904290
- [20] Chen SU, Yang YS. Slow freezing or vitrification of oocytes: Their effects on survival and meiotic spindles, and the time schedule for clinical practice. *Taiwanese Journal of Obstetrics & Gynecology*. 2009;**48**:15-22. DOI: 10.1016/S1028-4559(09)60030-9
- [21] Angel S, von Briesen H, Oh YJ, Baller MK, Zimmermann H, Germann A. Toward optimal cryopreservation and storage for achievement of high cell recovery and maintenance of cell viability and T cell functionality. *Biopreservation and Biobanking*. 2016;**14**(6):539-547. DOI: 10.1089/bio.2016.0046. Epub 2016 Oct 28
- [22] Mlotshwa M, Riou C, Chopera D, de Assis Rosa D, Ntale R, Treunicht F, et al. Fluidity of HIV-1-specific T-cell responses during acute and early subtype C HIV-1 infection and associations with early disease progression. *Journal of Virology*. 2010;**84**(22):12018-12029.

DOI: 10.1128/JVI.01472-10.
 Epub 2010 Sep 8

[23] Kwon SG, Kwon YW, Lee TW, Park GT, Kim JH. Recent advances in stem cell therapeutics and tissue engineering strategies. *Biomaterials Research*. 2018;**22**:36. DOI: 10.1186/s40824-018-0148-4

[24] Brandstadter JD, De Martin A, Lütge M, Ferreira A, Gaudette BT, Stanossek Y, et al. A novel cryopreservation and biobanking strategy to study lymphoid tissue stromal cells in human disease. *bioRxiv*. 2023:2023.02.06.525604. DOI: 10.1101/2023.02.06.525604. Update in: *Eur J Immunol*. 2023 Sep; **53**(9):e2250362. doi: 10.1002/eji.202250362

[25] Campbell LD, Astrin JJ, DeSouza Y, Giri J, Patel AA, Rawley-Payne M, et al. The 2018 revision of the ISBER best practices: Summary of changes and the editorial team's development process. *Biopreservation and Biobanking*. 2018;**16**(1):3-6. DOI: 10.1089/bio.2018.0001. Epub 2018 Feb 2

[26] Miyamoto Y. Cryopreservation of cell sheets for regenerative therapy: Application of vitrified hydrogel membranes. *Gels*. 2023;**9**(4):321. DOI: 10.3390/gels9040321

[27] Langer R, Vacanti JP. Tissue engineering. *Science*. 1993;**260**(5110): 920-926. DOI: 10.1126/science.8493529

[28] Vacanti CA, Vacanti JP. The science of tissue engineering. *The Orthopedic Clinics of North America*. 2000;**31**(3):351-356. DOI: 10.1016/s0030-5898(05)70155-3

[29] Chang T, Zhao G. Ice inhibition for cryopreservation: Materials, strategies, and challenges. *Advanced Science*. 2021;**8**(6):2002425. DOI: 10.1002/advs.202002425

[30] Uhrig M, Ezquer F, Ezquer M. Improving cell recovery: Freezing and thawing optimization of induced pluripotent stem cells. *Cells*. 2022;**11**(5):799. DOI: 10.3390/cells11050799

[31] Schliess F, Reinehr R, Häussinger D. Osmosensing and signaling in the regulation of mammalian cell function. *The FEBS Journal*. 2007;**274**(22):5799-5803. DOI: 10.1111/j.1742-4658.2007.06100.x. Epub 2007 Oct 17

[32] Ekpo MD, Boafu GF, Xie J, Liu X, Chen C, Tan S. Strategies in developing dimethyl sulfoxide (DMSO)-free cryopreservation protocols for biotherapeutics. *Frontiers in Immunology*. 2022;**13**:1030965. DOI: 10.3389/fimmu.2022.1030965

[33] Murray A, Kilbride P, Gibson MI. Trehalose in cryopreservation. Applications, mechanisms and intracellular delivery opportunities. *RSC Medicinal Chemistry*. 2024;**15**(9):2980-2995. DOI: 10.1039/d4md000174e

[34] Chaytor JL, Tokarew JM, Wu LK, Leclère M, Tam RY, Capicciotti CJ, et al. Inhibiting ice recrystallization and optimization of cell viability after cryopreservation. *Glycobiology*. 2012;**22**(1):123-133. DOI: 10.1093/glycob/cwr115. Epub 2011 Aug 17

[35] Choi JK, Yue T, Huang H, Zhao G, Zhang M, He X. The crucial role of zona pellucida in cryopreservation of oocytes by vitrification. *Cryobiology*. 2015;**71**(2):350-355. DOI: 10.1016/j.cryobiol.2015.08.012. Epub 2015 Aug 20

[36] Capes-Davis A, Freshney RI. *Freshney's Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications*. 8th ed. Hoboken, NJ: Wiley-Blackwell; 2021

- [37] Harbell JW. Best practices for cryopreserving, thawing, recovering, and assessing cells. *In Vitro Cellular and Developmental Biology - Animal*. 2017;**53**:880-889. DOI: 10.1007/s11626-017-0201-y
- [38] Wang L, Hückelhoven A, Hong J, Jin N, Mani J, Chen BA, et al. Standardization of cryopreserved peripheral blood mononuclear cells through a resting process for clinical immunomonitoring—Development of an algorithm. *Cytometry. Part A*. 2016;**89**(3):246-258. DOI: 10.1002/cyto.a.22813. Epub 2016 Feb 5
- [39] Kutscher S, Dembek CJ, Deckert S, Russo C, Körber N, Bogner JR, et al. Overnight resting of PBMC changes functional signatures of antigen specific T- cell responses: Impact for immune monitoring within clinical trials. *PLoS ONE*. 2013;**8**(10):e76215. DOI: 10.1371/journal.pone.0076215
- [40] de Camargo GC, da Cruz Landim-Alvarenga F, Maciel AP, Dos Santos DB, de Paula Freitas Dell'Aqua C, E Alvarenga ML, et al. Polyvinyl alcohol can replace the fetal bovine serum during cryopreservation of canine adipose mesenchymal stromal cells. *In Vitro Cellular & Developmental Biology. Animal*. 2025;**61**(4):369-373. DOI: 10.1007/s11626-025-01046-x. Epub 2025 May 21
- [41] Aldahmash A, Zaher W, Al-Nbaheen M, Kassem M. Human stromal (mesenchymal) stem cells: Basic biology and current clinical use for tissue regeneration. *Annals of Saudi Medicine*. 2012;**32**(1):68-77. DOI: 10.5144/0256-4947.2012.68
- [42] Baust JG, Gao D, Baust JM. Cryopreservation: An emerging paradigm change. *Organogenesis*. 2009;**5**(3):90-96. DOI: 10.4161/org.5.3.10021
- [43] Shu Z, Zhao X, Gao D. Cryobiology and biopreservation: Essential science and technology thriving from fundamental research to biomedical applications. *Biopreservation and Biobanking*. 2022;**20**(4):309-310. DOI: 10.1089/bio.2022.29109.editorial. Epub 2022 Jul 29
- [44] Murray KA, Gibson MI. Chemical approaches to cryopreservation. *Nature Reviews Chemistry*. 2022;**6**(8):579-593. DOI: 10.1038/s41570-022-00407-4. Epub 2022 Jul 18
- [45] Heumüller-Klug S, Maurer K, Tapia-Laliena MÁ, Sticht C, Christmann A, Mörz H, et al. Impact of cryopreservation on viability, gene expression and function of enteric nervous system derived neurospheres. *Frontiers in Cell and Development Biology*. 2023;**11**:1196472. DOI: 10.3389/fcell.2023.1196472
- [46] Saultz JN, Otegbeye F. Optimizing the cryopreservation and post-thaw recovery of natural killer cells is critical for the success of off-the-shelf platforms. *Frontiers in Immunology*. 2023;**14**:1304689. DOI: 10.3389/fimmu.2023.1304689
- [47] Guillouzo A, Rialland L, Fautrel A, Guyomard C. Survival and function of isolated hepatocytes after cryopreservation. *Chemico-Biological Interactions*. 1999;**121**(1):7-16. DOI: 10.1016/s0009-2797(99)00087-3
- [48] Daily MI, Whale TF, Partanen R, Harrison AD, Kilbride P, Lamb S, et al. Cryopreservation of primary cultures of mammalian somatic cells in 96-well plates benefits from control of ice nucleation. *Cryobiology*. 2020;**93**:62-69. DOI: 10.1016/j.cryobiol.2020.02.008. Epub 2020 Feb 21
- [49] Hermansen JU, Tjønnfjord GE, Munthe LA, Taskén K, Skånland SS.

Cryopreservation of primary B cells minimally influences their signaling responses. *Scientific Reports*. 2018;**8**(1):17651. DOI: 10.1038/s41598-018-36121-9

[50] Walker Z, Lanes A, Ginsburg E. Oocyte cryopreservation review: Outcomes of medical oocyte cryopreservation and planned oocyte cryopreservation. *Reproductive Biology and Endocrinology*. 2022;**20**(1):10. DOI: 10.1186/s12958-021-00884-0

[51] Taka S, Nikopoulou C, Polyzos A, Megremis S, Skevaki CL, Roumpedaki E, et al. Effects of cryopreservation on antiviral responses of primary airway epithelial cells. *Allergy*. 2020;**75**(6):1486-1489. DOI: 10.1111/all.14163. Epub 2020 Feb 8

[52] Hirsch A, Hirsh Raccah B, Rotem R, Hyman JH, Ben-Ami I, Tsafirir A. Planned oocyte cryopreservation: A systematic review and meta-regression analysis. *Human Reproduction Update*. 2024;**30**(5):558-568. DOI: 10.1093/humupd/dmae009. Erratum in: *Human Reproduction Update* 2024; 30(5):651. doi: 10.1093/humupd/dmae021

[53] Deller RC, Pessin JE, Vatish M, Mitchell DA, Gibson MI. Enhanced non-vitreous cryopreservation of immortalized and primary cells by ice-growth inhibiting polymers. *Biomaterials Science*. 2016;**4**(7):1079-1084. DOI: 10.1039/c6bm00129g. Epub 2016 May 6

[54] Akiyama Y, Shinose M, Watanabe H, Yamada S, Kanda Y. Cryoprotectant-free cryopreservation of mammalian cells by superflash freezing. *Proceedings of the National Academy of Sciences of the United States of America*. 2019;**116**(16):7738-7743. DOI: 10.1073/pnas.1808645116. Epub 2019 Apr 1

[55] Cui M, Liu L, Chen L, Han H, Zhan T, Dang H, et al. New cryoprotectant loading method for cell droplet vitrification with continuous evaporation. *Langmuir*. 2022;**38**(46):14129-14139. DOI: 10.1021/acs.langmuir.2c02082. Epub 2022 Nov 9

[56] Zhan L, Guo SZ, Kangas J, Shao Q, Shiao M, Khosla K, et al. Conduction cooling and plasmonic heating dramatically increase droplet vitrification volumes for cell cryopreservation. *Advanced Science*. 2021;**8**(11):2004605. DOI: 10.1002/advs.202004605

[57] de Vries RJ, Banik PD, Nagpal S, Weng L, Ozer S, van Gulik TM, et al. Bulk droplet vitrification for primary hepatocyte preservation. *Journal of Visualized Experiments*. 2019;(152):e60250. DOI: 10.3791/60250

[58] Hunt CJ. Cryopreservation: Vitrification and controlled rate cooling. *Methods in Molecular Biology*. 2017;**1590**:41-77. DOI: 10.1007/978-1-4939-6921-0_5

[59] Wolkers WF, Oldenhof H. Principles underlying cryopreservation and freeze-drying of cells and tissues. *Methods in Molecular Biology*. 2021;**2180**:3-25. DOI: 10.1007/978-1-0716-0783-1_1

[60] Ziliani F, Michalak-Micka K, Klar AS. Isolation and culture of human dermal fibroblasts. In: *Skin Tissue Engineering, Methods in Molecular Biology*. Vol. 2922. New York, NY, USA: Springer; 2025. pp. 75-83. DOI: 10.1007/978-1-0716-4510-9_6

[61] Haack-Sørensen M, Eklund A, Kastrup J. Cryopreservation and revival of human mesenchymal stromal cells. In: *Mesenchymal Stem Cells, Methods in Molecular Biology*. Vol. 1416. New York, NY, USA: Humana Press; 2016. pp. 357-374. DOI: 10.1007/978-1-4939-3584-0_21

[62] Lloyd TDR, Orr S, Skett P, Berry DP, Dennison AR. Cryopreservation of hepatocytes: A review of current methods for banking. *Cell and Tissue Banking*. 2003;**4**(1):3-15. DOI: 10.1023/A:1026392216017

[63] Kuleshova LL, Tan FCK, Magalhães R, Gouk SS, Lee KH, Dawe GS. Effective cryopreservation of neural stem or progenitor cells without serum or proteins by vitrification. *Cell Transplantation*. 2009;**18**(2):135-144. DOI: 10.3727/096368909788341298

[64] Hønge BL, Petersen MS, Olesen R, Møller BK, Erikstrup C. Optimizing recovery of frozen human peripheral blood mononuclear cells for flow cytometry. *PLoS ONE*. 2017;**12**(11):e0187440. DOI: 10.1371/journal.pone.0187440

[65] Lee BE, Lee BJ, Lee KJ, Lee M, Lim YJ, Choi JK, et al. A simple and efficient cryopreservation method for mouse small intestinal and colon organoids for regenerative medicine. *Biochemical and Biophysical Research Communications*. 2022;**595**:14-21. DOI: 10.1016/j.bbrc.2021.12.021. Epub 2022 Jan 15. Erratum in: *Biochem Biophys Res Commun*. 2022 Apr 30; 602:186. doi: 10.1016/j.bbrc.2022.02.111

[66] Shaik S, Devireddy R. Cryopreservation protocols for human adipose tissue–derived adult stem cells. In: *Adipose-Derived Stem Cells, Methods in Molecular Biology*. Vol. 1773. New York, NY, USA: Humana Press; 2018. pp. 231-259. DOI: 10.1007/978-1-4939-7799-4_19

[67] Watanabe K, Ueno M, Kamiya D, Nishiyama A, Matsumura M, Wataya T, et al. A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nature Biotechnology*. 2007;**25**(6):681-686. DOI: 10.1038/nbt1310

Chapter 2

Mycoplasma Contamination of Primary Cell Cultures

Oksana A. Leonovich

Abstract

Contamination of cells with mycoplasmas is a serious problem in modern cell biology. The special biological properties of mycoplasmas – small size (0.3–0.8 μm), lack of a rigid cell wall, and high adaptability – are the reasons for their contamination of 10% to 90% of cell lines used in biotechnology and scientific research. Primary cell cultures are especially vulnerable to contamination. Mycoplasmas are capable of changing cell metabolism, affecting their morphology and functional properties. This can lead to serious distortions of research results and cause significant damage to the production of biotechnological drugs. Various methods are used to detect mycoplasmas in cell cultures: cultural, microscopic, molecular biological, and immunological. A combined approach, incorporating various methods, is considered the most effective. Decontamination of mycoplasma contaminants includes mechanical removal, physical methods, and antibiotic therapy. However, treatment is associated with the risk of damaging the cell cultures themselves. The key element of protection against mycoplasma contamination is prevention, including strict control of incoming materials, organization of the workspace, regular monitoring, and professional training of personnel. Modern trends in the fight against mycoplasma contamination are aimed at the introduction of innovative technologies using artificial intelligence, nanotechnology, automated diagnostic systems, and new pharmacological drugs.

Keywords: mycoplasma, cell cultures, contamination, diagnostics, decontamination

1. Introduction

Cell technologies play a key role in modern biomedicine. Cell cultures have become an indispensable tool in various areas of pharmaceutical production. They are actively used in the creation of the most important medical immunobiological drugs, including vaccines against dangerous diseases such as measles, flu, and poliomyelitis, and also serve as a basis for obtaining a variety of biologically active compounds and drugs [1, 2]. Modern biotechnology allows the use of cell cultures to develop innovative genetically engineered drugs that open up new prospects for the treatment of various diseases [3]. Such a variety of applications makes the issue of cell culture purity critically important for ensuring the safety and effectiveness of the resulting biopreparations. Cell cultures obtained from human and animal tissues,

especially when used in the production of immunobiological drugs, must be sterile, viable, morphologically uniform, non-tumorigenic, and free of foreign agents.

In the mid-twentieth century, during an experiment on artificially infecting cell cultures with mycoplasmas to study their effect on eukaryotic cells, it was discovered that the culture under study already contained a mycoplasma infection [4]. This observation became the starting point in studying the problem of contamination of cell cultures and marked the beginning of systematic studies on the effect of mycoplasmas on cultured cells. This discovery served as the basis for the first official report on the problem of mycoplasma contamination in cell cultures [4].

Studies have shown that cell cultures obtained from a wide variety of eukaryotic organisms are at risk of contamination by these microorganisms [4, 5]. Such universality of pathogenic impact makes the problem of mycoplasma contamination especially important and most widespread in all areas of modern cell biology and biotechnology.

Specific biological characteristics of mycoplasmas, their wide prevalence in combination with a high reproduction rate and ability to effectively use components of the nutrient medium, allow them to easily penetrate cells, quickly and imperceptibly spread in laboratory conditions, avoiding detection by standard control methods used for other bacteria [6].

Mycoplasmas are capable of changing the metabolism of cells, affecting their morphology and functional properties, which can lead to serious distortions of research results and cause significant damage in the production of biotechnological drugs [6]. Primary cultures obtained directly from body tissues are especially vulnerable, since mycoplasma can be present in the body initially or may get into the primary cell culture during repeated manipulations with it [7].

This chapter examines a comprehensive study of the problem of mycoplasma contamination, including the biological characteristics of mycoplasmas, routes and sources of infection, diagnostic methods, and strategies for preventing this type of contamination. All data presented are based on modern scientific research and international standards.

2. Biology of mycoplasmas: Main species causing contamination of cell cultures

Mycoplasmas belong to the *Mycoplasmataceae* family, class *Mollicutes*. They are the smallest (0.3 to 0.8 μm) of all free-living bacteria capable of independent reproduction [6]. Distinctive properties of mycoplasmas include the absence of a traditional cell wall and the presence of a flexible lipoprotein membrane [6]. This gives them unique properties [8, 9]: it allows mycoplasmas to easily penetrate many bacterial filters used for sterilization and makes them resistant to some classes of antibiotics, such as beta-lactams, which act on most bacteria.

Mycoplasmas have a limited genome, which makes them dependent on the host cell for the synthesis of vital substances and determines their parasitic lifestyle [10, 11].

Mycoplasmas can both attach to the surface of cells and penetrate inside, actively using the metabolic processes of the host [12]. The absence of a rigid cell wall facilitates the formation of close contact between the membranes of the parasite and the host, which sometimes leads to the fusion [4, 13] of mycoplasma with the host cell.

Mycoplasmas are characterized by specific mechanisms of adaptation to unfavorable environmental factors, which significantly increase their virulence [14, 15]. The conditions for culturing eukaryotic cells provide a favorable environment for the reproduction of mycoplasmas *in-vitro* [4, 15, 16].

According to data obtained by various scientific groups, contamination of cultured cell lines with mycoplasmas varies from 10–15% to 45–90% [5, 6, 17, 18]. Studies of contaminated cell cultures have revealed more than two dozen different types of mycoplasmas [6, 17]. In most cases of infection (90–95%), mycoplasmas from humans, cattle, and pigs are detected in cell cultures [6, 19]. *Mycoplasma orale*, an opportunistic pathogen for humans [20], accounts for 20–40% of all mycoplasma infections in cell cultures [6]. A significant proportion (10–20%) is also made up of the species *Mycoplasma fermentans* and *Mycoplasma hominis*, which inhabit the urogenital tract and oropharynx of humans and have long been considered normal microflora of healthy people [21]. About a third of all isolated strains belong to the group of mycoplasmas associated with cattle: *Mycoplasma arginini* (20–30%) and *Acholeplasma laidlawii* (5–20%). A significant proportion (10–40%) of mycoplasmas detected in infected cell cultures are *Mycoplasma hyorhinis*, a common inhabitant of the porcine nasal cavity [6]. This diversity of mycoplasma species and potential interspecies contamination poses serious challenges in the prevention and control of cell culture purity.

3. Routes and sources of contamination

Mycoplasma infection in laboratory conditions occurs mainly through direct physical contact with contaminated surfaces, instruments, and consumables, as well as through contaminated serums and nutrient media [5]. Airborne spread of microorganisms in poorly ventilated rooms, as well as cross-infection during parallel work with different cell cultures, poses a significant risk [22].

Due to their microscopic size and ability to exist in a latent state, mycoplasmas can remain undetected for a long time, causing chronic contamination of cell populations [6, 13]. According to literature data, 10–15% of all cell lines used in the USA [23] and 15–35% of widely used cell lines in Germany are contaminated with mycoplasmas [24].

The main sources of contamination are biological media of animal origin used for cell cultivation. Cattle blood serum, trypsin, growth factors, and other biological preparations are especially dangerous [18, 25]. Studies show that up to 32% of cattle serum batches contain mycoplasma microorganisms [25].

Mycoplasmas often colonize the oral cavity and respiratory tract of humans, which facilitates their transmission through non-sterile hand surfaces or respiratory secretions during talking and coughing [20, 21]. Insufficient hygiene and carelessness when working with cultures increase the risk of contamination.

Aerosol particles formed during manipulations with cultures can contain a significant number of mycoplasmas (up to 0.5×10^6 cells) and quickly settle on the surfaces of the workspace, dishes, and hands of personnel, which leads to large-scale contamination even after working with single infected cultures [26].

Primary cell cultures require special attention due to their increased susceptibility to mycoplasma contamination and the possibility of unimpeded reproduction of parasites in them [7]. Primary cell cultures are obtained directly from body tissues,

which determine their unique biological properties and requires special cultivation conditions. Their increased vulnerability is associated with several key factors:

3.1 Initial contamination of donor tissues

When preparing primary cultures, mycoplasmas can penetrate cell isolates and remain undetected until cultivation, becoming a source of internal infection.

3.2 Lack of adaptive defense mechanisms

Primary cells retain most of the characteristics of the donor tissue and do not have stable defense mechanisms or resistance, which are formed during long-term culture. This makes them more vulnerable to the effects of mycoplasmas and their toxins.

3.3 Multi-stage technological processes

Obtaining a primary culture involves many procedures: tissue dissociation, filtration, washing, transplantation, and the use of various solutions and preparations.

3.4 Features of the nutrient medium

Primary cultures require rich nutrient media with the addition of serums and growth factors, which can be potential sources of mycoplasmas. An enriched medium also promotes the rapid reproduction of mycoplasmas, which compete with cells for resources and alter their metabolism.

3.5 Heterogeneity of the cell population

Unlike continuous lines, primary cultures are mixed populations that include different types and states of cells, which complicate the detection and destruction of mycoplasmas and also complicate the diagnosis of infection.

4. The effect of mycoplasma infection on cell culture: Consequences for scientific research and pharmaceutical production

Mycoplasmas can remain latent for a long time, causing serious pathological changes in the structure and function of cells [6]. The pathological effect of mycoplasmas on cells is manifested in metabolic disorders, changes in cell morphology and growth, suppression or, conversely, stimulation of proliferation [27]. Mycoplasmas can change the pH of culture media, which leads to the gradual degeneration of the cell culture [28].

It has been found that mycoplasmas affect the synthesis of proteins, nucleic acids, angiogenesis, cell membranes, induction of tumor-forming potential, and also cause cytopathic effects and cell death [25]. A series of special studies devoted to elucidating the features of transcriptional profile modulation in cell cultures contaminated with mycoplasmas showed that mycoplasma infection causes

a disruption in the expression of some genes in the host cell [28–31]. Changes in expression can be significant within a few hours after infection [31], and long-term cultivation of infected cells (18 weeks) can lead to their irreversible transformation (**Table 1** [29]).

A notable feature of mycoplasma infection is its ability to mask its presence even at significant concentrations of the pathogen (more than 10^7 CFU/ml) [32]. Obvious visual changes in the culture may be unnoticeable, while significant deviations are observed in the internal state of the cell culture. This can lead to false results in scientific studies, as well as negatively affect the production of biological products due to competition for nutrients in the environment or the release of toxic metabolic products [31]. Damage to the cell culture, up to its complete death, can be a serious consequence of contamination. Studies demonstrate serious consequences of contamination of cell lines for experimental work [32–34]. For example, when studying the metabolism of beta-amyloid peptide in Alzheimer's disease in cell models, it was found that mycoplasmas contaminating cell cultures are capable of actively destroying extracellular protein structures, which leads to distortion of research data (**Figure 1** [34]). Studies have shown such pathological effects of mycoplasma contamination of cells as the formation of vacuolated cells, disruption of the integrity and formation of a mesh structure of the monolayer, a decrease in proliferative activity, and possible death of the cell population (**Figure 2** [32]).

Contamination of cell cultures with mycoplasmas for the scientific community leads to significant time costs for repeated experiments, unjustified expenditure of material resources, the obtaining of unreliable research data, and the distortion of laboratory research results.

Production losses for biotechnological production include significant costs of diagnostic studies, costs of decontamination procedures, possible loss of valuable cell lines, and investments in preventive measures. The problem of mycoplasma contamination is becoming especially relevant due to the widespread use of primary cell cultures in the development of innovative drugs, vaccine production, and in the practice of regenerative medicine. Contamination of therapeutic biopreparations and vaccines for various purposes, as well as other medical products, poses a serious danger to human health [5, 6]. To ensure the safety of products obtained from cell cultures, most countries have introduced strict requirements for viral vaccines against measles, rubella, polio, and some other drugs. Mandatory control for the absence of mycoplasma contamination is an integral part of the production process and a guarantee of the safety of the final product for human health. This approach allows us to minimize risks and ensure high quality of biotechnological products that meet modern international standards.

5. Mycoplasma detection methods

The most important aspect of modern biotechnology and scientific research is to ensure a high degree of purity in cell cultures. Therefore, the development of effective methods for detecting mycoplasmas remains a priority task in this area.

The main methods for detecting mycoplasma in cell cultures include cultural, cytochemical (cytofluorimetry), molecular biological methods (PCR, hybridization, sequencing), immunological, and biochemical technologies. Each method has its own advantages and limitations in sensitivity and specificity, which determine their

Gene class	Screened	Expressed in control cells	Number of genes								
			Expressed and altered in cells following mycoplasmal infection								
			1 week			11 weeks			18 weeks		
			Expressed	Increased	Decreased	Expressed	Increased	Decreased	Expressed	Increased	Decreased
Apoptosis associated proteins	48	30	34	4	2	27	3	7	25	6	5
Cell adhesion receptors/proteins	100	61	60	6	1	61	9	10	69	12	3
Cell cycle associated proteins	45	34	31	2	4	26	2	9	35	2	5
Cell receptors (by activities)	162	74	90	16	5	90	26	16	70	9	14
Cell signaling, extracellular communication proteins	144	89	82	8	4	98	18	10	112	11	6
Cytoskeleton/motility proteins	59	39	41	4	3	37	8	8	51	6	7
DNA binding and chromatin proteins	44	23	29	5	1	29	6	1	31	8	0
Extracellular transport/carrier proteins	6	4	5	0	0	3	1	0	5	0	0
Extracellular matrix proteins	30	28	29	3	2	29	4	1	27	2	7
Functional unclassified	15	8	11	3	1	13	5	1	13	2	1
Intracellular transducers/ effectors/modulators	253	155	158	20	6	149	26	28	201	32	10
Membrane channels, transporters, and cell surface antigens	27	16	16	4	1	20	7	0	18	4	1

Gene class	Screened	Expressed in control cells	Number of genes Expressed and altered in cells following mycoplasmal infection									
			1 week		11 weeks		18 weeks		Expressed	Decreased	Increased	Decreased
			Expressed	Increased	Expressed	Increased	Expressed	Increased				
Metabolism-associated proteins	26	14	16	5	1	18	4	2	20	3	0	0
Oncogenes and tumor suppressors	65	43	46	3	1	41	6	7	53	8	2	2
Stress response proteins	39	24	22	1	1	21	3	2	26	2	3	3
Trafficking/targeting proteins	5	2	1	0	0	2	0	0	3	0	0	0
Transcription associated proteins	30	27	20	3	4	25	2	3	29	1	0	0
Translation, post-translation modification, and protein turnover	87	65	64	5	6	58	8	9	74	7	5	5
Total	1185	736	755	92	43	748	138	114	862	115	69	69

The expression of 1185 genes involved in various cellular functions was analyzed using the AtlasImage™ 2.01 software (Clontech Laboratories, Inc.) computer program. The study showed changes in gene function in C3H cells during infection by M. fermentans. The number of genes with increased activity (7.76%) rose one week after infection, reached a peak by week 11 (11.65%), and slightly decreased by week 18 of infection (9.53%). The number of genes with decreased expression increased over time, from 3.63% to 5.82% of the total.

Table 1.
Gene expression profile of C3H cells at different stages of mycoplasma-induced transformation [29].

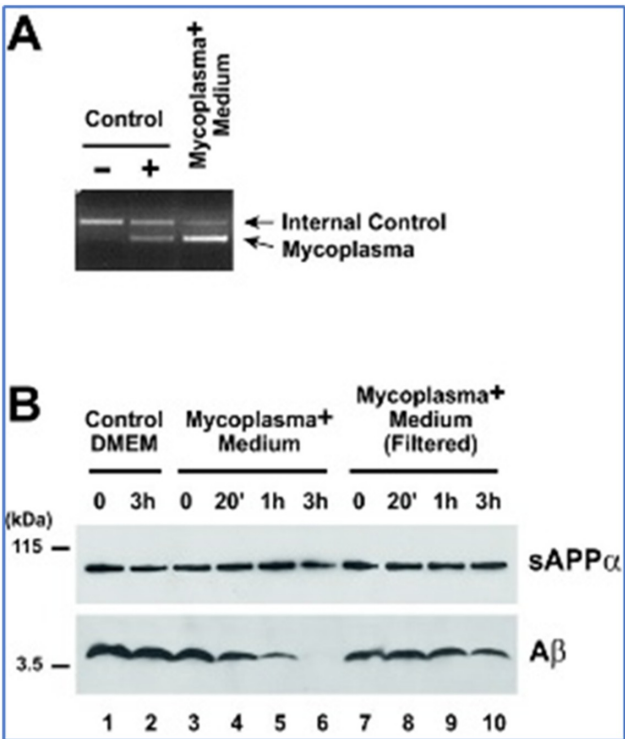


Figure 1. Degradation of extracellular A β protein by *M. Hyorhinis* contaminating HEK293 cells [34]. (A) PCR of mycoplasma-containing samples (Mycosensor kit, Stratagene); “control +” is *M. orale*. (B) Analysis of APP and A β levels by western blot. “DMEM control” is pure culture medium without mycoplasma; “mycoplasma + medium” is medium with mycoplasma; “mycoplasma + medium (filtered)” is filtered (pore size 0.2 μ m) medium from mycoplasma-infected cells. No A β accumulation was observed in the growth medium of cells transfected with amyloid β precursor protein (APP) and infected with mycoplasma. Elimination of mycoplasma from cells restored extracellular A β accumulation.

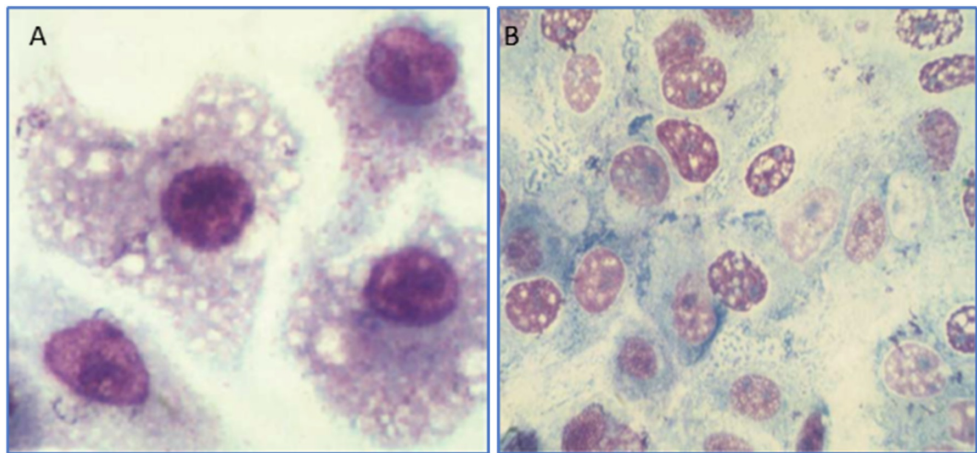


Figure 2. Cultures of PK-13 and Vero cells contaminated with mycoplasma [32]. (A) Pronounced vacuolization of the cytoplasm in PK-13 cells; (B) Vacuolization of the nucleus of Vero cells. Romanowsky-Giemsa staining, x250.

use depending on the specific objectives of the study and the requirements for diagnostic accuracy.

5.1 Classical microbiological method

Refers to the classical, direct method of detecting mycoplasmas [35, 36]. The essence of the method is to seed samples (medium or cell suspension) on selective media (PPLO broth, Sabouraud agar, etc.), incubate aerobically at 37°C, and then visually detect colonies. Hayflick and Kagan media are suitable for growing most mycoplasmas. When conducting the analysis, a liquid medium is used to accumulate mycoplasma, a semi-liquid medium for primary control (0.3% agar), and a solid medium (1.3% agar) to detect mycoplasma species. The composition of the basic liquid medium includes bovine heart hydrolyzate, meat extract or meat water, and yeast extract. Liquid and semi-liquid media are incubated in test tubes, and the solid medium is incubated on Petri dishes. The analysis time (incubation on selective media) is from 7 to 28 days, depending on the concentration of mycoplasma in the sample. On average, the final results are taken into account on the 21st day. Visual control is carried out on the 3rd, 7th, 10th, and 14th days by examining the test tubes in transmitted light (**Figure 3**). Mycoplasma growth in a semi-liquid medium is determined by characteristic turbidity and granularity; on a solid medium – by the formation of characteristic colonies on a dense medium. The analysis necessarily



Figure 3.
Detection of mycoplasma in the culture medium of Vero E6 cells. The analysis was carried out in semi-liquid Kagan medium in test tubes. On the seventh day, mycoplasma was clearly visible at the site of seeding.

includes the use of a test strain as a positive control, a negative control, and testing the growth properties of each batch of media. There are no strict requirements for the selection of a mycoplasma strain to control the sensitivity of the media and identify the inhibitory effect of the sample. It is recommended to use *M. orale*, *M. hominis*, and *M. fermentans* approved by the European Pharmacopoeia [36].

The sensitivity of the culturing method is approximately ~100–1,000 CFU/ml (colony-forming units/ml). The advantages of this method include the ability to detect live, reproducing mycoplasmas with the possibility of further species identification. The obvious disadvantages include a long analysis time (up to 3 weeks), possible false-negative results at low concentrations of mycoplasmas or the presence of antibiotics in the medium, and the inability to detect non-viable mycoplasmas. In addition, many mycoplasma species grow poorly on cell-free media, and some cannot be cultivated *in-vitro* at all. When performing this test, the medium may be contaminated with mycoplasmas from external sources, such as the researcher, medium components, and laboratory equipment.

5.2 Cytofluorometric method

Also refers to the standard for detecting mycoplasma, regulated by the main regulatory documents for detecting mycoplasmas [36]. The method involves treating cells with fluorescent dyes that bind DNA (DAPI, Hoechst, less often olivomycin), followed by visual detection of extranuclear mycoplasma signals using fluorescence microscopy. The cells to be examined are washed with saline, placed on glass slides, fixed (with ice-cold acetone, 96% ethanol), and stained according to the instructions for the dye. The samples are analyzed for mycoplasma contamination using a fluorescence microscope at a magnification of $\times 40$ –100 (**Figure 4** [37, 38]). The sensitivity of the method is about 1000 CFU/ml. The analysis time (cell staining, detection) is about 1–2 hours. The advantages of this method are its speed and the ability to detect mycoplasmas directly in cell cultures. Disadvantages include low sensitivity compared to other methods and dependence of the result on the personal experience of the operator (difficulty in visually distinguishing mycoplasmas from

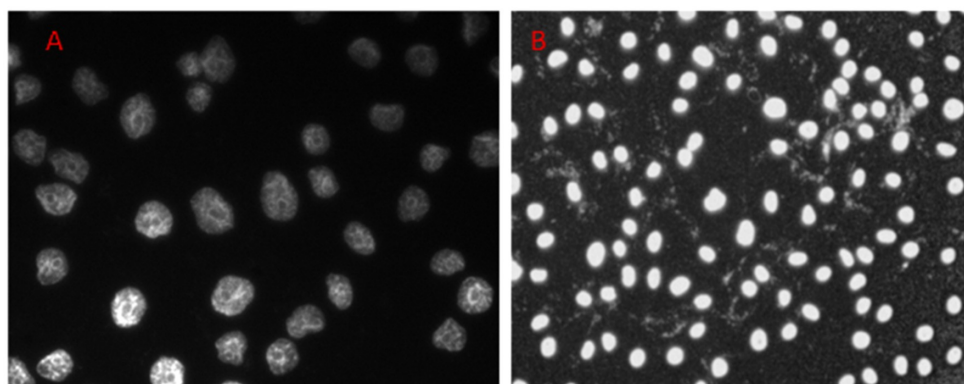


Figure 4. Cytochemical analysis using Hoechst 33,258 dye [37]. Vero cells: no mycoplasma (A), strong mycoplasma infection (B). Vero cell nuclei are visible as large spots, while mycoplasma appears as a web of fine granular spots. Image obtained using a fluorescence microscope with a 40x immersion objective.

other bacteria or cellular debris). Nevertheless, this method is very popular due to its simplicity and the possibility of detecting non-culturable mycoplasmas or mycoplasmas that grow poorly in cell-free media in cell cultures [39]. In this case, the test is carried out using an indicator cell culture free of mycoplasmas. The test sample is added to the indicator culture and grown at 37 °C in a humid environment on slides in special chambers or Petri dishes for 3 to 7 days until a monolayer of 50–70% (no more) is achieved. Then the cell monolayer is washed, fixed, and stained with a fluorescent dye in a standard manner. However, the duration of the test in this case is significantly increased, which also increases the risk of contaminant spread in the laboratory.

5.3 DNA hybridization (PCR)

The PCR method has become increasingly widespread in recent years for diagnosing mycoplasma infection [40, 41]. The principle of analysis is based on multiple amplification of a portion of the genome, with subsequent detection of the reaction product by different methods. Oligonucleotides for amplification of variable regions of 16S rDNA or rRNA, as well as sequences of intergenic regions 16–23S, are usually used as primers. Amplification of specific fragments of mycoplasma DNA, with subsequent visualization using gel electrophoresis (**Figure 5** [42, 43]) allows for the simultaneous detection of a wide range of mycoplasma species with high sensitivity of up to 1–10 CFU/ml (for real-time PCR) and specificity (>95%). The analysis time is 2–6 hours. The disadvantages of the method include the possibility of obtaining false positives (the method allows for the detection of not only living but also inactive or destroyed microorganisms) or false negatives (the results depend on the quality of the DNA samples) negatives. The method requires a highly skilled and qualified operator. The European Pharmacopoeia recommends the use of the method as a supplement to microbiological and cytochemical methods. However, the

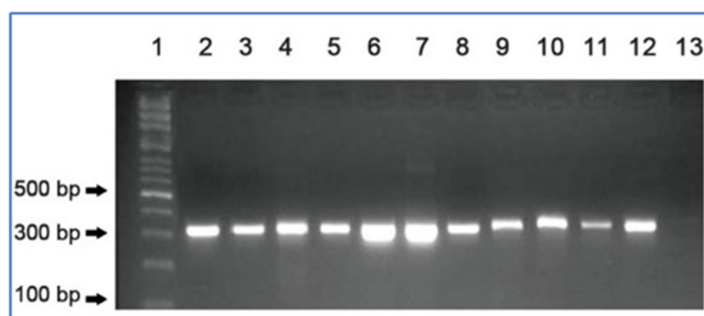


Figure 5. PCR analysis [42] with detection in 1% gel electrophoresis of DNA of different *Mycoplasma* strains using two universal and 11 species-specific *Mycoplasma* primers: (1) DNA size marker, 100 bp DNA ladder, Roche XIV, (2) *U. urealyticum*, (3) *m. fermentans*, (4) *M. oral*, (5) *M. salivarium*, (6) *M. hominis*, (7) *A. laidlawii*, (8) *M. pirum*, (9) *M. pneumoniae*, (10) *M. genitalium*, (11) *M. hyorhinitis*, (12) *M. arginini*, (13) water without DNA – negative control. PCR reaction mixtures were prepared in a total volume of 25 μ l containing 1 $\times$ PCR buffer, 50 μ M of each dNTP, 15 pmol of each primer, 1 U Taq DNA polymerase, 1.5 mM MgCl₂ and 0.1 μ g of mycoplasma genomic DNA (or 1 μ g of cell line sample DNA). PCR cycles were: initial denaturation at 94 °C for 3 min, 32 cycles consisting of denaturation at 94 °C for 60 s, annealing at 60 °C for 30 s, and extension at 72 °C for 60 s, followed by a final extension at 72 °C for 5 min. The annealing temperature of the universal primers was 55 °C [43].

PCR method is approved by international expert organizations [44]. Currently, there are a sufficient number of kits for testing cell cultures for mycoplasmas on the commercial market.

5.4 Enzyme-linked immunosorbent assay

The essence of the method is to detect mycoplasma antigens using specific antibodies [45]. The Mycoplasma Detection Kit from Roche, Germany, has proven itself well [45]. The kit contains four species-specific polyclonal antibodies to *A. laidlawii*, *M. arginini*, *M. hyorhinae*, and *M. orale*. The sensitivity of the method is approximately $\sim 10^3$ – 10^3 – 10^4 CFU/ml. The analysis time is 4–6 hours. The undoubted advantages of this method are the possibility of its automation and, as a result, the possibility of screening a large number of samples. The disadvantages include low specificity (reaction to partially destroyed or dead mycoplasmas), which requires validation taking into account the panel of antibodies used.

5.5 Bioluminescent (biochemical) test

The method is based on the detection of specific metabolites, such as substrate degradation by mycoplasma, followed by the recording of the light signal [46]. The MycoAlert Mycoplasma Detection Assay (Lonza), based on a biochemical test that utilizes mycoplasma enzyme activity, has proven effective [46]. During the test, viable mycoplasmas are lysed, and the released enzymes react with the MycoAlert substrate, catalyzing the conversion of ADP to ATP. The ATP level is measured using a bioluminescent reaction. By measuring the ATP level in the sample before and after adding the MycoAlert substrate, a ratio can be obtained, indicating the presence or absence of mycoplasma. The method has undoubted advantages: high sensitivity of $\sim 10^3$ CFU/ml, due to the detection of low concentrations of ATP, linear dependence of the signal on concentration, and the ability to work with sample dilutions up to 1:32. The advantages of biochemical tests are speed (no more than 3–5 hours), no need for complex expensive equipment and reagents, and the possibility of high-throughput screening (HTS). The disadvantages include the likelihood of host cell metabolism influencing the results.

In modern mycoplasmaology, there is a wide range of methods for detecting contamination of cell cultures. However, a comprehensive study of the effectiveness of several diagnostic strategies [47] showed that none of the methods considered provided completely satisfactory diagnostic effectiveness, which is confirmed by the data of other research groups [48, 49]. The study analyzed methods of specific staining of samples, methods involving sowing on special media, methods of immunological diagnostics, and electron microscopy [47]. Modern trends in the development of detection methods are aimed at increasing the sensitivity and speed of analysis while maintaining high specificity in detecting contamination with mycoplasmas. The most effective approach at present is considered to be combined diagnostics, including PCR, culture methods, and enzyme immunoassay [50].

For regular monitoring, PCR and enzyme immunoassay are preferable, providing an optimal combination of high specificity and sensitivity with process efficiency. Classical seeding on nutrient media retains its importance for the species identification of mycoplasmas and confirmation of their viability. When choosing

a diagnostic method in modern biotechnological production, it is also necessary to correlate the financial capabilities of the enterprise, the time constraints of the process, and the required accuracy of the results.

6. Problems of decontamination

When creating and maintaining unique cell lines that are highly sensitive to various viruses, producing high titers of vaccine strains of viruses, or cell cultures producing recombinant drugs, the problem of freeing cells from mycoplasmas arises. The difficulties of eliminating mycoplasmas are associated with the high resistance of mycoplasmas and the possibility of developing resistance to antibiotics, the risk of damaging the cell cultures themselves during processing, the need for a complete replacement of media and materials, as well as the time spent on cleaning and verification. Antibiotics, chemical reagents, physical, and mechanical methods are used to decontaminate cell cultures [51, 52]:

6.1 Mechanical removal

The method is based on multiple cell passages with medium changes [53]. Its use, especially for the decontamination of primary cultures, is extremely limited [54]. This method is not the primary method for removing mycoplasma from cell cultures; it is most often used in combination with other methods (e.g., antibiotics).

6.2 Physical methods

These include exposure to elevated temperatures (up to 40–42°C) [55], photoinactivation [56], and filtration through special 0.1 µm filters. Filters with a size of 0.22 µm can partially stop mycoplasma cells (e.g., antibiotics), but are not suitable for all of its species [57]. Physical methods are well-suited for decontaminating liquid media from mycoplasmas- but are rarely applicable to cell cultures, as they can damage the cells themselves [56].

6.3 Antibiotic therapy

It is one of the main methods of decontaminating cell cultures from mycoplasmas. Of all the existing methods, antibiotic therapy remains the most widespread and relatively effective [52]. The main difficulties during elution are associated with the possibility of mycoplasmas developing resistance to antibiotics and significant risks of damage to the cell cultures themselves during processing. Since mycoplasmas, due to the absence of a cell wall, are resistant to many typical antibiotics, there is a need for specialized antibiotic therapy [58]. Antibiotic therapy requires a comprehensive approach, including fairly long courses (from 1 week or more) and personalized selection of treatment regimens, taking into account the state of the cells and the genetic characteristics of the mycoplasma strains.

The main groups of antibiotics recognized as effective for the treatment of mycoplasma infection in cell cultures are:

- Quinolones (e.g., ciprofloxacin, ofloxacin, enrofloxacin) have a broad spectrum of antimycoplasma action, but there are restrictions on their use depending on the type of cell culture [59–61].
- Tetracyclines (e.g., doxycycline, minocycline) are commonly used, but resistance may develop in some species of mycoplasma [16, 62, 63].
- Macrolides (e.g., tylosin, clarithromycin) are used as an alternative when other groups of antibiotics are ineffective and in polymicrobial infections [16, 63].

Recently, complex therapy, including the sequential use of various drugs and combinations of drugs from different pharmacological groups (example protocol: enrofloxacin therapy for 10–14 days followed by replacement with tylosin), has increasingly demonstrated the best results in practice [64]. This approach significantly increases the efficiency of cell decontamination and reduces the risk of mycoplasma resistance.

Commercial new-generation drugs have proven themselves as ready-made solutions for mycoplasma decontamination: MycoZap [65], Plasmocin [49, 50], mycoplasma removal agent [52, 66], which also contain mixtures of antibiotics and are often effective against most mycoplasmas [67].

When using individual antibiotics, it is necessary to take into account a number of factors, which are especially important when processing primary cell cultures:

- It is necessary to minimize stress and toxic effects on cells: give preference to mild antibiotics, use minimal doses, and be sure to conduct preliminary testing for cell sensitivity to antibiotics.
- It is necessary to take into account the possibility of re-infection, which can be extremely high; it is important to maintain sterility, use tested reagents, and consumables.
- For primary cultures, the duration of therapy should be minimal reagents, since the risk of cytotoxicity is high.

According to researchers, the therapeutic effect of antibiotics (or other agents) on contaminated cultures is associated with serious risks. The use of antibiotics and other chemical agents can lead to the disruption of natural biological processes and the formation of new characteristics in the cell culture [68]. It is important not only to understand the effectiveness of decontamination methods but also to take into account the possible changes in cell properties after treatment with drugs. Stem, progenitor, or poorly differentiated cells contaminated with mycoplasmas, used to study the differentiation and metabolism of various cell types or their potential use in regenerative medicine, are not subject to decontamination and are necessarily rejected.

Decontamination of cell cultures from mycoplasmas remains a key task in cell biology, as mycoplasmas disrupt metabolic processes, alter morphological characteristics, and affect the sensitivity of cells to external factors. This is especially critical for primary cultures, which exhibit increased sensitivity to therapy and low resistance to aggressive effects. For primary cultures, it is extremely important to

select a drug and treatment regimen that prevents the loss of a valuable cell culture. The optimal strategy remains prevention (strict asepsis), rapid identification (regular monitoring and timely detection of contamination), and specific antibiotic therapy (most often a combination of quinolones, tetracyclines, and macrolides in non-cytotoxic doses). Successful work with cell cultures requires an integrated approach, where prevention plays a key role, and treatment is considered a necessary measure, taking into account all potential risks and consequences for valuable cell lines.

7. Preventive measures against mycoplasma contamination

In the modern conditions of biotechnological production and scientific research, it is critical to create a reliable system of protection against mycoplasma contamination. Effective prevention is a multi-level structure of measures, where each element plays a significant role in the overall safety system [6, 69].

The basis for protection against mycoplasma contamination is careful control of incoming materials. All new cell lines and reagents are carefully tested for the absence of mycoplasmas. Particular attention is paid to the selection of suppliers, giving preference to companies providing certified serums and nutrient media with documented quality.

Organization of a sterile workspace when working with cell lines is the most important element of contamination prevention. In the laboratory, it is necessary to create zones with different levels of cleanliness to minimize the risk of cross-contamination. The use of a full set of protective clothing by personnel is mandatory, including disposable gowns, gloves, and masks. Regular treatment of equipment with specialized disinfectants, including those containing DNase, is required. Modern air purification technologies are implemented through a system of HEPA filters in laminar boxes. The premises of the boxes and all work surfaces are sterilized with ultraviolet radiation.

Constant monitoring of the state of the culture is required [70], carried out using various diagnostic methods, among which PCR diagnostics occupies a special place. It is necessary to track and analyze suspicious changes in cell growth are sterilized and keep a clear control log.

Each newly received cell line must undergo a mandatory quarantine period, during which all necessary tests for the absence of mycoplasmas are carried out [16].

Professional training of employees plays a key role in the prevention system. Regular training in biosafety and modern control methods ensures a high level of staff competence.

If signs of infection are detected, an isolation protocol for the affected culture must be immediately implemented. After confirming contamination by several independent methods, a decision is made on the future fate of the cell line [71]. Having a reserve stock of frozen, mycoplasma-free cultures is also an important part of the safety strategy.

The practical application of the described system shows impressive results. The introduction of a comprehensive approach to prevention allows us to reduce the level of contamination to minimum levels. Regular PCR diagnostics, combined with a clear separation of work areas, ensures a reduction in the percentage of infections from 12% to less than 1% within a year. The use of modern screening systems, such as

Lonza MycoAlert, allows us to identify hidden cases of infection at an early stage, preventing the spread of infection.

Thus, only a systematic approach to the prevention of mycoplasma contamination, combining technical, organizational, and educational measures, can provide reliable protection for both laboratory research and industrial production of biopreparations.

8. Modern approaches to detection and control of mycoplasma contamination in cell cultures

In recent years, there has been significant progress in the methods of detection and elimination of mycoplasma infections in cell cultures [72–85]. Traditional methods are supplemented by innovative technologies that open up new possibilities in diagnostics and treatment.

Artificial intelligence is actively being introduced into monitoring processes. Modern machine learning algorithms analyze autofluorescence patterns of individual cells, which allow for the detection of mycoplasma-infected cells with high accuracy (more than 70%) [72]. This approach opens up new prospects in the early diagnosis of contamination.

Nanotechnology is becoming an important area in the fight against mycoplasma infection. Nanosensors and nanobiotech chips provide highly sensitive detection of pathogens [73], and nanotherapy offers innovative ways to kill them [74]. A study of andrographolide poloxamer nanoparticles (AND-ALG-POL/NP), obtained by wet milling, revealed a therapeutic effect against *M. pneumoniae* infection [74]. AND-ALG-POL/NP demonstrated slow release in-vitro and significant inhibitory activity against mycoplasma, with an average drug load of $13.17 \pm 0.54\%$. At the same time, the nanoparticles were not cytotoxic to normal cells, reduced apoptosis, and increased levels of inflammatory factors caused by mycoplasma infection.

Pharmacological innovations aim to develop new-generation multicomponent drugs. Modern broad-spectrum antibiotics can effectively combat various types of mycoplasmas while preserving valuable cell lines from destruction [75].

Molecular technologies open up new horizons in the fight against contamination. The possibilities of using antimicrobial peptides, silver nanoparticles, and CRISPR–Cas systems are being explored [76]. These methods allow for targeted action on mycoplasmas, minimizing the negative impact on cultured cells.

Automation of processes plays a key role in improving diagnostic accuracy. Robotic platforms, such as Tecan and Hamilton, provide automated sampling and testing, significantly reducing the likelihood of human error and increasing the reproducibility of results.

Experimental developments include the creation of genetically modified cell lines resistant to mycoplasma infection [77]. The method involves introducing an antibiotic resistance gene into cells. The resulting cell tolerance to high concentrations of antibiotics is sufficient to destroy the mycoplasma contaminating the cells without damaging the host cells themselves. After the removal of the vector, the cells recover and return to their original sensitivity. Successful curing of BHK-21 cells from *A. laidlawii* using the pUCSV-Neo vector for resistance to G418 has been demonstrated [77].

These innovative approaches together form a new level of control over mycoplasma contamination, allowing for more effective protection of valuable cell cultures and ensuring the reliability of biotechnological processes.

In modern mycoplasmaology, there is also a constant improvement in existing diagnostic methods, which significantly expands the possibilities for monitoring mycoplasma contamination, increasing the accuracy and speed of infection detection.

A significant breakthrough has occurred in the field of quantitative PCR diagnostics. Modern, next-generation test systems allow for the simultaneous detection of several mycoplasma species in cell cultures [78]. These detection systems are characterized by exceptional sensitivity – up to 10 colony-forming units per milliliter – and results are available in 1–2 hours. A wide range of solutions from leading manufacturers is available on the market, among which are the MycoSEQ (Life Technologies), the MycoTOOL real-time mycoplasma detection kit (Roche), the VenorGEM qOneStep kit (Minerva Biolabs), the ATCC universal mycoplasma detection kit, the Biofire mycoplasma assay (bioMérieux Industry), and the Minerva Biolabs Venor®GeM qEP kit [79].

Metagenomic studies open new horizons in contamination diagnostics [80]. NGS (next-generation sequencing) technologies allow not only the detection of mycoplasmas but also the identification of any foreign microorganisms in the culture. Large biotech companies and biobanks are actively implementing this method for regular monitoring of the purity of their collections.

Loop-mediated Isothermal Amplification (LAMP) technology is becoming a promising direction [81]. This innovative method is easy to implement and fast – results can be obtained in just 45 minutes. An important advantage is the lack of need for expensive equipment, which makes it ideal for rapid diagnostics both in production conditions and in field studies. Portable systems are being developed for on-site diagnostics [82].

Of considerable interest are developments in the field of mass spectrometry [83, 84]. Scientists are working on developing protocols that allow direct identification of mycoplasmas from a culture medium using MALDI-TOF MS, which opens up new possibilities for rapid and accurate diagnostics.

Of particular note is digital PCR, which allows for the detection of even minimal amounts of mycoplasmas. This method is especially valuable in the production of biopreparations, where an absolute guarantee of product purity is required. The high sensitivity of digital PCR makes it an indispensable tool in modern diagnostics of mycoplasma contamination [85].

The future of diagnostics and control of mycoplasma contamination is determined by several important areas of development. Automation of processes is becoming a priority vector for the development of the industry. The introduction of robotic control systems will significantly increase the efficiency of monitoring cell cultures and minimize the human factor in the diagnostic process. Comprehensive screening based on advanced technologies, especially NGS methods, opens up new opportunities for a comprehensive assessment of the purity of cell collections. This approach provides the most complete picture of the state of cultures and allows for identifying not only mycoplasmas for identifying but also other potential contaminants. In the field of contamination treatment, there is a shift toward innovative solutions [86]. Developers are increasingly turning to immunotherapy based on specific antibodies, actively

exploring the possibilities of genomic technologies using CRISPR systems, and creating new selective drugs. International standardization is becoming an important aspect of the industry's development. The unification of approaches to quality control and diagnostic methodology will ensure a single high level of safety in all countries.

Mycoplasma contamination continues to be one of the most serious threats to modern cell technology. Solving this problem requires continuous improvement of prevention methods, development of new approaches to detection, and creation of effective strategies for eliminating contamination. Only an integrated approach that includes all stages of working with cell cultures – from receipt to storage – can provide reliable protection against mycoplasma contamination. This requires not only the use of modern technologies detection, but also strict adherence to protocols, continuous training of personnel, and the introduction of innovative solutions into work practices. Thus, the future of the fight against mycoplasma contamination lies in the integration of advanced technologies, standardization of processes, and the development of an interdisciplinary approach to solving this urgent problem.

Author details

Oksana A. Leonovich

Chumakov Federal Scientific Center for Research and Development of Immune-and-Biological Products of Russian Academy of Sciences, Institute of Poliomyelitis, Moscow, Russia

*Address all correspondence to: leonovich_oa@chumakovs.su

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References

- [1] Neumann K-H, et al. Plant cell and tissue culture – A tool in Biotechnology. Basics and Application. 2020;**21**:459
- [2] Sommer L, Rao M. Neural stem cells and regulation of cell number. Progress in Neurobiology. 2002;**66**:1–18. DOI: 10.1016/s0301-0082(01)00022-3
- [3] Ozturk SS, Hu WS, Eds.. Cell Culture Technology for Pharmaceutical and Cell-Based Therapies. Vol. **XIII**. Boca Raton, FL, USA: CRC Press, Taylor & Francis Group; 2006. 755 p
- [4] Robinson LB, et al. Contamination of human cell cultures by pleuropneumonia-like organisms. Science. 1956;**124**:1147–1148
- [5] Rottem S, et al, Contamination of Tissue Cultures by Mycoplasmas, Biomedical Tissue Culture. Luca CN, and Barbara, M, editors. London: IntechOpen. 2012. DOI: 10.5772/51518
- [6] Drexler HG, Uphoff CC. Mycoplasma contamination of cell cultures: Incidence, sources, effects, detection, elimination, prevention. Cytotechnology. 2002;**39**:75–90. DOI: 10.1023/A:1022913015916
- [7] Weiskirchen S, et al. A beginner's guide to cell culture: Practical advice for preventing needless problems. Cells. 2023;**12**(5):682. DOI: 10.3390/cells12050682
- [8] Archer DB, Daniels MJ. Plant and Insect Mycoplasma Techniques. Daniels MJ, Markham PG, Eds. London: Springer; 1982. 9–39 p
- [9] Razin S. The Prokaryotes. Balows A, editor. New York: Springer-Verlag; 1991. 1937–1959 p
- [10] Fraser CM, et al. The Minimal Gene Complement of Mycoplasma genitalium. Science. 1995;**270** (5235):397–404. DOI: 10.1126/science.270.5235.397
- [11] Glass JI, et al. Essential genes of a minimal bacterium. Proceedings of the National Academy of Sciences. 2006;**103**(2):425–430. DOI: 10.1073/pnas.0510013103
- [12] Lo SC, et al. Mycoplasma penetrans sp. nov., from the Urogenital tract of patients with AIDS. International Journal of Systematic and Evolutionary Microbiology. 1992;**42**(3). DOI: 10.1099/00207713-42-3-357
- [13] Rottem S. Interaction of mycoplasmas with host cells. Physiological Reviews. 2003;**83**:417–432. DOI: 10.1152/physrev.00030.2002
- [14] Liu W, et al. Comparative genomics of Mycoplasma: Analysis of conserved essential genes and diversity of the Pan-Genome. PLoS One. 2012;**7** (4):e35698. DOI: 10.1371/journal.pone.0035698
- [15] Razin S, Hayflick L. Highlights of mycoplasma research—An Historical perspective. Biological. 2010;**38**(2): P.183–190. DOI: 10.1016/j.biologicals.2009.11.008
- [16] Uphoff CC, Drexler HG. Eradication of mycoplasma contaminations. Methods in Molecular Biology. 2011;**731**:105–117. DOI: 10.1007/978-1-61779-080-5_7

- [17] Hopert A, et al. Mycoplasma detection by PCR analysis. *In Vitro Cellular & Developmental Biology - Animal*. 1993;**29**:819–821
- [18] Rosengarten R. Quality Control — Mycoplasma Testing. 2006 [Internet]. Available from: <https://www.vu-wien.ac.at/i102/QCMycopl/qcmycoplE.htm>
- [19] Kong F, et al. Species-specific PCR for identification of common contaminant mollicutes in cell culture. *Applied and Environmental Microbiology*. 2001;**67**(7):3195–3200. DOI: 10.1128/AEM.67.7.3195-3200.2001
- [20] Kumagai K, et al. Incidence, species, and significance of Mycoplasma species in the mouth. *Journal of Infectious Diseases*. 1971;**123**:16–21
- [21] Taylor-Robinson D, Purcell RH. Mycoplasmas of the human urogenital tract and oropharynx and their possible role in disease: A review with some recent observations. *Proceedings of the Royal Society of Medicine*. 1966;**59**(11):1112–1116. DOI: 10.1177/003591576605911P121
- [22] McGarrity GJ. Spread and control of mycoplasmal infection of cell cultures. *In Vitro Cellular & Developmental Biology — Plant*. 1976;**12**:643–648. DOI: 10.1007/BF02797464
- [23] Tully JG. Diagnosis of mycoplasma infections of cell cultures. Introductory remarks. In: Tully JG, Razin S, eds. *Molecular and Diagnostic Procedures in Mycoplasmaology, Diagnostic Procedures*. Vol. 2. San Diego: Academic Press, Inc; 1996. p. 405–410
- [24] Uphoff CC, et al. Elimination of mycoplasma from leukemia-lymphoma cell lines using antibiotics. *Leukemia*. 2002;**16**(2):284–288. DOI: 10.1038/sj.leu.2402364
- [25] Lincoln C, et al. Cell culture contamination: Sources, consequences, prevention, and elimination. *Methods in Cell Biology*. 1998;**57**:49–65
- [26] Gignac SM, et al. Elimination of mycoplasma from infected leukemia cell lines. *Leukemia*. 1991;**5**:62–165
- [27] Feng N, Huang X, Jia Y. Mycoplasma contamination affects cell characteristics and decreases the sensitivity of BV2 microglia to LPS stimulation. *Cytotechnology*. 2019;**71**:623–634. DOI: 10.1007/s10616-019-00311-8
- [28] Macpherson I. Mycoplasmas in tissue culture. *Journal of Cell Science*. 1966;**I**:145–168
- [29] Zhang S, et al. Alteration of gene expression profiles during Mycoplasma-induced malignant cell transformation. *BMC Cancer*. 2006;**6**:116
- [30] Zhang S, et al. Mycoplasmal infections alter gene expression in cultured human prostatic and cervical epithelial cells. *FEMS Immunology & Medical Microbiology*. 2000;**27**(1): 43–50. DOI: 10.1111/j.1574-695X.2000.tb01410.x
- [31] Lavrič M, et al. Gene expression modulation in chicken macrophages exposed to Mycoplasma synoviae or Escherichia coli. *Veterinary Microbiology*. 2008;**126**:111–121
- [32] Shalunova NV, et al. Mycoplasma contamination of cell cultures. Biopreparations. Preventions, Diagnostics, Treatment. (In Russian).

2016;**16**(3):151–160.

Available from: <https://www.biopreparations.ru/jour/article/view/58/108>

[33] Winner F, RosengarTen R, Citti C. In Vitro Cell Invasion of Mycoplasma gallisepticum. *Infection and Immunity*. 2000;**68**(7):4238–4244. DOI: 10.1128/IAI.68.7.4238-4244.2000

[34] Zhao H, Dreses-Werringloer U, Davies P, Marambaud P. Amyloid-beta peptide degradation in cell cultures by mycoplasma contaminants. *BMC Research Notes*. 2008;**1**(38). DOI: 10.1186/1756-0500-1-38

[35] Barile MF, Rottem S. Mycoplasmas in Cell Culture. Rapid Diagnosis of Mycoplasmas. Vol. **62**. In: Kahane I, and Adoni A editors. Springer New York: Plenum Press. 1993;155–193. Available from: https://link.springer.com/chapter/10.1007/978-1-4615-2478-6_12 p

[36] European Pharmacopoeia (Ph. Eur.) 11 O. 2022 Mycoplasmas. p.210–215. Available from: <https://www.webofpharma.com/2023/06/european-pharmacopoeia-11th-edition-pdf.html>.

[37] Leonovich OA, et al. Experience in decontamination of viral strains from Mycoplasmas. *VetPat (In Russian)*. 2020;**3**:29–37

[38] Spierenburg GT, et al. Indicator cell lines for the detection of hidden mycoplasma contamination, using an adenosine phosphorylase screening test. *Journal of Immunological Methods*. 1988;**114**(1-2):115–119. DOI: 10.1016/0022-1759(88)90162-7

[39] Milne C, Daas A. Establishment of European pharmacopoeia Mycoplasma reference strains. *Pharmeuropa Bio*. 2000; **377**–72

[40] Harasawa R, et. al. Rapid detection and differentiation of the major mycoplasma contaminants in cell cultures using real-time PCR with SYBR Green I and melting curve analysis. *Microbiology and Immunology* 2005;**49**:859–863

[41] Han X, et al. Amplification of 16S rDNA by nested PCR for measurement of Mycoplasma pneumoniae DNA over time: Clinical application. *Journal of Medical Microbiology*. 2012;**61**:426–430

[42] Molla Kazemiha V, et al. Effectiveness of Plasmocure™ in Elimination of mycoplasma species from contaminated cell cultures: A comparative study versus other antibiotics. *Cell Journal*. 2019;**21**(2):143–149. DOI: 10.22074/cellj.2019.5996

[43] Molla Kazemiha V, et al. PCR-based detection and eradication of mycoplasmal infections from various mammalian cell lines: A local experience. *Cytotechnology*. 2009;**61**(3):117–124. DOI: 10.1007/s10616-010-9252-6

[44] Uphoff CC, Drexler HG. Detecting Mycoplasma contamination in cell cultures by polymerase chain reaction. *Methods in Molecular Medicine*. 2004; (88):319–326. DOI: 10.1385/1-59259-406-9:319

[45] Dvorakova H, et al. Detection of mycoplasma contamination in cell cultures and bovine sera. *Veterinární Medicína*. 2005;**50**(6):262–268. DOI: 10.17221/5622-VETMED

[46] Falagan-Lotsch P, et al. Performance of PCR-based and Bioluminescent assays for mycoplasma detection. *Journal of Microbiological Methods*. 2015;**118**:31–36

- [47] House W, et al Methods for detecting mycoplasma in cell cultures. Imperial Cancer Research Fund Reports. 1973;108–109
- [48] Drexler HG. Mycoplasma Contamination of Cell Cultures. In: The Encyclopedia of Cell Technology. Drexler HG, Uphoff CC, Spier RE, eds. New York: Wiley; 2000. p. 609–627
- [49] Uphoff CC, et al. Mycoplasma contamination in human leukemia cell lines. I. Comparison of various detection methods. Journal of Immunological Methods. 1992;149:43–53
- [50] Amanda Capes-Davis R, Ian F. Freshney's Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications. 8th edn. Wiley-Blackwell; 2021
- [51] Uphoff CC, et al. Treatment of Mycoplasma contamination in cell cultures with Plasmocin. Journal of Biomedicine and Biotechnology. 2012;2012:267678. DOI: 10.1155/2012/267678
- [52] Uphoff CC, Drexler HG. Eradication of mycoplasma contaminations. Methods in Molecular Biology. 2005;290:25–34. DOI: 10.1385/1-59259-838-2:025
- [53] Ogawa, et al. Decontamination of mycoplasma-contaminated Orientia tsutsugamushi strains by repeated passages through cell cultures with antibiotics. BMC Microbiology. 2013;13(32). Available from: <https://www.biomedcentral.com/1471-2180/13/32>
- [54] Huang X. Prevention, diagnosis, and elimination of mycoplasma infection of cell cultures. Journal of Biological Methods. 2023;10:e99010005. DOI: 10.14440/jbm.2023.407
- [55] Voronin ES. Contaminants of Veterinary Viral Vaccines. Vol. 60–62. Moscow: Agropromizdat; 1986. p. 88–95
- [56] Giulio J. Photodynamic therapy of microbial infections: Current status and prospects. Journal of Environmental Pathology, Toxicology and Oncology. 2006;25(1–2):505–519. DOI: 10.1615/JEnvironPatholToxicolOncol.v25.i1-2.320
- [57] Gaurivaud P. Mycoplasmas are no exception to extracellular vesicles release: Revisiting old concepts. PLOS ONE. 2018;13:e0208160. DOI: 10.1371/journal.pone.0208160
- [58] Chernova OA. Mycoplasmas and their resistance to antibiotics: Problems and prospects for controlling mycoplasma infections and contamination of cell cultures. Acta Naturae. 2016;8(2):27–38
- [59] Arközy GS. Quinolones: A class of antimicrobial agents. Veterinárni Medicina. 2001;46(9–10):257–274
- [60] Gohara Y. In vitro antimicrobial activities of new quinolone antibiotics against Mycoplasma pneumoniae. Nippon Saikingaku Zasshi. 1992;47(2):387–393. DOI: 10.3412/jsb.47.387
- [61] Soltanian B. Mycoplasma contamination in cell cultures treated with ciprofloxacin and enrofloxacin: Brief report. Tehran University Medical Journal (TUMJ). 2015;72(11):794–798
- [62] Wen X, et al. Tetracyclines resistance in Mycoplasma and Ureaplasma urogenital isolates derived from human: A systematic review and meta-analysis. Annals of Clinical

Microbiology and Antimicrobials.
2023;22:83

[63] Taylor-Robinson D, Bébéar C. Antibiotic susceptibilities of mycoplasmas and treatment of mycoplasmal infections. *Journal of Antimicrobial Chemotherapy*. 1997;40(5):622–630. DOI: 10.1093/jac/40.5.622

[64] Wang C, et al. In vitro susceptibility of avian mycoplasmas to enrofloxacin, sarafloxacin, tylosin, and oxytetracycline. *Avian Diseases* 2001;45:456–460

[65] Mariotti E, et al. Mollicutes contamination: A new strategy for an effective rescue of cancer cell lines. *Biologicals*. 2012;40(1):88–91. DOI: 10.1016/j.biologicals.2011.10.006

[66] Gignac SM, et al. Treatment of mycoplasma-contaminated continuous cell lines with mycoplasma removal agent (MRA). *Leukemia Research*. 1992;16(8):815–822. DOI: 10.1016/0145-2126(92)90161-y

[67] Uphoff CC, Drexler HG. Comparative antibiotic eradication of mycoplasma infections from continuous cell lines. *In Vitro Cellular & Developmental Biology - Animal*. 2002;38(2):86–89. DOI: 10.1290/1071-2690(2002)038<0086:CAEOMI>2.0.CO;2

[68] Ann H, et al. Use antibiotics in cell culture with caution: Genome-wide identification of antibiotic-induced changes in gene expression and regulation. *ViroLIEgy*. 2021; DOI: 10.1101/130484

[69] Nikfarjam L, Farzaneh P. Prevention and detection of Mycoplasma contamination in cell culture. *Cell Journal*. 2012;13(4):

203–212. Available from: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3583899>

[70] McGarrity GJ. Spread and control of mycoplasmal infection of cell cultures. *In Vitro Cellular & Developmental Biology—Plant*. 1976;12:643–647. DOI: 10.1007/BF02797464

[71] Hay RJ, et al. Mycoplasma infection of cultured cells. *Nature*. 1989; 339:487–488

[72] Bamba K, et al. Discrimination of mycoplasma infection using machine learning models trained on autofluorescence signatures of host cells. *Sensors & Diagnostics*. 2024;3:287–294

[73] Matini A, Naghib SM. The necessity of nanotechnology in Mycoplasma pneumoniae detection: A comprehensive examination. *Sensing and Bio-Sensing Research*. 2024;43:100631. DOI: 10.1016/j.sbsr.2024.100631

[74] Liling W, et al. In-vitro and in-vivo study of andrographolide nanoparticles for the treatment of Mycoplasma pneumoniae pneumonia. *Biochemical and Biophysical Research Communications*. 2024;698:149540. DOI: 10.1016/j.bbrc.2024.149540

[75] Malave-Ramos DR, et al. Safe, effective, and inexpensive clearance of Mycoplasma contamination from cultures of Apicomplexan Parasites with Sparfloxacin. *Microbiology Spectrum*. 2022;10(5):e0349722. DOI: 10.1128/spectrum.03497-22

[76] Lpoutcha, et al. Genome Editing of veterinary relevant Mycoplasmas using

a CRISPR-Cas Base Editor System. Applied and Environmental Microbiology. 2022;88:e0099622. DOI: 10.1128/aem.00996-22

[77] Gabriele M, et al. Permanent mycoplasma removal from tissue culture cells: A genetic approach. Biotechnology and Bioprocess Engineering. 2000;5:84–91

[78] Schnee C. A novel rapid DNA Microarray Assay Enables Identification of 37 Mycoplasma Species and Highlights Multiple Mycoplasma Infections. PLoS One. 2012;E7(3): e33237. DOI: 10.1371/journal.pone.0033237

[79] Totten AH, et al. Comparison of five commercial molecular assays for mycoplasma testing of cellular therapy products. Journal of Clinical Microbiology. 2023;61(2):e0149822. DOI: 10.1128/jcm.01498-22

[80] Wei G, et al. Clinical metagenomic next-generation sequencing for pathogen detection. Annual Review of Pathology. 2018;14:319–338

[81] Notomi T, et al. Loop-mediated isothermal amplification of DNA. Nucleic Acids Research. 2000;28(12): E63. DOI: 10.1093/nar/28.12.e63

[82] Wang A, et al. Rapid and sensitive screening system of Mycoplasma hyorhinis based on Sandwich-DNA hybridization with nano-amplifiers. Materials Today Advances. 2023;20:100437.1

[83] McDaniel AJ, Derscheid RJ. MALDI-TOF mass spectrometry and high-resolution melting PCR for the identification of Mycoplasma bovis isolates. BMC Veterinary Research. 2021;17:170

[84] Baudler L, et al. Identification and differentiation of avian Mycoplasma species using MALDI-TOF MS. Journal of Veterinary Diagnostic Investigation. 2019;31(4):620–624. DOI: 10.1177/1040638719856932

[85] Sidorova IA, et al. Detecting mycoplasma contamination of cell cultures by means of digital drop-PCR. Cell Therapy and Transplantation. 2022;11(3):1–132. DOI: 10.18620/ctt-1866-8836-2022-11-3-1-132

[86] Weng J, et al. Elimination of Mycoplasma contamination from infected Human Hepatocyte C3A cells by intraperitoneal injection in BALB/c Mice. Frontiers in Cellular and Infection Microbiology. 2017;7:440

Primary Cell Isolation from Solid Tissues and Current Understanding for Their Application in Personalized Medicine and Drug Development

Erika Berenyi

Abstract

Cells isolated from solid healthy or cancer tissues can provide more accurate and reliable information about the biology of the disease and thus can direct personalized cancer therapy. Cell cultures established from primary cells can model the *in-vivo* tissue biology, physiology and 3D structure. Nowadays, with the advancement of science and technology and the recognition of the importance of personalized medicine, the use of primary cell lines is increasingly coming to the forefront. Primary cells isolated from patients can be used in personalized medicine to establish patients' specific treatments in cancer, neurological disorders and other diseases. Also, these cells can form the base of cell therapies, new drug target identifications and drug developments.

Keywords: primary cells, primary cell isolation, personalized medicine, cancer treatment, drug development, cell therapy

1. Introduction

Since the maintenance of the first cells in a simple saline solution, cell lines have played an important role in research and in regenerative medicine. In 1885, Wilhelm Roux was the first who successfully maintained chicken embryo cells in a simple saline solution, thus laying the foundation for cell culture technology [1, 2]. In 1907, R. G. Harrison successfully cultivated small pieces of frog nerve tissue in hanging drop cell culture [3]. After these first steps, the technology of cell culture experiences enormous development. Nowadays, numerous immortalized cell lines are available for research to help understanding the biology of the human body, to develop new therapies, to discover new drug targets in cancer therapy and other diseases.

Primary cell culture is the *ex-vivo* culture of freshly isolated cells from malignant or healthy tissues. These cell cultures are more representative of *in-vivo* tissues than cell lines that poorly represent the heterogeneity, genotypic and phenotypic characteristics of the *in-vivo* cells, and their genetic aberrations increasing with every passage. The advantage of the primary cell cultures is that they recapitulate the tissue biology they were isolated from. 3D or spheroid cultures of primary cells can preserve the physiology and the structure of the *in-vivo* tissue, the natural crosstalk between cell-cell and cell-extracellular matrix (ECM). No two tumors are the same, not even the same type of tumor in different patients, and this genetic variability cannot be detected and studied by using a limited number of cancer cell lines. And the patient's reactions are also different to the same drug used to treat cancer [4]. For this reasons, isolation and short-term culture of primary cells gained from solid tumors or healthy tissues can form the basis of personalized cancer therapy, drug development and metabolism studies. In cell therapy, they can be used for the treatment of other non-cancerous diseases, for example, liver, kidney and neurological disorders, to study developmental biology and disease mechanisms. However, it is still challenging to culture and maintain primary cells *in-vitro* in an environment that reflects the *in-vivo* microenvironment and preserves the cells' genetic stability.

1.1 Primary cell isolation

The success of primary cell isolation is dependent on the used tissue digestion and enzymatic cell dissociation technique, which must preserve the viability of cells and the presence of specific cell surface receptors, membrane proteins and other molecules. Also, it is important to maintain the heterogeneity, phenotypic and genotypic characteristics of primary cells, which reflect the *in-vivo* features of the tissue they were isolated from. The length of storage, the temperature and the composition of storage media all affect the success of cell isolation and cell viability from both healthy and cancerous tissues. It is always best to use fresh tissue and media which do not contain any harmful agents or fixation chemicals [4].

To obtain cells from solid tissue and to disrupt cell-cell and cell-ECM adhesion, tissues are subject to a combination of mechanical and enzymatic digestion (**Tables 1 and 2**). The enzymatic digestion protocol, the type and concentration of the enzyme used in the process and the donor age and gender can affect the yield, phenotype, viability and heterogeneity of isolated cells. The isolated cells can be used for the establishment of primary cell cultures, single-cell sequencing, translational research, to study the epigenetic regulatory mechanism, proteomics, drug screening and/or drug validation. Optimization of cell isolation protocol, such as size of the tissue sample, enzyme concentration, length of digestion and temperature, the composition of used culture media and the culture conditions, is very important to preserve not just the cell viability but also the cell surface receptors, antigens and epitope structures for phenotypic characterization [15, 16].

There are tissues, for example, the skin, which is hard to digest and requires prolonged tissue dissociation. Lymphocyte isolation from human skin tissue using 30 min collagenase D and DNase I digestion at 37°C resulted in high cell yield, retained the functional activity of the T-cells and preserved the epitopes important for further analysis with flow cytometry [39].

Isolation of cells from healthy human lung tissue using either dispase, collagenase D or collagenase P can affect the cellular yield and cell surface marker composition of

Dissociation method	Advantages	Issues
Mechanical	• Quick • No enzymes-no enzyme-induced damage • Good for soft tissues (brain, spleen, liver, kidney) [5] • Cells preserve the tissue heterogeneity [6]	• Inefficient for hard, collagenous tissues [7] • Low cell viability [8] • Low cell yield [8]
Enzymatic	• Efficient breakdown of ECM and cell-cell junctions • High yield of cells [9] • High cell viability [9] • Preserve membrane integrity, intracellular components, cell surface molecules and metabolic activity when used in the right concentration [10] • Allows isolation of rare cell types, such as stem cells and immune cells [11] • Faster than mechanical dissociation • Ideal for single-cell preparation for FACS, single-cell RNA sequencing [8] • Can be automated (GMP cell therapy) [12] • Cells are better for culturing, transfection and functional assay	• Expensive • Enzyme batch-to-batch variability [13] • Difficult to reproduce and standardize [14] • Altered cell surface markers and low cell viability when using high concentrations of enzyme or improper temperature [10] • Digestion protocol (concentration, incubation time) must be optimized for different tissues and target cell types [15–17] • Enzyme preparation can be contaminated with endotoxins
Microfluidic	• All steps, such as digestion, disaggregation, filtration, staining and cell counting, can be done in one device [7, 18–20] • Enzyme-free • High cell viability and integrity [21] • Preserve cell surface markers [19] • Reproducible and standardizable • Compatible with small tissue sample [22] • Minimal reagents required	• Clogging and fouling [23] • Non-cross-linked PDMS can leak to the media, which can affect cell viability and downstream processes • Flow and fluid shear stress [23] • Need for tissue-specific optimization [7]

Table 1.
 Dissociation methods used for primary cell isolation from solid tissues.

the isolated cells, which influence the further phenotypic, translational and functional assays. Depending on the type of enzymes and protocol used, the composition of the cell yield and the phenotype of the isolated cells were different. Disperse in high concentration cleaved CD69, CD103 cell surface markers and CD4, CD8 markers in dose dose-dependent manner, increased the yield of TSPN8+ (tetraspanin8) and ITGA6+ (integrin alpha) epithelial cells and monocytes and decreased the number of CD66e + (carcinoembryonic antigen-related cell adhesion molecule 5) cells. The increase in TSPN8+ and ITGA6+ and decrease in CD66e + cells suggest the preferential deliberation of goblet and secretory cells at the expense of basal cells with disperse. Using collagenase D and P resulted in a higher number of endothelial cells compared to disperse [9, 40].

	Enzyme	Mechanical
Brain [5, 24–26]	• Papain, trypsin, accutase: can lead to substantial artefacts • Large temperature shifts can elicit transcriptional changes • Can alter cell surface markers • Collagenase: brain endothelial cell isolation	• Dounce, pipette: Preserve transcriptome integrity, reduce viability of cells,
Heart [27]	• Collagenase type II from Worthington: popular choice for cardiomyocyte isolation as it has greater clostripain activity • Collagenase B and D • Proteases, pancreatin • Langendorff method: retrograde perfusion of the heart with enzyme containing solution, reproducible but technically demanding	• Mincing, perfusion • fewer and less pure cardiomyocyte • Altered cell morphology
Lung [28, 29]	• Collagenase/dispase, DNase I • The lung contains different cell populations which have different susceptibilities and resistance to the digestive enzymes. Therefore, isolation of different cells requires different enzymes and protocols.	• Mild trituration after enzymatic digestion
Liver [30–32]	• Collagenase IV perfusion: The enzyme has lower tryptic activity, which limits its membrane protein and receptor-damaging properties. High cell viability, but technically demanding. The perfusion can cause tissue damage, which alters the gene expression profiles of isolated cells • Collagenase H/P: using together with foetal calf serum allows mild digestion and results in high cell yield	• When perfusion is not possible • Mincing, trituration • Dounce homogenizer • Low cell yield, extensive cell membrane damage, loss of function of isolated hepatocytes.
Spleen [33, 34]	• Collagenase D, Dispase, DNase	MACS dissociator
Skin [35]	• Dispase, trypsin • Simultaneous digestion: DNase, Hyaluronidase, collagenase IV • Sequential digestion: Dispase I, Liberase, Dnase—greatest cell yields and viability • Overnight digestion: Collagenase IV, Dnase I,	Scraping, gentleMACS
Kidney [36–38]	• The kidney contains anatomically different segments and specialized cells • Collagenase, dispase, DNase, FCMase Tissue Digestive Enzyme • Collagenase II: highest cell viability • Combination of collagenases has a different effect on the final cell count • DNase I reduced the cell debris formation • Cold dissociation kit: improves cellular viability, reduces cellular aggregation, maintains RNA stability and reduces cell stress in comparison to warm enzymes.	• Mincing, pipetting • Lower cell yield • Preserve more cell types and epitopes

Table 2.
Dissociation method used for different tissues with advantages and issues.

1.2 Mechanical tissue dissociation

To obtain cells from solid tissues, the tissue samples are subject to mechanical and enzymatic dissociation. Before enzymatic digestion, the tissue should be minced to increase the surface area and the contact between enzymes and tissue, which leads to more efficient and faster digestion of the extracellular matrices. The mechanical disaggregation can be chopping, slicing, sieving, syringing or pipetting. Isolating cells from soft tissues such as brain, spleen, liver and soft tumors by slicing and sieving the dissected tissue through a progressively smaller mesh size of sieves can provide single cells. Harsh mechanical dissociation of the tissue may result in low cell viability and low yield of primary cells. The tissue digestion and enzymatic cell dissociation must preserve the viability of cells and the presence of specific cell surface receptors, membrane proteins and other molecules. Avoiding the use of enzymes and heat during single-cell isolation preserves cell surface receptor expression and provides more representative cell populations, including sensitive cells. Furthermore, enzymatic digestion is most of the time not reproducible and time-consuming compared to traditional mechanical methods. Cells isolated with mechanical digestion are more heterogeny and provide more accurate information about the phenotypic composition of healthy or malignant tissue samples. Single-cell analysis data provided from these cells provide more accurate and reliable information for personalized therapeutic decisions [6].

1.3 Enzymatic tissue dissociation

The length of incubation time of enzymatic digestion and the type of enzyme used for the process depend on the type of tissue sample. The digestion protocol must be designed to consider the composition of the ECM and to preserve the viability and phenotype of the cells. Each tissue has a unique extracellular matrix composition and physiology that scaffold and support the cells within all tissues and organs and take part in tissue morphogenesis, differentiation and homeostasis. The composition and the mechanical properties of normal, aged and tumor tissue ECM are different, which can affect the success of their enzymatic dissociation [17].

There are different types of enzymes used for the digestion of the extracellular matrix, such as collagenase, dispase, Accutase, hyaluronidase, Liberase™, trypsin and DNase. Collagenase recognizes the Pro-X-Gly-Pro sequence and cleaves the peptide between the amino acid(X) and glycine (Gly). The type of collagenase used for digestion depends on the specific tissue type [41]. Because of their impurities, crude collagenase enzyme preparations have decreased stability compared to the purified enzymes [13]. Dispase, which is produced in *Bacillus polymyxa*, provides rapid and effective ECM digestion with mildness cell damage. Dispase is a neutral protease and cleaves the peptide chain at the N-terminus of non-polar amino acids, and mostly cleaves collagen type IV and fibronectin. Dispase cleaves the attachment between cells and ECM and is useful to dissociate tissue pieces into small clumps of cells [40, 42]. However, compared to the collagenase, which has only a minor effect on cell surface molecules, dispase is able to cleave specific surface molecules that affect further cell analysis assays [10]. The Liberase™ enzyme technology combines an optimal ratio of highly purified clostridial collagenases and dispase to increase the quality and reproducibility of tissue digestion and improve the viability of the isolated cells. This

mixture of enzymes provides increased cell yields, improved cell viability and greater cell functionality. Trypsin, a serine protease, is the most widely used in laboratories to resuspend cells adherent to the cell culture dish. Trypsin is well tolerated by most of the cell types, but long incubation with high concentrations of trypsin can damage and kill the cells, which leads to cell aggregation due to the DNA released from damaged cells. Also, trypsin can damage cell surface proteins. Adding EDTA (disodium ethylenediaminetetraacetic acid) to the trypsin increases its effectiveness by chelating with the divalent cations (Ca^{2+} , Mg^{2+}) needed to maintain the cell-cell and cell-ECM interactions [43]. Accutase enzyme has proteolytic, collagenolytic and DNase activity. Accutase mimics the action of trypsin and collagenase, but compared to those, it is gentler with cells, less toxic and can be used in lower concentration [14]. Hyaluronidases cleave the (1-4) linkages between N-acetylglucosamine and glucuronate and catalyze the degradation of hyaluronic acid [44]. DNase degrades free DNA released from damaged cells and prevents cell aggregation [45].

1.4 Microfluidic cell dissociation

Single-cell isolation from solid tissues to further analysis or personalized treatment requires many steps, which not just affect the number and quality of cells but can also introduce batch-to-batch differences. Lab-on-chip microfluidic platforms, as new technologies, can combine digestion, disaggregation and filtration steps to help avoid these problems, standardize the single-cell isolation methods and provide higher viability in a faster manner compared to the current methods. Using microfluid devices, filtration, centrifugation, washing, pipetting, staining, cell counting and vortexing can be done in one uninterrupted process, which helps to eliminate operator and laboratory-dependent deviations [46].

These microfluidic devices are made from a mineral-organic polymer, PDMS (Polydimethylsiloxane). This polymer is inexpensive, deformable, transparent, features that facilitate the observation of contents visually or through a microscope, has low autofluorescence, bio-compatible and can be bound to glass or another PDMS layer with a single plasma treatment. Disadvantages of the PDMS are that it is permeable to water, absorbs hydrophobic molecules and can release non-cross-linked PDMS molecules into the medium, which can be a problem for some biological studies. Generally, these devices are designed to have an inlet channel to upload the tissue sample which is excised into smaller fragments to match the chamber capacity. The digestion happens in the tissue dissociation chamber by flushing the tissue with enzyme solution. Exposing the tissue sample to flow and increased fluid shear can enhance cell dissociation. After digestion, the cells pushed through a filter in a microchannel to capture tissue debris and connective tissue fibers. Cells trapped at the end of the device can be stained, imaged and counted [23].

2. Culture of primary cells

Primary cell cultures can be established from healthy and cancer cells derived from the patient's tissue.

Removing cancerous or healthy tissue from patients requires morphological, histological classification and patients' specific characterization. The tissue handling, the optimization of the isolation methods, the type of tissue digestion techniques (enzymatic, mechanical), the composition of culture media and the culture conditions

all affect the success of establishing primary cell cultures [16]. For primary cell line establishment, the genotypic, phenotypic and morphological characterization of the cells is indispensable to preserve the phenotypes and heterogeneity of the *in-vivo* tissue. Primary cancer cell lines can preserve the stem-like phenotype, which is important for drug resistance assays [47]. Establishment of primary cell cultures from patient's tissue is very difficult and has a very low success rate, and these cells often fail to grow *in-vitro*, and they have a finite lifespan [48, 49].

Primary cell cultures have many advantages compared to cancer cell lines, but they also have limitations. They have limited cell division before they enter senescence; they require specific culture conditions and supplements to maintain their *in-vivo* genotype and phenotype. But, despite their limited lifespan and short-term culture, these cell cultures gained significant importance in personalized cancer and other disease therapies, and in studying drug responsiveness and resistance [50]. Primary cells isolated from cancer tissue are very heterogeneous and contain different cell types, such as ECM components, immune cells and cancer-specific stem cells (CSCs). Before establishing a primary cell lines, the cells must be characterized using cell surface and intracellular markers. The most frequently used assays for the characterization are immunohistochemistry, western blot, PCR (polymerase chain reaction), sequencing and flow cytometry [51]. Many times, heterologous cell populations originated from solid tissue are cultured together in 3D or spheroid cell culture to mimic the tissue *in-vivo* structure and microenvironment, cell-cell, cell-extracellular matrix interactions and to discover new genes and target molecules which have a role in tissue and cancer development, drug resistance, tumor recurrence and metastasis formation and can be new targets in drug development [52–54].

Primary cells isolated from healthy solid tissue have a slow growth rate and a finite lifespan. These cells are contact inhibited, which means when they reach each other during cell culture and cannot attach further to the culture plate, they die by anoikis [49].

The media used for primary cell culture must preserve the heterogeneity and genetic stability of the parental tissue and support the growth of cells. Primary cells can be cultured in 2D monolayer or 3D spheroid forms. While it is easier to work with monolayers, the spheroid culture preserves the structure of the tissue, the polarization of the cells and the cell-ECM, cell-cell interactions. These preserved *in-vivo* features provide more understanding of tissue/cancer biology, drug metabolism and sensitivity, which are very important to establish a specifically targeted personalized therapy [55].

2.1 2D cell culture

Most research in cancer biology still uses 2D cell culture for *in-vitro* research. The handling of these cultures is simple, and they have low-cost maintenance. As cells isolated from solid tissue are anchorage-dependent, these culture flasks and plates must be coated with collagen, fibronectin or other components to facilitate cell attachment. The disadvantages of these cultures are that they do not simulate the architectural diversity of the tissue, the cell-ECM connections, the cell polarity and cell morphology of the tissue they originated from. These connections are especially important in cancers where the tumor microenvironment impacts the tumor differentiation, epigenetics, immune invasion and their response to cancer treatments. Results obtained from using 2D cell culture must be interpreted carefully, as these results may not represent the same effect in *in-vivo* circumstances [55, 56]. For example, primary

oral cancer cells cultured in 2D, 3D and spheroid culture showed significantly different metabolic activity, proliferation rate and drug chemosensitivity [57].

2.2 3D cell culture

The first 3D culture was made using soft agar by Hamburg and Salmon in the 1970s [58]. There are three main types of 3D cell culture methods. In the first one, the cells are grown in non-adherent plates and form a spheroid-like structure after 3 days of culture. The second method using low melting soft agarose gel or Matrigel allows tumor cells to divide in an anchorage-dependent way and form a spheroid in about 7 days after culture. The third type of 3D culture cells are cultured in a structured environment, or scaffold, which closely resembles the ECM. The material of the scaffold can be natural (e.g. collagen, gelatine, silk) or synthetic (e.g. Polylactic acid, polyglycolic acid). This structure reflects the physical and psychological features of the solid tissue or tumor mass. Moreover, 3D cell cultures are powerful tools in drug screening and development as these cell cultures closely mimic the physiological environment where cells have diverse phenotypes and polarity and can exist in different cell physiology stages such as necrosis, quiescent and anoikis [59, 60]. Depending on where the cells are located within the spheroids, their metabolisms and genotypes may vary. Cells found on the surface of a tumor behave similarly to cells in 2D cell cultures. These cells are more accessible to chemotherapeutic drugs, and their metabolism is also ensured since these areas are well supplied with blood vessels. In contrast, the cells in the deeper layers of the tumor tissue do not receive adequate nutrients and oxygen due to inadequate blood supply, which leads to changes in both their phenotypes and genotypes. These cells respond differently to treatments and are much more resistant to cancer therapies. Erlichman and colleagues demonstrated that the effect of Adriamycin is much stronger on those cells that are present on the surface of the spheroids, or which were cultured in 2D cell culture, compared to the cells located in the inner part of the spheroid, which showed resistance to the Adriamycin treatment. Fluorescence assay showed that Adriamycin did not penetrate into the deeper layer of the spheroid and was only detected in cells close to the surface of the spheroids. These results are consistent with the fact that Adriamycin is only effective on proliferating cells and its penetration to the deeper layers of the spheroids is inhibited [61].

2.3 Xenografts

The first patient-derived xenograft (PDX) model was documented in 1969 when malignant tumor tissue, originated from patients with colon adenocarcinoma, was implanted into 8-week-old “Nude”, highly immunodeficient mice [62]. These xenografts derived from patients excellently model the 3D structure of tumor tissue and the tumor microenvironment, preserving its heterogeneity, genotype, epigenetic, morphology, and provide very useful insight into the tumor progression, drug resistance, metastasis and intratumoural cell-cell and cell-ECM communications. These PDX models also enable the discovery and identification of various biomarkers, predict the outcomes of preclinical drug tests and show drug response patterns that closely resemble those seen in patients [63]. In personalized medicine, the PDX is called the “Avatar” model as they allow the patient’s own tumor to grow *in-vivo* in a mouse to establish a personalized, targeted cancer therapy [64]. The effectiveness of several anticancer drugs/drug cocktails can be tested with this method for personalized anticancer treatment. These “Avatars” also allow to study the tumor at the

molecular level and determine the intracellular pathways responsible for the tumor growth, drug metabolism and resistance [64]. However, there are many disadvantages of these PDX models that limit their widespread adoption in translational research. For example, the metabolism of drugs may differ in mice from human metabolism, so there have been cases when the response of PDX for drug treatments was false negative/positive, and not all patient-derived human tissue is suitable for PDX assay. Also, they are very expensive, require specialized facilities and not all tumor types can engraft successfully, and their establishment takes weeks or months, which is very long for a patient with a fast-growing cancer [65, 66]. Organoids can be a faster and cheaper option to obtain an “Avatar” model [67].

3. Personalized cancer treatment

For effective personalized cancer treatment, identifying the somatic genetic alterations driving cancer development and drug resistance is essential. To match the right medicine to the right patient is impossible without understanding the biological relationship between cancer genotype and phenotype. Cancer cell lines still play an important role in understanding cancer and disease biology, as well as drug resistance, as they are easy to handle, have limitless growth potential and their homogeneous character makes them a choice for many large high-throughput experiments. However, these cells are often lacking the complex heterogeneity of the *in-vivo* tissue, and with the increasing passage numbers, these cells lose their original genotype and phenotype and do not truly represent the biology, heterogeneity and drug resistance of the tissue they originated from, which limits their clinical use, especially in personalized medicine [68]. These established cell lines also evolve in culture; they are not stable genetically [69].

Malignant tumors are heterogeneous at genotypic and phenotypic levels, and even the same tumor from different patients shows differences and different responses to the same drug during cancer treatment [70]. In personalized cancer treatment, the aim is to find these specific molecular alterations that drive the cancer development and progression in each patient. *Ex-vivo* culture of primary cancer cells isolated from cancer patients can overcome these problems and help to establish a personalized treatment based on cancer genotype [4]. Furthermore, the establishment of primary cancer cell culture can provide a robust platform for preclinical drug testing and bench-to-bedside translational medicine.

Tumors form a very complex ecosystem and primary cancer cells isolated from patients' tumor tissue are very heterogenic in karyotype, protein and biomarker expression. Also, they are surrounded by non-malignant stroma, the tumor microenvironment (TME), which includes immune cells, blood vessels, fibroblasts, exosomes and ECM. The communication between tumor cells and TME cells supports cancer cell survival, promotes angiogenesis to restore oxygen supply, supports cancer invasion and metastasis and influences the response of tumor cells to anticancer treatments [71].

Immune cells either promote or suppress cancer development. Presence of cytotoxic T-cells (CD8⁺) and NK cells is often associated with good prognosis, while B cells and macrophages can have a protumour effect and support the cancer aggression by IL-10 and IL-4 cytokine production, respectively [71]. The presence of a large number of CD8⁺ cells in rectal cancer before neoadjuvant chemoradiotherapy is a positive sign of successful treatment and cancer regression [72]. Using patient-derived

cancer tumoroids to test and evaluate the tumor infiltration lymphocyte's (TILs) function can predict the patient's response to cancer therapy [73]. In many cases, cancer cells evade the immune surveillance either by downregulating the expression of their cell surface antigens, what makes them invisible for the immune cells, or by expressing immune checkpoint inhibitors such as PD-1 (programmed cell death protein-1), PD-L1 (programmed cell death protein ligand-1) and CTLA4 (cytotoxic T-lymphocyte-associated protein 4) to suppress the immune response [74]. With the discovery of CATL-4 and PD-1/PD-1 L by James Allison and Tasuku Honjo, immunotherapy rapidly developed in cancer treatment. Targeting and inhibiting these checkpoint proteins by monoclonal antibodies prevent cancer cells to "turn off" T-cells, and the cancer cells can be destroyed by the immune system [75]. Immune checkpoint inhibitor therapies (ICI) revolutionized the strategy for cancer treatment. Clinical studies prove the effectiveness of this treatment in hematological and solid tumor cases and in tumors with a high mutational burden, but unfortunately, only a small percentage of patients can achieve a long-lasting response. Co-culturing patients-derived primary tumor organoids with T-cells can help to understand the mechanism behind ICI and drug resistance, the role of immune cells in tumor progression and metastasis and why some patients' cancer does not respond to the treatment [54]. Many types of co-culture systems exist, and they all have their benefits and limitations. Establishing a co-culture system in 2D does not fully represent the complexity and structure of the TME. 3D co-cultures better represent the TME structure, but they are difficult to handle, and with the increase in the size of the spheroids, the diffusion of drugs becomes increasingly difficult, leading to the formation of a concentration gradient through the tumor tissue [54].

In the tumor environment, endothelial cells support angiogenesis, and the transformation of endothelial cells to mesenchymal cells and the formation of cancer-associated fibroblasts (CAFs) during tumor progression support the migration and metastasis formation of cancerous cells [72]. Exosomes, by carrying oncogenic molecules, microRNAs and other small molecules, can contribute to cancer progression, but on the other hand, they can be used for drug delivery specifically to cancer cells [76]. The cancer cells are continuously in contact with the TME, and this dynamic relationship heavily influences the cancer progression, growth, prognosis and treatment options. Hypoxia, oxidative stress and acidosis modulate the TME and ECM composition and stiffness, which promotes cancer cell proliferation and resistance to treatment via activating the STAT3 pathway [77].

Cancer cells can be obtained with core biopsies, fine-needle aspirates, resections and autopsy specimens [70]. Computed tomography-guided biopsies from malignant tissues can provide samples free from normal epithelial cells [70]. The success of isolation of cancer cells and establishing a cancer cell monoculture is affected by the digestion methods, the number of cells collected, the presence of fibroblast and tumor stromal cells, tissue viability, the presence of microbial contamination and the biology of the cancer [4, 78]. Establishment of primary cancer cell lines is challenging; they often fail to adhere to the cell culture dish and lose the proliferative capability after a few passages. Non-tumor cells, stromal cells, can overgrow the slow-growing tumor cells. Resection from necrotic tissue may provide only a few viable cells, which can cause complications during cell culture [4, 78]. In case of treating cancer patients, time is an important factor. In most cases to establish a primary cancer cell culture originating from the patient's tissue can take weeks and months, and the success rate of the process is different and depends on the cancer type. When establishing primary cancer cell culture, it is important to maintain the phenotypic

and genotypic features of the primary tumor and grow them indefinitely without genetic manipulation [78].

Culturing isolated NSCLC (non-small cell lung cancer) and patient-derived colorectal tumor cells by using irradiated fibroblast feeder cells in the presence of Rho-associated kinase (ROCK) inhibitor (Y27632), which disrupts the actin cytoskeleton and inactivates Rho, resulted in successful primary cancer cell line establishment [79–81]. This conditional reprogramming technology (CR) works well for all types of human epithelial cells, such as human cervical cells, human bronchial cells and human prostate cells, and also can be used for other animal species such as mouse, rat, pig, horse, rabbit, dog, fish and others [82]. Although these cells have characteristics of adult stem cells, they do not express stem cell factors, e.g. SOX2, Oct4, Nanog, Klf4 [70]. These cells can later be cultured without feeder cells, can be cryopreserved, thawed and re-grown, and most importantly, these cells maintain genetic stability that features the initial tissue [69]. The effect of the Y27632 can be completely reversible by removing it from the media. With this method, cancer primary cells can be grown and tested for drug sensitivity within weeks from the biopsy and can be used for personalized cancer therapies and biobanking [78, 79].

When co-culturing primary cancer cells with feeder cells, it is important that the feeder cells do not affect cell viability assessment and do not mediate the resistance to targeted therapies. During drug sensitivity testing, the assay conditions and the composition of the culture media must be considered and tested, as some additives can change the sensitivity of cancer cells to treatment. Growth factors such as insulin and exogenous EGF, for example, can reduce the sensitivity of ALK translocated and EGFR mutant lung cancer cells to EGFR and ALK tyrosine kinase inhibitors [69].

Tumors contain a heterogeneous population of different cell types; the largest portion of cells are non-tumorigenic, differentiated cells, benign cancer-associated fibroblast, tumor-associated macrophages and stromal cells [72]. The cancer has its own CSCs population that is capable of unlimited proliferation, disseminates the cancer, remodels the tumor microenvironment, drives tumor progression and self-renewal and is responsible for the resistance mechanism developed for cytotoxic drugs and radiotherapies. Identification and understanding of the biology of CSCs are promising targets for cancer therapies [83].

Traditional culture systems mostly support the proliferation of benign cells, stromal cells or differentiated, non-tumorigenic cells, leading to the loss of the CSCs population. To establish a pure CSCs population by depleting benign cells from the culture takes several weeks and specific culture media, which are commercially available [84]. Clonal expansion of tumor cells as well as intra- and intertumoural heterogeneity of the malignant tissue, differences among patients in disease progression, clinical response and sensitivity for anticancer treatment make the cancer treatment more challenging [85]. Intertumoural heterogeneity refers to the heterogeneity of the same histological tumor type between patients. Intratumoural heterogeneities can be observed as the presence of different tumor cell subpopulations in the same patients or even in the same site of the disease [80, 86, 87]. Intratumoural heterogeneity can be spatial, when genetically different tumor subpopulations are unevenly distributed in different or in the same disease site, or can manifest as temporal heterogeneity when there is a dynamic variation in the different developmental stages of tumor cells in the single lesion over time [80, 82, 85, 86]. This heterogeneity can also emerge from the dynamic development of the tumor and the disease progression. With the increasing heterogeneity, the resistance is also increasing and these cancer cells with different molecular signatures have different sensitivity

to anticancer treatments. Heterogeneity is present not only within the tumor but also between the tumor and its metastasis, as well as among metastases. There is a growing demand for detecting these heterogeneities, to identify new cancer-specific target molecules and to understand the underlying pathological mechanisms for personalized cancer treatment [86].

Cells, healthy or malignant, isolated from a multicellular organism are heterogeneous. These cells are characterized with cell-to-cell variations in morphology, function and gene expression profile. No matter the size of the tissue, analyzing the pool of cells gives a weighted average information of that population's cellular, functional and physiological features. This heterogeneity is especially significant in tumor tissues, where malignant and non-malignant cells form a cellular ecosystem and their interaction affects the tumor progression and response to treatment. The heterogeneity of these tumour tissues makes the establishment of a primary cancer culture difficult. Classical media formulations support the growth of non-malignant cells, stromal cells, which can lead to the loss of original cancer cells, especially the slow-growing tumor cells [88].

2D primary cancer cell cultures do not reflect the *in-vivo* cancer environment, the cell-cell and cell-tumor microenvironment connections and communications. These disadvantages of the 2D cell culture can be addressed by co-culturing the tumor cells with fibroblast or myoepithelial cells. However, co-culture can influence the growth of cancer cells and their response to anticancer drug treatment [89]. Also, it was shown that normal fibroblasts and fibroblasts originated from TME (tumor matrix environment) have different effects on cancer cells. Normal fibroblasts can inhibit cancer cell proliferation and metastasis during early cancer development [90].

3D cell culture models of primary cells increase the efficiency and accuracy of drug screening and development, as these cell cultures have the ability to remodel the *in-vivo* 3D structure, heterogeneity of the tissues and reduce the use of laboratory animals. Tumor cells growing in solid tumors often outgrow the blood supply and grow under hypoxic conditions, which change the metabolism, gene expression and sensitivity for anticancer treatment [91]. Dormant cells in the hypoxic tumor environment can survive the anticancer treatments aimed at proliferating cells and are responsible for the resistance of anticancer drugs. Also, penetration of anticancer drugs to the inner part of the tumor is not always efficient. Using spheroid cell cultures from cell lines and primary colon cancer cell lines, Wenzel et al. identified drugs that specifically target these dormant cells in the inner part of tumor spheroids. These drug targets would not have been identified using 2D cell culture [92].

Liver cancer is a major cause of death in many countries, and in 2020, an estimated 830,200 people died from liver cancer, and the number is increasing globally [93]. Broutier et al. developed an organoid culture of primary liver cancer cells originated from patients with primary liver cancer [94]. During the isolation of primary cancer cells, the samples can be contaminated with non-tumor cells, which can outgrow the cancer cells. To prevent this, the primary cell isolation conditions must be modified such as increasing the time of digestion, modifying the composition of the culture media and monitoring the development of the organoid. The cells in the organoid maintained the histological architecture of the tumor they originated from, resembled the *in-vivo* cancer genetic complexity and structure and retained the differences between patients and tumor subtypes even after ~1-year expansion. Compared the gene expression pattern between cancerous and healthy liver cells, they identified 11 genes that had never been associated with PLC [15].

A successful 3D Organoids model must

- present the patient-specific heterogeneity of the tumor tissue;
- maintain tumor-specific morphology;
- express differentiation markers regardless of the developmental stage of the tumor;
- retain histological characteristics and diagnostic marker expression;
- retain the gene expression profile present in the original tissue; and
- preserve tumor-initiating capability.

The TME and its components, such as T and B lymphocytes, tumor-associated macrophages (TAMs), natural killer cells (NK), mesenchymal cells, cancer-associated fibroblast (CAFs) and other non-cellular components, play a critical role in cancer development, proliferation, metastasis formation and influence the cancer sensitivity and resistance to the different anticancer therapies. The cancer cells are continuously in contact with the TME, and this dynamic relationship heavily influences the cancer progression, growth, prognosis and treatment options [77].

3.1 Role of CSCs in cancer resistance and therapy failures

The current cancer therapies are intended to target rapidly dividing, mature cancer cells and are not effective against the small number of resistant CSCs subpopulation residing within the tumor tissue. The CSCs were discovered in 1997 by Bonnet and Dick in acute myeloid leukemia [95]. The presence and variability of CSCs are different not just between tumor types but also between tumors in the same origin [96, 97]. The CSCs have unique properties, such as contributing to tumor progression, metastasis formation, responsible for the resistance to chemo and radiotherapy and tumor recurrence after cancer treatment. These cells are pluripotent, and this feature contributes to the maintenance of tumor heterogeneity by generating different lineages of cancer cells [98, 99]. CSCs are responsible for the resistance of cancer to common therapeutic agents and failure of the cancer therapies and clinical improvements. CSCs can overexpress genes with role in DNA repair during chemotherapy [100], multidrug efflux pumps such as ATP binding cassette (ABC) transporters [101] and overexpress ALDH, which involved in detoxification of aldehydes, reactive oxygen species and chemotherapeutic agents [102]. Taken together, isolation and culture of patients-specific CSCs can help to understand the biology of the cancer, the disrupted intracellular pathways driving the CSCs behavior and the resistance mechanisms of CSCs. Detecting and selectively targeting those pathways and resistance mechanisms responsible for the CSCs proliferation, stemness potential, invasion and metastasis formation, chemo and radiotherapy resistance, immune evasion and angiogenesis and targeting these routes in patient-specific manner hold great promise to improve the efficiency of cancer treatment and reduce the risk of cancer recurrence.

CSCs exist in almost every tumor, and unlike the normal stem cells, they carry multiple genetic mutations and have a strong resistance mechanism to survive cancer

therapies, which leads to disease relapse [98, 103, 104]. CSCs can be identified by their sphere-forming and tumor-initiating property, expression of cancer-specific transcription factors and signaling molecules. Their isolation and identification are difficult, regardless of their very low presence in the cancer tissue. CSCs are of great interest for designing personalized treatments and establishing drug therapies specially targeting CSCs surviving initial cancer therapy. For CSCs identification, flow cytometry is the most widely used technology where the cells are identified and selected using antibodies specific for cell surface CD (cluster of differentiation) molecules [105, 106].

Isolation and enrichment of CSCs from patients' cancer tissues, along with identification and therapeutic targeting of genes and proteins responsible for the CSCs resistance mechanism against anticancer treatment, can lead to a successful elimination of tumor and help prevent the recurrence of cancer [83]. However, culture of CSCs is difficult and time-consuming due to the low viability of cells, low cell number, necrosis of the tumor sample and the overgrowth of non-tumorigenic cells in the culture [4].

Expression and co-expression of CSCs surface molecules determine the cell's phenotype and tumorigenicity, and inhibition of their signaling pathway with specific antibodies may help eradicate cancer. Stem cell-specific immunotherapy is complicated by the fact that these cell surface markers are also present on healthy stem cells. Expression of these markers is different among cancer types. CD133, also known as prominin-1, is one of the most studied CSCs cell surface antigens [107]. Presence of CD133 positive CSCs in hepatocellular, prostate, colon and ovarian cancers indicates resistance to chemotherapeutic agents and poor prognosis. In these cells, the activation of antiapoptotic Bcl-2 and Akt/PKB pathways leads to the survival of anticancer treatment [108]. For identification of other CSCs from breast, gastric, pancreatic, prostate and head and neck cancer, mainly the CD44 cell surface marker is used, combined with other markers to identify subpopulations of CSCs. Many other CD markers are under investigation and identification [109].

3.2 Isolation of CSCs and primary cells based on cell surface markers

There are different methods for identification, isolation and enrichment of CSCs and primary cells such as using cell surface-specific markers, side population analysis, spheroid formation assay, ALDH activity, hypoxia and chemo resistance assay [110].

Isolation and enrichment of CSCs and primary cells based on their cell surface markers are mainly done by FACS (fluorescence-activated cell sorting) or MACS (magnetic-associated cell sorting) methods.

3.2.1 FACS

FACS is a specialized type of flow cytometry and the most sophisticated and user-friendly technique to characterizing cell types in a heterogeneous cell population. During FACS analysis, primary cells can be separated from other cell types by the presence or lack of specific cell surface markers detected by fluorescent antibody ligands [111]. This is similar to the isolation of stem cells from other tissues by using cell-specific cell surface cluster of differentiation (CD) markers.

To obtain high-quality flow cytometric data, the dissection, dissociation and enzymatic digestion of the solid tissue sample must be well controlled and quick. Processing the sample less than 30 min after removal gives the best result. ECM

composition, origin and type of tissue must be considered when designing protocols to maintain high cell viability, preserve cell surface molecules and relevant antigens. Protocols may also be modified regardless of the tumor type [112].

For the preparation of single cells for flow cytometric analysis, dispase, trypsin and papain should be avoided [10, 113]. Trypsin and papain have a harsh effect on the cell membrane and can induce cell lysis and cell aggregation due to the free DNA released from damaged cells. There are commercially developed enzyme cocktails such as Accutase and TrypLE, which provide higher cell yield, improved antigen preservation and cell viability [14]. Freezing the tissue, using forceful mechanical dissociation, such as tissue homogenizer and vortexing, must be avoided to prevent cell degradation and increase cell viability. During centrifugation, high speed should be avoided. In general, 300–900 RCF (relative centrifugal force) is suitable to separate cells from debris. High speed of centrifugation can damage cell membrane, while too low a speed does not provide successful separation, and cells will be lost during the supernatant aspiration. High concentration of enzyme and long incubation time during enzymatic digestion decrease cell viability, increase cell debris formation and damage cell surface markers important for FACS analysis. DNase and EDTA (ethylenediaminetetraacetic acid) should be used in the buffers during the digestion. DNase helps to prevent cell aggregation via digestion of free DNA, while EDTA, as a chelating agent, sequesters divalent cations important for integrin-mediated cell-ECM adhesion [8]. During FACS analysis, it is also possible to measure many biological parameters such as cell size, granularity, DNA, RNA, membrane potential, enzymatic activities, intra- and extracellular receptors and protein phosphorylation, and can detect multiple targets using different antibodies at the same time [111, 114].

3.2.2 MACS

MACS is a routine methodology for the separation of key cell populations for biomedical use, diagnostic and regenerative medicine by using magnetic beads conjugated monoclonal antibodies corresponding to cell surface antigens. These monoclonal antibodies bind to the cell surface molecules and are then attracted to a magnet, while the other, non-marked cells will wash out from the system. The disadvantage of this system compared to the FACS is that it can only use one parameter, the cell surface antigens, to select and separate cells [115].

3.2.3 Culture of CSCs

CSCs reside in niches, in a specialized microenvironment that supports their survival, protects the CSCs from the immune system and preserves their phenotypic plasticity. Niches also support the normal adult stem cell fate, for example, in the intestine, neural, epidermal and hematopoietic systems. Guo et al. cultured ALDH⁺ liver CSCs on mouse embryonic fibroblast (MEF) feeder cells and serum-free suspension culture. The two different culture conditions affected the size of the spheroids, the rate of the ALDH⁺ cells after 7 days of culture and the expression of stemness-associated genes. Cells cultured in suspension culture formed different sizes and shapes of spheroids compared to the MEF, where the spheroids were more uniform. This and the gene expression results indicate that CSCs cultured on MEF had more stem cell characteristics compared to the cells cultured in suspension, and the presence of MEF feeder cells in this case provided a niche to the CSCs to help maintain their stemness potential [116].

3.3 Primary cells in cancer precision medicine

Despite the groundbreaking discoveries in cancer genetics, the inter- and intratumor heterogeneity and the presence of genetically different cell populations complicate cancer therapy, and many patients have minimal or no response to the same treatment. Single-cell transcriptomics can provide detailed information about the complexity of the tumor, identify genes driving oncogenesis and drug resistance and can predict the effectiveness of the anticancer treatment. Identifying the specific molecular features of the patient's tumor and tailoring the treatment based on this information are the basis of cancer precision medicine. Molecular technologies such as genomics, transcriptomics, epigenetics and immunophenotyping together improve the precision, clinical effectiveness and utility of personalized cancer treatment.

Single-cell RNA (scRNA) sequencing can reveal more information about developmental processes, transcriptional differences, cell lineage segregation, clonal evolution of tumor cells, tumor microenvironment, disease progression and drug resistance compared to bulk transcriptomes [117]. Identification of genetic mutations in cancer cells with single-cell RNA sequencing helps to understand cancer heterogeneity, drug sensitivity, tumor microenvironment, tumor immune cell interactions and provides a more successful personalized cancer treatment [118].

Primary single-cell isolation for RNA sequencing (scRNA-seq) can be assessed for transcriptome analysis to gain information about cellular traits and cellular state, and permits the comparison of the transcriptome of individual cells. For example, single cells isolated from liver tissue provide more accurate information about drug metabolism compared to immortalized liver cell lines. Single cancer cell drug sensitivity testing (SCC-DST) together with scRNA-seq, epigenetic and omics data gives more detailed information about the drug sensitivity and resistance of the patient's tumor and provides more accurate therapeutic decisions for personalized cancer treatment [119].

In the tumor, the various cells communicate by ligand-receptor interactions, which implicate the mechanism of tumor progression and resistance to cancer therapy. For example, immune checkpoint inhibitors target these interactions, but most of the patients do not respond to these treatments. Identification and understanding of these cell-cell, receptor-ligand interactions by scRNA-seq can provide information about patient outcome and help to discover new molecules that can be targeted in immune therapies [120].

The success of the scRNA sequencing is affected by the period between tissue acquisition variability and processing, the applied mechanical and enzymatic digestion protocol, the type of tumor, the composition of its extracellular environment, sample processing steps, the quality of mRNA, sequencing depth and pipetting accuracy. Also, different tumor samples require different protocols for single-cell isolation, depending on their ECM composition and stiffness. Prolonged enzymatic digestion time and mechanical stress can negatively affect the native expression profile and transcriptome analysis [12]. These technical variables are the sources of batch effect during single-cell preparations, which make the analysis particularly challenging. Each step should be carefully optimized to avoid these variables and artefacts [121].

Recently, scRNA-seq is the most widely used technology for classifying tumors and identifying biomarkers for precision medicine. However, in the future, this technology can be used to match therapies in personalized cancer therapies and monitor resistance. Researchers and computational biologists, working together to develop

computational programs, are able to analyze scRNA-seq data and identify the patients who will respond and who will be resistant to the treatment [122]. Implementing precision medicine in personal cancer treatment is challenging and puts pressure for the healthcare system to adapt its infrastructure. Despite the rapidly growing number of new biomarker-based drug developments, many of the cancer patients still do not receive treatment based on genetic testing [123].

4. Primary cells in drug development

Drug development is a multiple and time-consuming process involving target identification, screening, optimization and clinical trials. Before a drug is marketed, it undergoes numerous tests that examine possible side effects and liver toxicity. These tests often still use animal models, even though the expression and activity of enzymes involved in drug metabolism can vary significantly between humans and animals, which can lead to termination of drug during clinical development using results based on animal models [124].

Many protocols in drug development and screening still use immortalized and genetically engineered cells to detect drug efficiency and potential drug target proteins. However, these cells physiologically and clinically are less relevant to primary cells which endogenously express the target molecule, and because of it, these assays many times give false positive/negative results. With the development of technology, many drug discovery programs utilize primary cells, normal or cancerous, isolated from patients, which phenotypically more closely resemble the cells found *in-vivo* and more closely reflect the human cell physiology [125].

Drug absorption, distribution, metabolism and toxicology (ADMET) assays are essential for drug developmental studies [126]. Hepatic CYP450s enzymes have main role in drug metabolism and hepatic clearance, and many drug toxicity assays use hepatocytes [127]. Most of the ADMET assays use primary hepatocytes instead of immortalized cell lines, which can predict the drug toxicity and metabolism before entering clinical stages [128]. Hepatotoxicity is still one of the most frequent causes for late-stage failure in drug development [129]. Results obtained from EDMA assay using animal models are limited due to the interspecies differences in the expression and activity of enzymes with a role in drug metabolism. Using primary human hepatocytes (PHH) can overcome these problems and represent a gold standard model for HT testing [130].

Using primary cells as complex biological systems for ADMET and drug-induced liver injury assays can provide more accurate information about the drug-cell interaction, drug metabolism and the overall cellular response *in-vivo* than immortalized cell lines, single enzyme or subcellular fractions [131]. However, *in-vitro* culture of primary hepatocytes for drug screening, tissue engineering and cell therapies is still challenging. 2D culture of primary hepatocytes does not reflect the *in-vivo* phenotypes of cells and does not give reliable information about the liver biology and drug metabolism [132, 133].

Isolation and *in-vitro* culture of primary hepatocytes for drug screening, tissue engineering and cell therapies is still challenging. Variations in the isolation techniques and differences in the sample types all affect the culture process. PHH cultured in suspension or 2D monolayers dedifferentiate and lose their hepatic function, and there is a high drop in phase I and II enzyme activity within hours; however, culturing PHH in 3D spheroid culture can prevent this process and maintain the molecular

phenotype of the hepatocytes. These 3D spheroid models can be established by using either a synthetic scaffold that mimics the ECM or inhibits the attachment of the hepatocyte to the cell culture flask surface [55]. There are many techniques to establish scaffold-based cell cultures, such as electrospinning, Solvent-casting Particle-leaching or using polyhydroxybutyrate for scaffold fabrication [134–136]. Using hydrogel scaffold extracted from murine Engelbreth-Holm-Swarm tumor affects the cell morphology, gene expression pattern, migration and cell proliferation. When using this scaffold for human cell culture, the results must be interpreted carefully. Among these materials, polyhydroxybutyrate is the best choice as it is a natural material found in bacteria, highly flexible, biocompatible and biodegradable [137]. Fontoura et al. compared 2D and 3D cell culture models, and it has been shown that the physiology and gene expression pattern of cells grown in a 3D model resembled the original tissue much more closely than that of a 2D model [138]. Proteome analysis data revealed differences in the expression of genes that play role in phase I and II reactions (CYP1A2, CYP2C8, CYP29C, CYP2D6 and CYP3A4) and hepatocyte metabolism between 2D sandwich and 3D culture of primary hepatocytes originated from the same donor [139]. In a toxicology study, PHH in 3D spheroid culture maintained the high level of expression of proteins with a role in drug metabolism, and their protein expression was stable over the 14-day culture compared to the 2D cultures. These spheroid cultures enable the evaluation and study of drug toxicity and metabolism for drug development and personalized medicine, as it considers the differences between patients [140].

Hepatocyte spheroids can be formed by culturing the cells in hanging drops, in bioreactors or in culture flasks where their attachment is inhibited [141, 142]. Tostões et al. successfully cultured primary hepatocyte spheroids for 3–4 weeks without serum in a perfusion bioreactor, and the cells maintained the expression of drug metabolizing phase I and II enzymes such as CYP450, GSTA1 and UGT2B7 [143].

In a new methodology, Wei et al. used self-assembling nucleic acid nanostructures to decorate living cells (NACs) and generate designer spheroids. This technology allows the positioning of multiple cell types in the spheroids to mimic natural tissue anatomy. Generation of heterogenic NAC spheroids using millions of human primary hepatocytes, stromal cells and immune cells provides a detailed, standardizable modeling of liver diseases and improves drug screening and development efficiency. This technology may open the way to establish spheroids from primary cells in personalized medicine, which completely mimic the *in-vivo* structure and environment of tissues. Also, assembling these spheroids, we can generate functional organs for disease treatments, drug screening and the study of the molecular basis of different diseases [144].

These models unfortunately cannot yet be used for testing drugs that target immune cells, because these tumoroid systems do not support the proliferation of immune and stromal cells. Another method to establish 3D cell culture, which preserves the structure of the original tissue, is the *in-vitro* culture of patient-derived tumor slices. These 3D tumoroid models can be used for evaluating the immune cell populations infiltrating the tumor, to study the efficiency of new drugs and chemotherapy treatments in a heterogeneous environment, which contains not just the tumor cells but also cells from the TME. Finnberg et al. developed a 3D culture system using colon, lung, pancreas, rectum and liver cancer tissues, which enabled maintenance of the complex tumor tissue for 44 days. These 3D cultures retain tumor

markers, CSCs, stromal and immune cells and the vasculature of the *in-vivo* tumor. This model, along with the sequencing data, can provide information about how these tumors respond to individual drug treatments and can predict drug resistance, which information can help clinicians to decide which is the most effective treatment for the patient in personalized therapies [145]. Stromal cells, recruited from the bone marrow, are also important to the tumor development, as they are responsible for several biological processes that contribute to the survival of cancer cells: the formation of metastases, evasion of immune system, angiogenesis, resistance to apoptosis, maintenance of proliferative signals and evasion of growth suppressors [146].

Using primary cells for genome, transcriptome, epigenome, proteome and metabolome analysis provides more precise, cell-specific data and helps to validate and identify new drug targets in cancer, neurological disease, infectious disease, cardiovascular disease and immunological disorders and to improve the effectiveness and safety of drugs [147].

The heterogeneity of tumor and normal tissue and the different sensitivity of individual cells for drugs make the drug development and personalized cancer treatment challenging. These models, whether 2D, 3D, or tumoroid, must preserve the original tissue's genotype and phenotype, and they also need to reflect the *in-vitro* environment, which not only influences the tumor development but also the response to chemo- and radiotherapy, the treatment outcome and the emergence of resistance mechanisms. Also, this culture must maintain the tumor markers and CSCs and support the proliferation of immune and stromal cells. Using tumor cells and tissues from patients, we can consider the differences between patients, as well as the intra- and intertumor heterogeneity and based on these, these culture systems enable the development of personalized therapy. For this method to become available to all patients in clinical practice, it still requires a lot of development. In the treatment of cancer patients, time is a very important factor, and the creation of these primary cell cultures often takes weeks. Furthermore, the development of such synthetic culture media becomes necessary, which maintain and preserve the *in-vivo* biological and physiological features of the original cells and tissues.

4.1 Single-cell RNA-seq in drug development

Using scRNA-seq and public bulk RNA sequencing data, Zhang et al. identified 10 overexpressed genes in malignant type 2 alveolar (AT2) cells originated from lung adenocarcinoma (LUAD) tissue sample. AT2 cells, as secretory and regenerative cells, play a role in maintaining the homeostasis in the alveolus of the lung and may be the cells of origin of LUAD. Among these genes, AQP5 and KPNA2 can potentially serve as a drug target and early diagnostic marker of lung cancer [148]. Identification of epithelial club cells population in prostate cancer, which are characterized by lower expression *SCGB3A1* and *LCN2* and higher androgen responsiveness, may play a role in supporting cancer development [149]. ScRNA-seq helped to identify proximal tubule (PT) cells as the cell-of-origin of cancer in one of the most heterogeneous and extremely difficult to treat papillary renal cell carcinoma (pRCC). In tumors, the same genetic mutation in different cell lineages can lead to different phenotypes, morphology and malignancy. Identifying the cell-of-origin of heterogeneous tumors helps to understand the progress of the tumor development, response to treatments and can provide new targets for drug development [150].

5. Primary cells in regenerative medicine

In the last years, cell therapy has undergone significant development in terms of both safety and efficiency. The market analysis report predicts a 22.66% increase in the global cell therapy market size by 2030 compared to 2024 [151]. Regenerative medicine relies on primary cells isolated from different tissues, for example, liver, kidney, neural tissues, skin and cartilage. The high demand for available organs and the low number of organ donations result in a long waiting list in transplantation medicine, and many patients do not receive the necessary organs in time, and many of them die before a suitable organ becomes available for them.

5.1 Cell therapy for kidney disorders

Treatment of kidney failure by kidney transplantation is very successful, with 5-year survival of >80% for recipients, but the patients require lifelong immunosuppression [152]. In Europe, 10,002 patients waited for a kidney transplant in 2024 [153]. Kim et al. isolated primary renal epithelial cells from a cadaveric kidney not suitable for transplant to prepare a bioartificial kidney to mimic human nephron physiology. The primary kidney cells were cultured in a mini-bioreactor system in such a way, which allowed the gas and nutrient transport but protected the cells from the host immune system to prevent rejection. This implantable bioartificial kidney (iBAK) was implanted into a porcine model, where the cells survived 7 days without any hyperacute immune reaction against the human kidney cells. However, still much research is required for the further development of these mini bioreactor systems. This technology, using primary cells, is very promising not only for kidney but also for the treatment of other organ system failures until suitable donor tissue is available [154].

5.2 Cell therapy for liver disorders

Like the kidney, liver also has many vital functions, and liver failure has life-threatening consequences. Liver diseases can be classified into three groups: acute liver disease, chronic liver disease and inherited metabolic disorders [155–157].

In case of liver failure, the only available treatment is the liver transplant. In Europe, 1497 patients waited for liver transplant in 2024 [154]. After acute liver disease, the hepatocytes retain their ability to proliferate, and the liver can regenerate itself. But the loss of liver function during the disease causes serious systemic complications. To overcome these complications and help the liver to regenerate, Dhawan et al. microencapsulated primary hepatocytes into alginate beads and transplanted them into pediatric patients with acute liver failure and with an average age of 14.5 days. With this technique, hepatocytes isolated from one donor could be transplanted intraperitoneally into many children, as the alginate capsulation protected the hepatocytes from the donor immune system while allowing protein and substrate transport. This procedure helped the children to recover from acute liver failure or to survive until a suitable organ becomes available. The treatment was successful in seven out of eight infants. One child who died was not suitable for liver transplant, and his care was electively withdrawn [158].

Isolation and culture of primary hepatocytes are still challenging. Variations in the isolation techniques and differences in the sample types all affect the culture process. PHH cultured in suspension or 2D monolayers dedifferentiate and lose their hepatic

function, and there is a high drop in phase I and II enzyme activity within hours; however, culturing PHH in 3D spheroid culture can prevent this process. In a toxicology study, PHH in 3D spheroid culture maintained the high level of expression of proteins with a role in drug metabolism, and their protein expression was stable over the 14-day culture compared to the 2D cultures [140].

5.3 Cell therapy for neurological disorders

Neurological disorders, for example, Alzheimer's disease (AD), stroke, Huntington's disease (HD), Parkinson's disease (PD), motoneuron diseases, multiple sclerosis and brain and spinal cord injuries, are leading causes of disability and second leading causes of death worldwide [159]. There are two methods that can be applied to treat neurological disorders: in the first one, cells such as mesenchymal cells are injected into the patient's tissue to aid its regeneration, while in the other method, the so-called cell replacement therapy (CRT), the patient's diseased cells are replaced with healthy cells. This second approach can provide long-term benefits and recovery for the patients [159].

In Parkinson's and Huntington's diseases, the core pathologies are well defined, loss of dopaminergic nigrostriatal tract and loss of the GABAergic striatal output neurons, respectively. Primary and multipotent progenitor cells derived from embryos may be suitable for replacing the function of diseased cells. Neural cell lines differ greatly from primary nerve cells, and because they possess oncogenic properties, they cannot be used for cell therapy. Currently, the only source for primary, non-transformed nerve extraction is embryonic tissue [160]. The disadvantage of these cell cultures is that they cannot be maintained *in-vitro* for long, and in the case of cell therapy, the patient requires immunosuppressive treatment, and, in some countries, the use of embryonic tissues raises ethical questions. The solution may be to reprogram patient-derived cells, such as fibroblast cells, into pluripotent cells capable of differentiating into neuronal cells [161, 162]. This autologous transplantation no longer requires immunosuppressive treatment. In the case of Parkinson's disease, the goal is to replace dopamine-producing cells [163]. The production of these dopamine (DA) progenitor cells can be achieved in three ways. In the first step, cells are isolated from a donor with the same HLA type as the patient, and these cells are differentiated into DA progenitors using iPS technology. In the second method, they use the same iPS technology but with cells originated from the patients [164]. In the third method, embryonic stem cells isolated from blastocyst are differentiated into DA progenitors. In iPS technology, the pluripotent stem cells are generated from somatic cells, such as fibroblasts, using specific transcription factors, and these pluripotent stem cells can be later differentiated into any type of cell. This technology holds big promise in regenerative medicine [165].

In the case of Huntington's disease, the mutation of the Huntington gene leads to the loss of GABA-producing cells. In the treatment of this disease, they also attempt cell therapy derived from foetal tissue, but clinical studies do not demonstrate long-term effects. Park et al. successfully transplanted iPSC-derived neural precursor cells into mouse HD model (YAC128), where these cells differentiated mainly astrocytes and showed neuroprotective effects [166]. These animals have higher expression of DARPP-32 (dopamine and cAMP-regulated phosphoprotein Mr. 32 kDa) and showed improved motor and cognitive function [166].

Unlike PD and HD, AD affects all the entire area of the brain, which is associated with the loss of nerve cells and a decrease in their number [163, 167]. In AD, the

accumulation of amyloid plaques and neurofibrillary tangles interferes with normal cell function, which leads to irreversible neuron degeneration [168]. Therefore, this disease cannot be localized to a specific area of the brain or neuron type. In the treatment of this disease, stem cell therapy has also been attempted, some of which have proven promising as they reduced neurological symptoms in mouse experiments. Although the brain is immune-privileged due to the blood-brain barrier, there is considerable evidence that immune cells are present in the brain. By using the patient's own cells, the development of an immune response against the transplanted cells can be eliminated [169].

6. Primary cells is biotechnology

Bioreactors are widely used essential tools for expansion and cultivation of cell lines for gene and cell therapy and stem cell treatment. In bioreactors, cells are maintained in well-controlled microenvironment to regulate cell growth, differentiation and tissue development. Bioreactors can be customized to fit the requirements of the cells cultured to support their maximal growth and metabolism under well-controlled conditions. Bioreactors can be found in almost every size, from small microfluidic systems to hundreds of liters, adjusted to the size of the cell culture [170]. In regenerative medicine and cell therapy, bioreactors are essential tools to provide a reproducible, standardized technology to establish a physiological environment for cell growth and tissue formation. In personalized cell therapy, the cells are very different; the viability and quality of the cells are influenced not only by the patient's genetics but also by the type of disease they have and what treatments they have received. This means that the culture conditions must also be tailored to meet the needs of the patients' cells [171]. Bioreactors also have the advantages of 3D tissue culture, which can closely mimic the *in-vivo* tissue development, provide more reliable results for individual drug testing, drug development and may help to reduce the use of laboratory animals. There are microbioreactor systems that are capable of maintaining heterogeneous primary cells in 3D or spheroid culture and provide a more biologically relevant environment. However, cell therapy and tissue engineering require a large number of patient cells that are difficult to obtain from a small tissue sample. Culture of primary cells requires optimized growth conditions, specific cytokines and growth factors. In bioreactors, the pH, temperature and oxygen concentration can be well controlled. Using a perfusion system, it is possible to remove the metabolic waste and provide fresh media for the cells [172]. Expansion of cells using a multistep conventional method can cause batch variability, a decrease in cell quality and viability and changes in the cell identity and gene expression [173]. There are certain types of cells, whose physiology can also be regulated by mechanical stress, such as osteocytes, chondrocytes, cardiomyocytes and skeletal muscle cells [174]. For example, culturing chondrocytes together is a scaffold polymer in a static, mixed flask or a rotating vessel can affect the size and the mechanical properties of the tissue. Compared to the static and mixed flask culture, the rotating method resulted in cartilage tissue with the best mechanical properties and the highest fraction of glycosaminoglycan and collagen [174]. It occurs in many cases that the donor lung intended for transplantation is not suitable. Since there are still very few transplantable donor lungs, these unsuitable lungs can be saved using genetic methods. By removing the cells from the donor lung, the remaining scaffold can be repopulated with new, functional lung cells [175]. Bioreactor technology can provide a suitable physical and biochemical environment

for the developing lung. Culture together the lung scaffold and the healthy epithelial and endothelial cells in a bioreactor, which provides well-controlled culture conditions, recapitulates the need for lung physiological development and will lead to the reepithelization of the scaffold and development of a functioning lung together with its vasculature [175].

Serra et al. used computer-controlled stirred bioreactors to expand CSCs isolated from patients with non-small cell lung cancer (NSCLC) and colorectal cancer (CRC). Their results showed that the growth and the ability of aggregate formation of NSCLC stem cells were not affected by the presence or lack of serum in the culture media, however, culturing the CRC cells in serum-free media resulted in a higher number of aldehyde dehydrogenase positive (ALDH⁺) cell populations. ALDH is a cancer prognostic and cancer stem cell marker [176]. Both cell lines were successfully grown in microcarriers; however, NSCLC cells showed higher expansion ratios when compared to aggregate culture. On the other hand, the vessel design and the impeller geometry influenced the growth kinetics and the size of aggregates. The size of aggregates limits the O₂ diffusion to the inner cells detected by the presence of hypoxic regions in the inner part of the aggregates. These aggregated structures well represent the *in-vivo* conditions of the solid tumor, where outside of the central necrotic area, there is a middle layer with quiescent cells and an outer layer with highly proliferating cells [177]. Harvesting, enzymatic dissociation of these aggregates resulted in 7–15% cell viability and 74–83% ALDH positivity. Among the used digestion enzymes, the trypsin 0,05% and the A + C + D (Accutase+Collagenase III + DNase) showed the highest ALDH positivity compared to TrypLE [176].

Author details

Erika Berenyi
University of Debrecen, Debrecen, Hungary

*Address all correspondence to: berenyi.era@gmail.com

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References

- [1] Kearn M. Wilhelm Roux (1850-1924). In: Embryo Project Encyclopedia. Arizona State University, School of Life Sciences, Center for Biology and Society; 2009. ISSN: 1940-5030. Available from: <https://hdl.handle.net/10776/1753>
- [2] Moro LG, Guarnier LP, Azevedo MF, Fracasso JAR, Lucio MA, Castro MV, et al. A brief history of cell culture: From Harrison to organs-on-a-chip. *Cells*. 2024;**13**(24):2068. DOI: 10.3390/cells13242068
- [3] Michael A. Ross Granville Harrison, 1870-1959. *Biographical Memoirs of Fellows of the Royal Society*. 1961;**7**:110-126. DOI: 10.1098/rsbm.1961.0009
- [4] Mitra A, Mishra L, Li S. Technologies for deriving primary tumor cells for use in personalized cancer therapy. *Trends in Biotechnology*. 2013;**31**(6):347-354. DOI: 10.1016/j.tibtech.2013.03.006. Epub 2013 Apr 16
- [5] Herron S, Delpech JC, Madore C, Ikezu T. Using mechanical homogenization to isolate microglia from mouse brain tissue to preserve transcriptomic integrity. *STAR Protocols*. 2022;**3**(4):101670. DOI: 10.1016/j.xpro.2022.101670. Epub 2022 Sep 14
- [6] Hu P, Zhang W, Xin H, Deng G. Single cell isolation and analysis. *Frontiers in Cell and Development Biology*. 2016;**4**:116. DOI: 10.3389/fcell.2016.00116
- [7] Aliaghaei M, Haun JB. Optimization of mechanical tissue dissociation using an integrated microfluidic device for improved generation of single cells following digestion. *Frontiers in Bioengineering and Biotechnology*. 2022;**10**:841046. DOI: 10.3389/fbioe.2022.841046
- [8] Reichard A, Asosingh K. Best practices for preparing a single cell suspension from solid tissues for flow cytometry. *Cytometry. Part A*. 2019;**95**(2):219-226. DOI: 10.1002/cyto.a.23690. Epub 2018 Dec 6
- [9] Bondonese A, Craig A, Fan L, Valenzi E, Bain W, Lafyatis R, et al. Impact of enzymatic digestion on single cell suspension yield from peripheral human lung tissue. *Cytometry. Part A*. 2023;**103**(10):777-785. DOI: 10.1002/cyto.a.24777. Epub 2023 Jul 27
- [10] Autengruber A, Gereke M, Hansen G, Hennig C, Bruder D. Impact of enzymatic tissue disintegration on the level of surface molecule expression and immune cell function. *European Journal of Microbiology & Immunology (Bp)*. 2012;**2**(2):112-120. DOI: 10.1556/EuJMI.2.2012.2.3. Epub 2012 Jun 13
- [11] Alstrup T, Eijken M, Bohn AB, Møller B, Damsgaard TE. Isolation of adipose tissue-derived stem cells: Enzymatic digestion in combination with mechanical distortion to increase adipose tissue-derived stem cell yield from human aspirated fat. *Current Protocols in Stem Cell Biology*. 2019;**48**(1):e68. DOI: 10.1002/cpsc.68. Epub 2018 Oct 26
- [12] Nguyen QH, Pervolarakis N, Nee K, Kessenbrock K. Experimental considerations for single-cell RNA sequencing approaches. *Frontiers in Cell and Development Biology*. 2018;**6**:108. DOI: 10.3389/fcell.2018.00108
- [13] Cavanagh TJ, Lakey JR, Wright MJ, Fetterhoff T, Wile K. Crude collagenase loses islet-isolating efficacy regardless

of storage conditions. Transplantation Proceedings. 1997;**29**(4):1942-1944.
 DOI: 10.1016/s0041-1345(97)00291-1

[14] Bajpai R, Lesperance J, Kim M, Terskikh AV. Efficient propagation of single cells Accutase-dissociated human embryonic stem cells. Molecular Reproduction and Development. 2008;**75**(5):818-827. DOI: 10.1002/mrd.20809

[15] Li L, Knutsdottir H, Hui K, Weiss MJ, He J, Philosophe B, et al. Human primary liver cancer organoids reveal intratumor and interpatient drug response heterogeneity. JCI Insight. 2019;**4**(2):e121490. DOI: 10.1172/jci.insight.121490

[16] Piwocka O, Musielak M, Ampuła K, Piotrowski I, Adamczyk B, Fundowicz M, et al. Navigating challenges: Optimising methods for primary cell culture isolation. Cancer Cell International. 2024;**24**(1):28. DOI: 10.1186/s12935-023-03190-4

[17] Frantz C, Stewart KM, Weaver VM. The extracellular matrix at a glance. Journal of Cell Science. 2010;**123**(Pt. 24):4195-4200. DOI: 10.1242/jcs.023820

[18] Qiu X, Westerhof TM, Karunaratne AA, Werner EM, Pourfard PP, Nelson EL, et al. Microfluidic device for rapid digestion of tissues into cellular suspensions. Lab on a Chip. 2017;**17**(19):3300-3309. DOI: 10.1039/c7lc00575j

[19] Qiu X, De Jesus J, Pennell M, Troiani M, Haun JB. Microfluidic device for mechanical dissociation of cancer cell aggregates into single cells. Lab on a Chip. 2015;**15**(1):339-350. DOI: 10.1039/c4lc01126k

[20] Varma S, Voldman J. Caring for cells in microsystems: Principles and practices

of cell-safe device design and operation. Lab on a Chip. 2018;**18**(22):3333-3352. DOI: 10.1039/c8lc00746b

[21] Lombardo JA, Aliaghaei M, Nguyen QH, Kessenbrock K, Haun JB. Microfluidic platform accelerates tissue processing into single cells for molecular analysis and primary culture models. Nature Communications. 2021;**12**(1):2858. DOI: 10.1038/s41467-021-23238-1

[22] Qiu X. Microfluidic Devices for Digestion, Dissociation, and Filtration of Tissues into Single Cell Suspensions. UC Irvine, ProQuest ID: Qiu_uci_0030D_14705, Merritt ID. Irvine: University of California; 2017. ark:/13030/m54b7ww4. Available from: <https://escholarship.org/uc/item/1p68plqh>

[23] Celeste Welch E, Tripathi A. Preparation of tissues and heterogeneous cellular samples for single-cell analysis. In: Sample Preparation Techniques for Chemical Analysis. London, UK: IntechOpen; 2021. DOI: 10.5772/intechopen.100184.me

[24] Cumba Garcia LM, Huseby Kelcher AM, Malo CS, Johnson AJ. Superior isolation of antigen-specific brain infiltrating T cells using manual homogenization technique. Journal of Immunological Methods. 2016;**439**:23-28. DOI: 10.1016/j.jim.2016.09.002. Epub 2016 Sep 10

[25] Mattei D, Ivanov A, van Oostrum M, Pantelyushin S, Richetto J, Mueller F, et al. Enzymatic dissociation induces transcriptional and Proteotype bias in brain cell populations. International Journal of Molecular Sciences. 2020;**21**:7944. DOI: 10.3390/ijms21217944

[26] Czapalla CJ, Yousef H, Wyss-Coray T, Butcher EC. Collagenase-based

- single cell isolation of primary murine brain endothelial cells using flow cytometry. *Bio-Protocol*. 2018;**8**(22):e3092. DOI: 10.21769/BioProtoc.3092
- [27] Louch WE, Sheehan KA, Wolska BM. Methods in cardiomyocyte isolation, culture, and gene transfer. *Journal of Molecular and Cellular Cardiology*. 2011;**51**(3):288-298. DOI: 10.1016/j.yjmcc.2011.06.012. Epub 2011 Jun 24
- [28] Nakano H, Nakano K, Cook DN. Isolation and purification of epithelial and endothelial cells from mouse lung. *Methods in Molecular Biology*. 2018;**1799**:59-69. DOI: 10.1007/978-1-4939-7896-0_6
- [29] Baumann Z, Wiethe C, Vecchi CM, Richina V, Lopes T, Bentires-Alj M. Optimized full-spectrum flow cytometry panel for deep immunophenotyping of murine lungs. *Cell Reports Methods*. 2024;**4**(11):100885. DOI: 10.1016/j.crmeth.2024.100885. ISSN 2667-2375
- [30] Bhogal RH, Hodson J, Bartlett DC, Weston CJ, Curbishley SM, Haughton E, et al. Isolation of primary human hepatocytes from normal and diseased liver tissue: A one hundred liver experience. *PLoS One*. 2011;**6**(3):e18222. DOI: 10.1371/journal.pone.0018222
- [31] Charni-Natan M, Goldstein I. Protocol for primary mouse hepatocyte isolation. *STAR Protocols*. 2020;**1**(2):100086. DOI: 10.1016/j.xpro.2020.100086
- [32] Kaur I, Vasudevan A, Rawal P, Tripathi DM, Ramakrishna S, Kaur S, et al. Primary hepatocyte isolation and cultures: Technical aspects, challenges and advancements. *Bioengineering*. 2023;**10**:131. DOI: 10.3390/bioengineering10020131
- [33] Jungblut M, Oeltze K, Zehnter I, Hasselmann D, Bosio A. Preparation of single-cell suspensions from mouse spleen with the gentle MACS dissociator. *Journal of Visualized Experiments*. 2008;**22**:1029. DOI: 10.3791/1029
- [34] Fujiyama S, Nakahashi-Oda C, Abe F, Wang Y, Sato K, Shibuya A. Identification and isolation of splenic tissue-resident macrophage sub-populations by flow cytometry. *International Immunology*. 2019;**31**(1):51-56. DOI: 10.1093/intimm/dxy064
- [35] Clister T, Fey RM, Garrison ZR, Valenzuela CD, Bar A, Leitenberger JJ, et al. Optimization of tissue digestion methods for characterization of photoaged skin by single cell RNA sequencing reveals preferential enrichment of T cell subsets. *Cells*. 2024;**13**(3):266. DOI: 10.3390/cells13030266
- [36] Yaigoub H, Tirichen H, Xin X, Shi S, Wu C, Li R, et al. Isolation of viable single cells with high yield and purity using a small amount of human kidney tissue biopsy. *Frontiers in Cell and Development Biology*. 2022;**10**:822275. DOI: 10.3389/fcell.2022.822275
- [37] Cold Dissociation for Improved Kidney Tissue Cell Capture and Viability, while Reducing Aggregation and Cell Stress. Cytiva; 2025. Available from: <https://www.cytivalifesciences.com/en/us/solutions/genomics/knowledge-center/cold-dissociation-for-improved-kidney>
- [38] Schaffer S, Maul-Pavicic A, Voll RE, Chevalier N. Optimized isolation of renal plasma cells for flow cytometric analysis. *Journal of Immunological Methods*. 2019;**474**:112628. DOI: 10.1016/j.jim.2019.06.019. Epub 2019 Jun 26

- [39] Polakova A, Hudemann C, Wiemers F, Kadys A, Gremke N, Lang M, et al. Isolation of lymphocytes from human skin and murine tissues: A rapid and epitope-preserving approach. *JID Innovations: Skin Science from Molecules to Population Health*. 2022;**3**(1):100155. DOI: 10.1016/j.xjidi.2022.100155
- [40] Stenn KS, Link R, Moellmann G, Madri J, Kuklinska E. Dispace, a neutral protease from *Bacillus polymyxa*, is a powerful fibronectinase and type IV collagenase. *Journal of Investigative Dermatology*. Aug 1989;**93**(2):287-290. DOI: 10.1111/1523-1747.ep12277593
- [41] Wang ZZ, Wang K, Xu LF, Su C, Gong JS, Shi JS, et al. Unlocking the potential of collagenases: Structures, functions, and emerging therapeutic horizons. *Biodesign Research*. 2024;**6**:0050. DOI: 10.34133/bdr.0050
- [42] Abreu-Velez AM, Howard MS. Collagen IV in Normal skin and in pathological processes. *North American Journal of Medical Sciences*. 2012;**4**(1):1-8. DOI: 10.4103/1947-2714.92892
- [43] Tsuji K, Ojima M, Otabe K, Horie M, Koga H, Sekiya I, et al. Effects of different cell-detaching methods on the viability and cell surface antigen expression of synovial mesenchymal stem cells. *Cell Transplantation*. 2017;**26**(6):1089-1102. DOI: 10.3727/096368917X694831. Epub 2017 Jan 31
- [44] Stern R, Jedrzejewski MJ. Hyaluronidases: Their genomics, structures, and mechanisms of action. *Chemical Reviews*. 2006;**106**(3):818-839. DOI: 10.1021/cr050247k
- [45] Potter AS, Steven Potter S. Dissociation of tissues for single-cell analysis. In: Vainio S, editor. *Kidney Organogenesis. Methods in Molecular Biology*. Vol. 1926. New York, NY: Humana Press; 2019. DOI: 10.1007/978-1-4939-9021-4_5
- [46] Al-Mofty S, Elsayed M, Ali H, Ahmed O, Altayyeb A, Wahby A, et al. A microfluidic platform for dissociating clinical scale tissue samples into single cells. *Biomedical Microdevices*. 2021;**23**(1):10. DOI: 10.1007/s10544-021-00544-5
- [47] Shibue T, Weinberg RA. EMT, CSCs, and drug resistance: The mechanistic link and clinical implications. *Nature Reviews. Clinical Oncology*. 2017;**14**(10):611-629. DOI: 10.1038/nrclinonc.2017.44. Epub 2017 Apr 11
- [48] Janik K, Popeda M, Peciak J, Rosiak K, Smolarz M, Treda C, et al. Efficient and simple approach to *in-vitro* culture of primary epithelial cancer cells. *Bioscience Reports*. 2016;**36**(6):e00423. DOI: 10.1042/BSR20160208
- [49] Mei J, Jiang XY, Tian HX, Rong DC, Song JN, Wang L, et al. Anoikis in cell fate, physiopathology, and therapeutic interventions. *MedComm* (2020). 2024;**5**(10):e718. DOI: 10.1002/mco2.718
- [50] Weiskirchen S, Schröder SK, Buhl EM, Weiskirchen R. A Beginner's guide to cell culture: Practical advice for preventing needless problems. *Cells*. 2023;**12**(5):682. DOI: 10.3390/cells12050682
- [51] Richter M, Piwocka O, Musielak M, Piotrowski I, Suchorska WM, Trzeciak T. From donor to the lab: A fascinating journey of primary cell lines. *Frontiers in Cell and Development Biology*. 2021;**9**:711381. DOI: 10.3389/fcell.2021.711381
- [52] Fang Y, Eglen RM. Three-dimensional cell cultures in drug discovery and development. *SLAS*

- Discovery. 2017;**22**(5):456-472. DOI: 10.1177/1087057117696795. Erratum in: SLAS Discov. 2021 Oct;**26**(9):NP1. doi: 10.1177/2472555218787198
- [53] Lee SY, Koo IS, Hwang HJ, Lee DW. In vitro three-dimensional (3D) cell culture tools for spheroid and organoid models. SLAS Discovery. 2023;**28**(4):119-137. DOI: 10.1016/j.slasd.2023.03.006. Epub 2023 Mar 28
- [54] Jeong SR, Kang M. Exploring tumor-immune interactions in co-culture models of T cells and tumor organoids derived from patients. International Journal of Molecular Sciences. 2023;**24**(19):14609. DOI: 10.3390/ijms241914609
- [55] Kapałczyńska M, Kolenda T, Przybyła W, Zajackowska M, Teresiak A, Filas V, et al. 2D and 3D cell cultures - A comparison of different types of cancer cell cultures. Archives of Medical Science (Poland: Termedia Publishing House). 2018;**14**(4):910-919. DOI: 10.5114/aoms.2016.63743
- [56] Labani-Motlagh A, Ashja-Mahdavi M, Loskog A. The tumor microenvironment: A milieu hindering and obstructing antitumor immune responses. Frontiers in Immunology. 2020;**11**:940. DOI: 10.3389/fimmu.2020.00940
- [57] Hsieh CH, Chen YD, Huang SF, Wang HM, Wu MH. The effect of primary cancer cell culture models on the results of drug chemosensitivity assays: The application of perfusion microbio reactor system as cell culture vessel. BioMed Research International. 2015;**2015**:470283. DOI: 10.1155/2015/470283. Epub 2015 Jan 14
- [58] Hamburger A, Salmon SE. Primary bioassay of human myeloma stem cells. The Journal of Clinical Investigation. 1977;**60**(4):846-854. DOI: 10.1172/JCI108839
- [59] Theodoridis K, Aggelidou E, Manthou ME, Keklikoglou K, Tsimponis A, Demiri E, et al. An effective device and method for enhanced cell growth in 3D scaffolds: Investigation of cell seeding and proliferation under static and dynamic conditions. Materials Science & Engineering. C, Materials for Biological Applications. 2020;**114**:111060. DOI: 10.1016/j.msec.2020.111060. Epub 2020 May 11
- [60] Micek HM, Visetsouk MR, Masters KS, Kreeger PK. Engineering the extracellular matrix to model the evolving tumor microenvironment. iScience. 2020;**23**(11):101742. DOI: 10.1016/j.isci.2020.101742
- [61] Erlichman C, Vidgen D. Cytotoxicity of adriamycin in MGH-U1 cells grown as monolayer cultures, spheroids, and xenografts in immune-deprived mice. Cancer Research. 1984;**44**(11):5369-5375
- [62] Rygaard J, Povsen CO. Heterotransplantation of a human malignant tumour to “nude” mice. 1969. APMIS. 2007;**115**(5):604-606; discussion 607-8. DOI: 10.1111/j.1600-0463.2007.apm_689a.x
- [63] Izumchenko E, Paz K, Ciznadija D, Sloma I, Katz A, Vasquez-Dunddel D, et al. Patient-derived xenografts effectively capture responses to oncology therapy in a heterogeneous cohort of patients with solid tumors. Annals of Oncology. 2017;**28**(10):2595-2605. DOI: 10.1093/annonc/mdx416
- [64] Malaney P, Nicosia SV, Davé V. One mouse, one patient paradigm: New avatars of personalized cancer therapy. Cancer Letters. 2014;**344**(1):1-12.

DOI: 10.1016/j.canlet.2013.10.010. Epub 2013 Oct 22

[65] Peterson JK, Houghton PJ. Integrating pharmacology and *in-vivo* cancer models in preclinical and clinical drug development. *European Journal of Cancer*. 2004;**40**(6):837-844. DOI: 10.1016/j.ejca.2004.01.003

[66] Abdolahi S, Ghazvinian Z, Muhammadnejad S, Saleh M, Asadzadeh Aghdai H, Baghaei K. Patient-derived xenograft (PDX) models, applications and challenges in cancer research. *Journal of Translational Medicine*. 2022;**20**(1):206. DOI: 10.1186/s12967-022-03405-8

[67] Vlachogiannis G et al. Patient-derived organoids model treatment response of metastatic gastrointestinal cancers. *Science*. 2018;**359**:920-926. DOI: 10.1126/science.aao2774

[68] Gillet J, Calcagno AM, Varma S, Marino M, Green LJ, Vora MI, et al. Redefining the relevance of established cancer cell lines to the study of mechanisms of clinical anti-cancer drug resistance. *Proceedings. National Academy of Sciences. United States of America*. 2011;**108**(46):18708-18713. DOI: 10.1073/pnas.1111840108

[69] Ben-David U, Siranosian B, Ha G, et al. Genetic and transcriptional evolution alters cancer cell line drug response. *Nature*. 2018;**560**:325-330. DOI: 10.1038/s41586-018-0409-3

[70] Kodack DP, Farago AF, Dastur A, Held MA, Dardaei L, Friboulet L, et al. Primary patient-derived cancer cells and their potential for personalized cancer patient care. *Cell Reports*. 2017;**21**(11):32980-33309. DOI: 10.1016/j.celrep.2017.11.051

[71] Anderson NM, Simon MC. The tumor microenvironment. *Current*

Biology. 2020;**30**(16):R921-R925. DOI: 10.1016/j.cub.2020.06.081

[72] Shinto E, Hase K, Hashiguchi Y, et al. CD8+ and FOXP3+ tumor-infiltrating T cells before and after chemoradiotherapy for rectal cancer. *Annals of Surgical Oncology*. 2014;**21**(Suppl. 3):414-421. DOI: 10.1245/s10434-014-3584-y

[73] Kong JCH, Guerra GR, Millen RM, Roth S, Xu H, Neeson PJ, et al. Tumor-infiltrating lymphocyte function predicts response to Neoadjuvant Chemoradiotherapy in locally advanced rectal cancer. *JCO Precision Oncology*. 2018;**2**:1-15. DOI: 10.1200/PO.18.00075

[74] Mundhara N, Sadhukhan P. Cracking the codes behind cancer cells' immune evasion. *International Journal of Molecular Sciences*. 2024;**25**(16):8899. DOI: 10.3390/ijms25168899

[75] Altmann DM. A Nobel prize-worthy pursuit: Cancer immunology and harnessing immunity to tumour neoantigens. *Immunology*. 2018;**155**(3):283-284. DOI: 10.1111/imm.13008

[76] Sergazy S, Seydahmetova R, Gulyayev A, Shulgau Z, Aljofan M. The role of exosomes in cancer progression and therapy. *Biology*. 2025;**14**(1):27. DOI: 10.3390/biology14010027

[77] Schrader J, Gordon-Walker TT, Aucott RL, van Deemter M, Quaas A, Walsh S, et al. Matrix stiffness modulates proliferation, chemotherapeutic response, and dormancy in hepatocellular carcinoma cells. *Hepatology*. 2011;**53**(4):1192-1205. DOI: 10.1002/hep.24108

[78] Meditz K, Rinner B. Establishment of tumor cell lines: From primary tumor cells to a tumor cell line. In: Kasper C, Charwat V, Lavrentieva A, editors. *Cell*

Culture Technology. Learning Materials in Biosciences. Cham: Springer; 2018

[79] Hong HK, Pyo DH, Kim TW, Yun NH, Lee YS, Song SJ, et al. Efficient primary culture model of patient-derived tumor cells from colorectal cancer using a rho-associated protein kinase inhibitor and feeder cells. *Oncology Reports*. 2019;**42**(5):2029-2038. DOI: 10.3892/or.2019.7285 Epub 2019 Aug 20

[80] Gao B, Huang C, Kernstine K, Pelekanou V, Kluger Y, Jiang T, et al. Non-malignant respiratory epithelial cells preferentially proliferate from resected non-small cell lung cancer specimens cultured under conditionally reprogrammed conditions. *Oncotarget*. 2017;**8**(7):11114-11126. DOI: 10.18632/oncotarget.14366

[81] Hynds RE, Ben Aissa A, KHC G, TBK W, Bosshard-Carter L, Rowan AJ, et al. Expansion of airway basal epithelial cells from primary human non-small cell lung cancer tumors. *International Journal of Cancer*. 2018;**143**(1):160-166. DOI: 10.1002/ijc.31383. Epub 2018 Apr 6

[82] Liu X, Ory V, Chapman S, Yuan H, Albanese C, Kallakury B, et al. ROCK inhibitor and feeder cells induce the conditional reprogramming of epithelial cells. *The American Journal of Pathology*. 2012;**180**(2):599-607. DOI: 10.1016/j.ajpath.2011.10.036. Epub 2011 Dec 18

[83] Chu X, Tian W, Ning J, et al. Cancer stem cells: Advances in knowledge and implications for cancer therapy. *Signal Transduction and Targeted Therapy*. 2024;**9**:170. DOI: 10.1038/s41392-024-01851-y

[84] Herreros-Pomares A. Identification, culture and targeting of cancer stem cells. *Life (Basel)*. 2022;**12**(2):184. DOI: 10.3390/life12020184

[85] Dagogo-Jack I, Shaw A. Tumour heterogeneity and resistance to cancer therapies. *Nature Reviews. Clinical Oncology*. 2018;**15**:81-94. DOI: 10.1038/nrclinonc.2017.166

[86] Zhang X, Xie J, Yang Z, Yu CKW, Hu Y, Qin J. Tumour heterogeneity and personalized treatment screening based on single-cell transcriptomics. *Computational and Structural Biotechnology Journal*. 2024;**27**:307-320. DOI: 10.1016/j.csbj.2024.12.020

[87] Graf JF, Zavodszky MI. Characterizing the heterogeneity of tumor tissues from spatially resolved molecular measures. *PLoS One*. 2017;**12**(11):e0188878. DOI: 10.1371/journal.pone.0188878

[88] Meijer TG, Naipal KA, Jager A, van Gent DC. *Ex vivo* tumor culture systems for functional drug testing and therapy response prediction. *Future Science OA*. 2017;**3**(2):FSO190. DOI: 10.4155/fsoa-2017-0003

[89] Koh B, Jeon H, Kim D, Kang D, Kim KR. Effect of fibroblast co-culture on the proliferation, viability and drug response of colon cancer cells. *Oncology Letters*. 2019;**17**(2):2409-2417. DOI: 10.3892/ol.2018.9836. Epub 2018 Dec 14

[90] Itoh G, Takagane K, Fukushi Y, Kuriyama S, Umakoshi M, Goto A, et al. Cancer-associated fibroblasts educate normal fibroblasts to facilitate cancer cell spreading and T-cell suppression. *Molecular Oncology*. 2022;**16**(1):166-187. DOI: 10.1002/1878-0261.13077. Epub 2021 Nov 5

[91] Liberti MV, Locasale JW. The Warburg effect: How does it benefit cancer cells? *Trends in Biochemical Sciences*. 2016;**41**(3):211-218. DOI: 10.1016/j.tibs.2015.12.001. Epub

- 2016 Jan 5. Erratum in: Trends Biochem Sci. 2016 Mar;41(3):287. Erratum in: Trends Biochem Sci. 2016 Mar;41(3):287. doi: 10.1016/j.tibs.2016.01.004
- [92] Pinto B, Henriques AC, Silva PMA, Bousbaa H. Three-dimensional spheroids as In vitro preclinical models for cancer research. *Pharmaceutics*. 2020;**12**(12):1186. DOI: 10.3390/pharmaceutics12121186
- [93] Rumgay H, Arnold M, Ferlay J, Lesi O, Cabasag CJ, Vignat J, et al. Global burden of primary liver cancer in 2020 and predictions to 2040. *Journal of Hepatology*. Dec 2022;**77**(6):1598-1606. DOI: 10.1016/j.jhep.2022.08.021. Epub 2022 Oct 5
- [94] Broutier L, Mastrogiovanni G, Verstegen M, et al. Human primary liver cancer-derived organoid cultures for disease modeling and drug screening. *Nature Medicine*. 2017;**23**:1424-1435. DOI: 10.1038/nm.4438
- [95] Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nature Medicine*. 1997;**3**(7):730-737. DOI: 10.1038/nm0797-730
- [96] Plaks V, Kong N, Werb Z. The cancer stem cell niche: How essential is the niche in regulating stemness of tumor cells? *Cell Stem Cell*. 2015;**16**(3):225-238. DOI: 10.1016/j.stem.2015.02.015
- [97] Prager BC, Xie Q, Bao S, Rich JN. Cancer stem cells: The architects of the tumor ecosystem. *Cell Stem Cell*. 3 Jan **24**(1):41-53. DOI: 10.1016/j.stem.2018.12.009
- [98] Batlle E, Clevers H. Cancer stem cells revisited. *Nature Medicine*. 2017;**23**(10):1124-1134. DOI: 10.1038/nm.4409
- [99] Chang JC. Cancer stem cells: Role in tumor growth, recurrence, metastasis, and treatment resistance. *Medicine (Baltimore)*. 2016;**95**(1 Suppl. 1):S20-S25. DOI: 10.1097/MD.00000000000004766
- [100] Desai A, Webb B, Gerson SL. CD133+ cells contribute to radioresistance via altered regulation of DNA repair genes in human lung cancer cells. *Radiotherapy and Oncology*. 2014;**110**(3):538-545. DOI: 10.1016/j.radonc.2013.10.040 Epub 2014 Jan 16
- [101] McIntosh K, Balch C, Tiwari AK. Tackling multidrug resistance mediated by efflux transporters in tumor-initiating cells. *Expert Opinion on Drug Metabolism & Toxicology*. 2016;**12**(6):633-644. DOI: 10.1080/17425255.2016.1179280. Epub 2016 May 4
- [102] Lavudi K, Nuguri SM, Pandey P, Kokkanti RR, Wang QE. ALDH and cancer stem cells: Pathways, challenges, and future directions in targeted therapy. *Life Sciences*. 2024;**356**:123033. DOI: 10.1016/j.lfs.2024.123033. Epub 2024 Aug 31
- [103] Lytle NK, Barber AG, Reya T. Stem cell fate in cancer growth, progression and therapy resistance. *Nature Reviews. Cancer*. 2018;**18**(11):669-680. DOI: 10.1038/s41568-018-0056-x
- [104] Yuan S, Norgard RJ, Stanger BZ. Cellular plasticity in cancer. *Cancer Discovery*. 2019;**9**(7):837-851. DOI: 10.1158/2159-8290.CD-19-0015. Epub 2019 Apr 16
- [105] Abbaszadegan MR, Bagheri V, Razavi MS, Momtazi AA, Sahebkar A, Gholamin M. Isolation, identification, and characterization of cancer stem cells: A review. *Journal of Cellular Physiology*.

2017;**232**(8):2008-2018. DOI: 10.1002/jcp.25759. Epub 2017 Feb 28

[106] Thakur R, Kumari N, Mishra DP. Chapter 6 - Isolation and characterization of cancer stem cells. In: Misra G, Rajawat J, editors. *Protocol Handbook for Cancer Biology*. Academic Press; 2021. pp. 87-105. DOI: 10.1016/B978-0-323-90006-5.00007-0. ISBN 780323900065

[107] Rappa G, Fodstad O, Lorico A. The stem cell-associated antigen CD133 (Prominin-1) is a molecular therapeutic target for metastatic melanoma. *Stem Cells*. 2008;**26**(12):3008-3017. DOI: 10.1634/stemcells.2008-0601

[108] Sun JH, Luo Q, Liu LL, Song GB. Liver cancer stem cell markers: Progression and therapeutic implications. *World Journal of Gastroenterology*. 2016;**22**(13):3547-3557. DOI: 10.3748/wjg.v22.i13.3547

[109] Dzobo K, Sinkala M. Cancer stem cell marker CD44 plays multiple key roles in human cancers: Immune suppression/evasion, drug resistance, epithelial-mesenchymal transition, and metastasis. *OMICS*. 2021;**25**(5):313-332. DOI: 10.1089/omi.2021.0025

[110] Yehya A, Youssef J, Hachem S, Ismael J, Abou-Kheir W. Tissue-specific cancer stem/progenitor cells: Therapeutic implications. *World Journal of Stem Cells*. 2023;**15**(5):323-341. DOI: 10.4252/wjsc.v15.i5.323

[111] Greve B, Kelsch R, Spaniol K, Eich HT, Götte M. Flow cytometry in cancer stem cell analysis and separation. *Cytometry. Part A*. 2012;**81**(4):284-293. DOI: 10.1002/cyto.a.22022. Epub 2012 Feb 6

[112] Dobbin ZC, Landen CN. Isolation and characterization of potential cancer

stem cells from solid human tumors—potential applications. *Current Protocols in Pharmacology*. 2013;**63**:14.28.1-14.28.19. DOI: 10.1002/0471141755.ph1428s63

[113] Gronke K, Kofoed-Nielsen M, Diefenbach A. Isolation and flow cytometry analysis of innate lymphoid cells from the intestinal lamina Propria. *Methods in Molecular Biology*. 2017;**1559**:255-265. DOI: 10.1007/978-1-4939-6786-5_17

[114] Drescher H, Weiskirchen S, Weiskirchen R. Flow cytometry: A blessing and a curse. *Biomedicine*. 2021;**9**(11):1613. DOI: 10.3390/biomedicines9111613

[115] Plouffe BD, Murthy SK, Lewis LH. Fundamentals and application of magnetic particles in cell isolation and enrichment: A review. *Reports on Progress in Physics*. 2015;**78**(1):016601. DOI: 10.1088/0034-4885/78/1/016601. Epub 2014 Dec 4

[116] Guo T, Zhang S, Zeng W, Liang Y, Xie J, Liu S, et al. Isolation and identification of patient-derived liver cancer stem cells and development of personalized treatment strategies. *Journal of Translational Medicine*. 2024;**22**(1):1036. DOI: 10.1186/s12967-024-05870-9

[117] Yuan GC, Cai L, Elowitz M, et al. Challenges and emerging directions in single-cell analysis. *Genome Biology*. 2017;**18**:84. DOI: 10.1186/s13059-017-1218-y

[118] Tang C, Fu S, Jin X, et al. Personalized tumor combination therapy optimization using the single-cell transcriptome. *Genome Medicine*. 2023;**15**:105. DOI: 10.1186/s13073-023-01256-6

- [119] Yang C, Yang C, Yarden Y, To KKW, Fu L. The prospects of tumor chemosensitivity testing at the single-cell level. *Drug Resistance Updates*. 2021;**54**:100741. ISSN 1368-7646
- [120] Kumar MP, Du J, Lagoudas G, Jiao Y, Sawyer A, Drummond DC, et al. Analysis of single-cell RNA-Seq identifies cell-cell communication associated with tumor characteristics. *Cell Reports*. 2018;**25**(6):1458-1468.e4. DOI: 10.1016/j.celrep.2018.10.047
- [121] Tung PY, Blischak J, Hsiao C, et al. Batch effects and the effective design of single-cell gene expression studies. *Scientific Reports*. 2017;**7**:39921. DOI: 10.1038/srep39921
- [122] Sinha S, Vegesna R, Mukherjee S, et al. PERCEPTION predicts patient response and resistance to treatment using single-cell transcriptomics of their tumors. *Nature Cancer*. 2024;**5**:938-952. DOI: 10.1038/s43018-024-00756-7
- [123] Paz-Ares L, Gonds A, Saldana D, Thomas M, Mascaux C, Bubendorf L, et al. Genomic testing among patients with newly diagnosed advanced non-small cell lung cancer in the United States: A contemporary clinical practice patterns study. *Lung Cancer*. May 2022;**167**:41-48. DOI: 10.1016/j.lungcan.2022.01.021. Epub 2022 Feb 3
- [124] Olson H, Betton G, Robinson D, Thomas K, Monro A, Kolaja G, et al. Concordance of the toxicity of pharmaceuticals in humans and in animals. *Regulatory Toxicology and Pharmacology*. 2000;**32**(1):56-67. DOI: 10.1006/rtph.2000.1399
- [125] Eglen R, Reisine T. Primary cells and stem cells in drug discovery: Emerging tools for high-throughput screening. *Assay and Drug Development Technologies*. 2011;**9**(2):108-124. DOI: 10.1089/adt.2010.0305. Epub 2010 Dec 27
- [126] Ferreira LLG, Andricopulo AD. ADMET modeling approaches in drug discovery. *Drug Discovery Today*. 2019;**24**(5):1157-1165. DOI: 10.1016/j.drudis.2019.03.015. Epub 2019 Mar 16
- [127] Zhao M, Ma J, Li M, Zhang Y, Jiang B, Zhao X, et al. Cytochrome P450 enzymes and drug metabolism in humans. *International Journal of Molecular Sciences*. 2021;**22**(23):12808. DOI: 10.3390/ijms222312808
- [128] Lyubimov AV, Li AP. Hepatocyte-based metabolism, drug-drug interaction, and toxicity assays in drug discovery and drug development to minimize attrition. In: Lyubimov AV, editor. *Encyclopedia of Drug Metabolism and Interactions*. John Wiley & Sons, Inc.; 2015. DOI: 10.1002/9780470921920.edm138
- [129] Miller RT. Risk assessment for hepatobiliary toxicity liabilities in drug development. *Toxicologic Pathology*. 2023;**51**(7-8):432-436. DOI: 10.1177/01926233231223751 Epub 2024 Jan 20
- [130] Bhakuni GS, Bedi O, Bariwal J, et al. Animal models of hepatotoxicity. *Inflammation Research*. 2016;**65**:13-24. DOI: 10.1007/s00011-015-0883-0
- [131] Sinz MW, Kim S. Stem cells, immortalized cells and primary cells in ADMET assays. *Drug Discovery Today: Technologies*. Spring 2006;**3**(1):79-85. DOI: 10.1016/j.ddtec.2006.03.006
- [132] Li AP. In vitro human hepatocyte-based experimental systems for the evaluation of human drug metabolism, drug-drug interactions, and drug toxicity in drug development. *Current Topics in Medicinal Chemistry*. 2014;**14**(11):

1325-1338. DOI: 10.2174/

1568026614666140506114411

[133] Ueyama-Toba Y, Tong Y, Yokota J, Murai K, Hikita H, Eguchi H, et al. Development of a hepatic differentiation method in 2D culture from primary human hepatocyte-derived organoids for pharmaceutical research. *iScience*. 2024;**27**(9):110778. DOI: 10.1016/j.isci.2024.110778

[134] Sola A, Bertacchini J, D'Avella D, Anselmi L, Maraldi T, Marmioli S, et al. Development of solvent-casting particulate leaching (SCPL) polymer scaffolds as improved three-dimensional supports to mimic the bone marrow niche. *Materials Science & Engineering. C, Materials for Biological Applications*. 2019;**96**:153-165. DOI: 10.1016/j.msec.2018.10.086. Epub 2018 Nov 7

[135] Hong J, Yeo M, Yang GH, Kim G. Cell-electrospinning and its application for tissue engineering. *International Journal of Molecular Sciences*. 2019;**20**(24):6208. DOI: 10.3390/ijms20246208

[136] Uddin MM, Jahan UM, Mohakar V, Talukder A, Absalan Y, Blevins B, et al. Fabrication of aligned polyhydroxybutyrate fibrous scaffolds via a touchspinning apparatus. *ACS Omega*. 2025;**10**(22):22735-22746. DOI: 10.1021/acsomega.4c11296

[137] Reusch RN. Physiological importance of poly-(R)-3-hydroxybutyrate. *Chemistry & Biodiversity*. 2012;**9**:2343-2366. DOI: 10.1002/cbdv.201200278

[138] Fontoura JC, Viezzer C, Dos Santos FG, Ligabue RA, Weinlich R, Puga RD, et al. Comparison of 2D and 3D cell culture models for cell growth, gene expression and drug resistance. *Materials Science & Engineering*

C-Materials for Biological Applications. Feb 2020;**107**:110264. DOI: 10.1016/j.msec.2019.110264. Epub 2019 Oct 14

[139] Bell CC, Dankers ACA, Lauschke VM, Sison-Young R, Jenkins R, Rowe C, et al. Comparison of hepatic 2D Sandwich cultures and 3D spheroids for long-term toxicity applications: A Multicenter study. *Toxicological Sciences*. 2018;**162**(2):655-666. DOI: 10.1093/toxsci/kfx289

[140] den Braver-Sewradj SP, den Braver MW, Vermeulen NP, Commandeur JN, Richert L, Vos JC. Inter-donor variability of phase I/phase II metabolism of three reference drugs in cryopreserved primary human hepatocytes in suspension and monolayer. *Toxicology In Vitro*. 2016;**33**:71-79. DOI: 10.1016/j.tiv.2016.02.013. Epub 2016 Feb 26

[141] Friend JR, Wu FJ, Hansen LK, Remmel RP, Hu WS. Formation and characterization of hepatocyte spheroids. In: Morgan JR, Yarmush ML, editors. *Tissue Engineering Methods and Protocols. Methods in Molecular Medicine™*. Vol. 18. Totowa, N.J: Humana Press; 1999. p. 245. DOI: 10.1385/0-89603-516-6

[142] Ahmed HMM, Salerno S, Piscioneri A, Khakpour S, Giorno L, De Bartolo L. Human liver microtissue spheroids in hollow fiber membrane bioreactor. *Colloids and Surfaces B: Biointerfaces*. 2017;**160**:272-280. ISSN 0927-7765. DOI: 10.1016/j.colsurfb.2017.09.024

[143] Tostões RM, Leite SB, Serra M, Jensen J, Björquist P, Carrondo MJ, et al. Human liver cell spheroids in extended perfusion bioreactor culture for repeated-dose drug testing. *Hepatology*. 2012;**55**(4):1227-1236. DOI: 10.1002/hep.24760

- [144] Wei J, Sun Y, Wang H, Zhu T, Li L, Zhou Y, et al. Designer cellular spheroids with DNA origami for drug screening. *Science Advances*. 2024;**10**(29):eado9880. DOI: 10.1126/sciadv.ado9880. Epub 2024 Jul 19
- [145] Finnberg NK, Gokare P, Lev A, Grivennikov SI, MacFarlane AW 4th, Campbell KS, et al. Application of 3D tumoroid systems to define immune and cytotoxic therapeutic responses based on tumoroid and tissue slice culture molecular signatures. *Oncotarget*. 2017;**8**(40):66747-66757. DOI: 10.18632/oncotarget.19965
- [146] Hanahan D, Coussens LM. Accessories to the crime: Functions of cells recruited to the tumor microenvironment. *Cancer Cell*. 2012;**21**(3):309-322. DOI: 10.1016/j.ccr.2012.02.022
- [147] Zheng Z, Chen J, Chen X, Huang L, Xie W, Lin Q, et al. Enabling single-cell drug response annotations from bulk RNA-Seq using SCAD. *Advanced Science (Weinh)*. 2023;**10**(11):e2204113. DOI: 10.1002/advs.202204113. Epub 2023 Feb 10
- [148] Zhang L, Zhang Y, Wang C, et al. Integrated single-cell RNA sequencing analysis reveals distinct cellular and transcriptional modules associated with survival in lung cancer. *Signal Transduction and Targeted Therapy*. 2022;**7**:9. DOI: 10.1038/s41392-021-00824-9
- [149] Song H, Weinstein HNW, Allegakoen P, Wadsworth MH 2nd, Xie J, Yang H, et al. Single-cell analysis of human primary prostate cancer reveals the heterogeneity of tumor-associated epithelial cell states. *Nature Communications*. 2022;**13**(1):141. DOI: 10.1038/s41467-021-27322-4
- [150] Wang Q, Zhang Y, Zhang B, Fu Y, Zhao X, Zhang J, et al. Single-cell chromatin accessibility landscape in kidney identifies additional cell-of-origin in heterogenous papillary renal cell carcinoma. *Nature Communications*. 2022;**13**(1):31. DOI: 10.1038/s41467-021-27660-3
- [151] Cell Therapy Market Size, Share & Trends Analysis Report by Therapy Type (Autologous (Stem Cell Therapies, Non-stem Cell Therapies), Allogeneic), by Therapeutic Area, by Region, and Segment Forecasts, 2024 – 2030. San Francisco, CA, United States: Grand View Research, Inc.
- [152] Szymańska A, Mucha K, Kosieradzki M, Nazarewski S, Pączek L, Foronczewicz B. Organization of post-transplant care and the 5-year outcomes of kidney transplantation. *International Journal of Environmental Research and Public Health*. 2010;**2022**:19. DOI: 10.3390/ijerph19042010
- [153] Eurotransplant - Statistics. Leiden, The Netherlands: Eurotransplant
- [154] Kim EJ, Chen C, Gologorsky R, et al. Feasibility of an implantable bioreactor for renal cell therapy using silicon nanopore membranes. *Nature Communications*. 2023;**14**:4890. DOI: 10.1038/s41467-023-39888-2
- [155] Trebicka J, Xie Q. Acute and acute-on-chronic liver failure: Pathogenesis, management and perspectives. *Liver International*. 2025;**45**:e16003. DOI: 10.1111/liv.16003
- [156] Vargas M, Cardoso Toniasso SC, Riedel PG, Baldin CP, Dos Reis FL, Pereira RM, et al. Metabolic disease and the liver: A review. *World Journal of Hepatology*. 2024;**16**(1):33-40. DOI: 10.4254/wjh.v16.i1.33

- [157] Guilder L, Pula S, Pierre G. Metabolic disorders presenting as liver disease. *Paediatrics and Child Health*. 2017;**27**(12):533-539. ISSN 1751-7222,. DOI: 10.1016/j.paed.2017.07.007
- [158] Dhawan A, Chaijitraruch N, Fitzpatrick E, Bansal S, Filippi C, Lehec SC, et al. Alginate microencapsulated human hepatocytes for the treatment of acute liver failure in children. *Journal of Hepatology*. 2020;**72**(5):877-884. DOI: 10.1016/j.jhep.2019.12.002. Epub 2019 Dec 13
- [159] Feigin VL, Vos T, Nichols E, Owolabi MO, Carroll WM, Dichgans M, et al. The global burden of neurological disorders: Translating evidence into policy. *Lancet Neurology*. 2020;**19**(3):255-265. DOI: 10.1016/S1474-4422(19)30411-9. Epub 2019 Dec 5
- [160] Svendsen CN, ter Borg MG, Armstrong RJ, Rosser AE, Chandran S, Ostensfeld T, et al. A new method for the rapid and long-term growth of human neural precursor cells. *Journal of Neuroscience Methods*. 1998;**85**(2):141-152. DOI: 10.1016/s0165-0270(98)00126-5
- [161] Xu Z, Su S, Zhou S, et al. How to reprogram human fibroblasts to neurons. *Cell & Bioscience*. 2020;**10**:116. DOI: 10.1186/s13578-020-00476-2
- [162] Yang Y, Chen R, Wu X, Zhao Y, Fan Y, Xiao Z, et al. Rapid and efficient conversion of human fibroblasts into functional neurons by small molecules. *Stem Cell Reports*. 2019;**13**(5):862-876. DOI: 10.1016/j.stemcr.2019.09.007. Epub 2019 Oct 17
- [163] Tenchov R, Sasso JM, Zhou QA. Alzheimer's disease: Exploring the landscape of cognitive decline. *ACS Chemical Neuroscience*. 2024;**15**(21):3800-3827. DOI: 10.1021/acscchemneuro.4c00339. Epub 2024 Oct 11
- [164] Barker RA, Parmar M, Studer L, Takahashi J. Human trials of stem cell-derived dopamine neurons for Parkinson's disease: Dawn of a new era. *Cell Stem Cell*. 2017;**21**(5):569-573. DOI: 10.1016/j.stem.2017.09.014
- [165] Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006;**126**(4):663-676. DOI: 10.1016/j.cell.2006.07.024. Epub 2006 Aug 10
- [166] Park HJ, Jeon J, Choi J, Kim JY, Kim HS, Huh JY, et al. Human iPSC-derived neural precursor cells differentiate into multiple cell types to delay disease progression following transplantation into YAC128 Huntington's disease mouse model. *Cell Proliferation*. 2021;**54**
- [167] Mangalmurti A, Lukens JR. How neurons die in Alzheimer's disease: Implications for neuroinflammation. *Current Opinion in Neurobiology*. 2022;**75**:102575. DOI: 10.1016/j.conb.2022.102575. Epub 2022 Jun 10
- [168] Hampel H, Hardy J, Blennow K, et al. The amyloid- β pathway in Alzheimer's disease. *Molecular Psychiatry*. 2021;**26**:5481-5503. DOI: 10.1038/s41380-021-01249-0
- [169] Goldberg NRS, Marsh SE, Ochaba J, Shelley BC, Davtyan H, Thompson LM, et al. Human neural progenitor transplantation rescues behavior and reduces α -synuclein in a transgenic model of dementia with Lewy bodies. *Stem Cells Translational Medicine*. 2017;**6**(6):1477-1490. DOI: 10.1002/sctm.16-0362. Epub 2017 Feb 22
- [170] Regonesi G. BIOREACTORS: A Complete Review. Giuliano

Regonesi: MicroalgaeX Innovation Center - Istanbul; 2023. DOI: 10.13140/RG.2.2.11630.79685

[171] Eaker S, Abraham E, Allickson J, Brieva TA, Baksh D, Heathman TRJ, et al. Bioreactors for cell therapies: current status and future advances. *Cytotherapy*. 2017;**19**(1):9-18. DOI: 10.1016/j.jcyt.2016.09.011

[172] Schmid A, Kreidl E, Bertschinger M, Vetsch P. Benchtop bioreactors in mammalian cell culture: Overview and guidelines. *Methods in Molecular Biology*. 2022;**2436**:1-15. DOI: 10.1007/978-1-0716-441-4_41

[173] Nath SC, Harper L, Rancourt DE. Cell-based therapy manufacturing in stirred suspension bioreactor: Thoughts for cGMP compliance. *Frontiers in Bioengineering and Biotechnology*. 2020;**8**:599674. DOI: 10.3389/fbioe.2020.599674

[174] Vunjak-Novakovic G, Martin I, Obradovic B, Treppo S, Grodzinsky AJ, Langer R, et al. Bioreactor cultivation conditions modulate the composition and mechanical properties of tissue-engineered cartilage. *Journal of Orthopaedic Research*. 1999;**17**(1):130-138. DOI: 10.1002/jor.1100170119

[175] Shakir S, Hackett TL, Mostaçõ-Guidolin LB. Bioengineering lungs: An overview of current methods, requirements, and challenges for constructing scaffolds. *Frontiers in Bioengineering and Biotechnology*. 2022;**10**:1011800. DOI: 10.3389/fbioe.2022.1011800

[176] Serra AT, Serra M, Silva AC, Brckalo T, Seshire A, Brito C, et al. Scalable culture strategies for the expansion of patient-derived cancer stem cell lines. *Stem Cells International*. 2019;**2019**:8347595. DOI: 10.1155/2019/8347595

[177] Däster S, Amatruda N, Calabrese D, Ivanek R, Turrini E, Droeser RA, et al. Induction of hypoxia and necrosis in multicellular tumor spheroids is associated with resistance to chemotherapy treatment. *Oncotarget*. 2017;**8**(1):1725-1736. DOI: 10.18632/oncotarget.13857

Generation of Functional β Cells from Pluripotent Stem Cells: Advances and Applications

Zenith Khashim and Quinn P. Peterson

Abstract

The generation of functional pancreatic β -cells from pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), offers transformative opportunities in diabetes research and regenerative medicine, as primary human pancreatic β -cells are scarce and difficult to maintain *in vitro*. Stem cell-based differentiation has emerged as a scalable and renewable strategy to produce insulin-secreting β -like cells. This chapter provides an overview of the stepwise differentiation protocols that mimic human embryonic pancreatic β -cell development, highlighting the key signaling pathways and transcription factors involved at each stage. It also addresses critical challenges in achieving full β -cell maturation, including strategies such as 3D culture, co-culture systems, and environmental conditioning. Methods for isolating and enriching functional stem cell-derived β cells (SC- β), such as surface marker-based sorting, metabolic assays like glucose-stimulated insulin secretion (GSIS), and reaggregation into islet-like clusters, are discussed in detail. Functional validation techniques, including immunostaining, GSIS, and mitochondrial assessments, are emphasized to confirm SC- β cells identity and physiological function. Finally, this chapter highlights the use of SC- β cells in drug screening, disease modeling, and cell replacement therapies for diabetes. Continued advancements in differentiation protocols are positioning SC- β cells as vital resources for deepening our knowledge of diabetes and driving the development of effective cell replacement therapies.

Keywords: pluripotent stem cells induced pluripotent stem cells, pancreatic β -cells, insulin, differentiation, definitive endoderm, glucose-stimulated insulin secretion, diabetes, cell replacement therapy, regenerative medicine

1. Introduction

Primary cells play a crucial role in both basic and translational research by offering physiologically relevant models to investigate tissue-specific functions, disease pathways, and responses to therapies. In diabetes research, the pancreas plays a vital role in maintaining glucose balance, primarily through the secretion of hormones by the [1, 2]. Among these hormones, insulin, secreted by pancreatic β -cells, plays

a crucial role in lowering blood glucose levels. In human islets, β -cell account for roughly 55–70% of the total cell population and release insulin in response to rising glucose concentrations [3–5]. Although functional human β -cells are essential for both research and therapy, their acquisition remains a major challenging. Harvesting islet from pancreatic tissue is limited by scarce donor availability, poor cell viability during culture process, and difficulties in expanding cell numbers to meet experimental or clinical demands. These constraints have impeded progress in developing cell replacement therapies, a promising strategy to restore insulin production in individuals with diabetes by transplanting functional β -cells [6, 7]. The discovery of pluripotent stem cells, like embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), offers a renewable and scalable source for generating stem cell-derived β (SC- β) cells *in vitro* [8]. Using stepwise differentiation protocols that recapitulate embryonic pancreatic development, ESCs and iPSCs can be directed to form insulin producing β -like cells with both physiological relevance and therapeutic potential. These advances not only accelerate the development of cell replacement therapies but also expand opportunities for disease modeling, drug screening, and personalized regenerative medicine. This chapter examines the evolution from traditional islet isolation methods to the generation of SC- β cells from ECS/iPSC, emphasizing recent progress in differentiation techniques, functional assessment, and the growing utility of SC- β cells in both diabetes research and therapeutic applications. It also discusses current challenges, technological innovations, and future perspectives aimed at improving SC- β cell maturation, scalability, and clinical translation for effective diabetes treatment.

2. Islet isolation: Overview and limitations

The isolation of pancreatic islets has long served as a critical technique for studying β -cell biology, diabetes pathophysiology, and potential therapeutic interventions. Traditionally, human and animal islets are isolated from donor pancreata using enzymatic and mechanical methods that separate the islets from the surrounding exocrine tissue. The most employed protocol involves collagenase digestion, which enzymatically disrupts the extracellular matrix, followed by density gradient centrifugation to enrich islet populations based on their buoyant density. After centrifugation, islets are typically handpicked under a microscope to ensure purity for downstream applications [9–11]. Islet isolation remains technically challenging despite decades of optimization, with extracting viable islets from donor pancreases particularly difficult. As a result, only about 50% of processed pancreata yield enough islets suitable for transplantation [12, 13]. The limited availability of donor organs continues to severely constrain the supply of primary islets for both clinical use and research. Even when donor tissue is obtainable, isolated β -cells experience rapid declines in viability and function due to physical stress, restricting their application in long-term cultures and functional assays [14, 15]. In addition, this approach lacks the scalability needed to meet the demands of widespread therapeutic use, especially since each clinical transplant requires millions of functional β -cells [16, 17]. These limitations highlight the urgent need for alternative, renewable sources capable of producing high-quality β -cells at a scale suitable for clinical applications. In this context, the development of pluripotent stem cell technologies, including ESCs and iPSCs, offers a promising solution. These platforms allow the *in vitro* generation of SC- β cells through directed differentiation protocols that mimic developmental cues, thereby providing an expandable, donor-independent source of insulin-producing cells [8, 18, 19]. As a result, stem cell-based methods are

gaining popularity as a more effective alternative to overcome the challenges of isolating primary islets. They offer new opportunities for studying disease, testing drugs, and developing cell replacement therapies to treat diabetes.

3. Pluripotent stem cells as sources for pancreatic β -cell generation

The discovery of human pluripotent stem cells (hPSCs) has transformed regenerative medicine and drug development by enabling the *in vitro* generation of replacement cells and tissues for therapeutic use [8]. Owing to their unique ability to differentiate into any cell type in the human body, pluripotent stem cells offer significant promise for advancing diabetes research. Importantly, these pluripotent stem cells can be directed to generate functional pancreatic β -cells, which are essential for insulin production and maintaining blood glucose homeostasis [8, 20]. They broadly fall into two categories: those derived from early-stage embryos (ESCs) and those reprogrammed from adult somatic cells (iPSC). ESCs are derived from an inner cell mass (ICM) of the human blastocyst, a hollow structure that forms approximately 5–6 days after fertilization. At this stage, the human embryo contains about 70–100 cells and is composed of two main components: ICM, which gives rise to the embryo proper and serves as the source of ESCs, and the trophoblast, which develops into the placenta [21, 22]. ESCs are naturally pluripotent and were the first human stem cells to be studied extensively in research. They have the remarkable ability to self-renew indefinitely and to differentiate into all three germ layers: ectoderm, mesoderm, and endoderm [21, 23, 24]. Importantly, since the endoderm gives rise to pancreatic tissue, ESCs serve as an ideal source for generating insulin-producing β -like cells *in vitro* [8] for diabetes research and potential therapy (**Figure 1**).

iPSCs are generated by reprogramming adult somatic cells, such as skin fibroblasts or blood cells, back into a pluripotent state. This is typically achieved by introducing

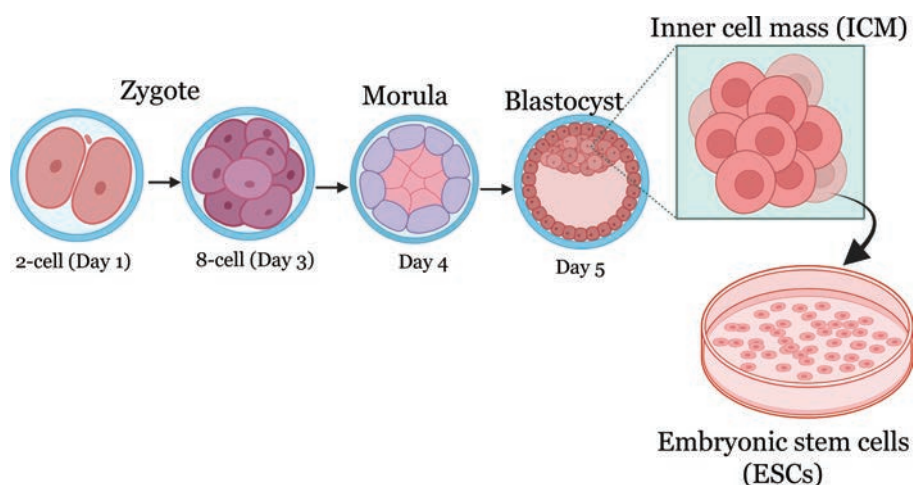


Figure 1. Derivation of embryonic stem cells (ESCs) from early human embryo development. The image illustrates the sequential stages of preimplantation embryonic development, starting from the 2-cell zygote (Day 1), progressing through the 8-cell stage (Day 3) and morula (Day 4), and culminating in the formation of the blastocyst (Day 5). The blastocyst's inner cell mass (ICM) gives rise to ESCs when isolated and cultured *in vitro*. ESCs derived from the ICM are pluripotent and capable of differentiating into all three germ layers.

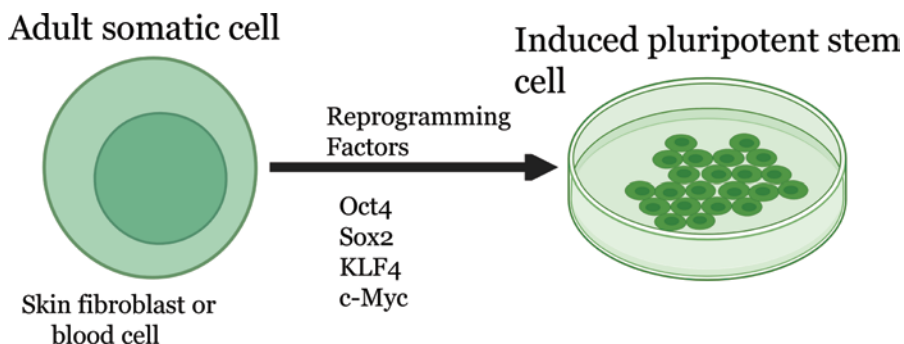


Figure 2.

Generation of induced pluripotent stem cells (iPSCs) from adult somatic cells. This schematic illustrates the reprogramming of an adult somatic cell, such as a skin fibroblast or blood cell, into an iPSC. The reprogramming is achieved by adding transcription factors that revert the somatic cell to a pluripotent state. The resulting iPSCs can differentiate into different cell types.

a set of transcription factors, most commonly Oct4 (Octamer-binding transcription factor 4), Sox2 (SRY-box transcription factor 2), Klf4 (Kruppel-like factor 4), and c-Myc (cellular Myc proto-oncogene), which reset the cell's gene expression and epigenetic profile [25, 26]. This technology offers several important advantages.

iPSCs present a key ethical advantage by circumventing the use of human embryos, thus enhancing their acceptance in both clinical and research contexts. Moreover, iPSCs enable the generation of patient specific cell lines, which are valuable in the field of personalized medicine [27]. In the context of diabetes, somatic cells from affected individuals can be reprogrammed into iPSCs and differentiated into pancreatic SC- β cells [28, 29]. These personalized cells provide a powerful platform for uncovering disease mechanisms, evaluating individual drug responses, and serving as autologous grafts for transplantation markedly reducing the risk of immune rejection (**Figure 2**).

PSCs hold immense promise in regenerative medicine, especially for developing personalized therapies aimed at chronic diseases such as diabetes, cardiovascular disorders, and neurodegenerative conditions. Progress in reprogramming techniques, gene editing technologies like CRISPR-Cas9, and optimized differentiation protocols has expanded the clinical and research utility of iPSC-based systems [30, 31]. Like ESCs, iPSCs exhibit self-renewal and pluripotency, enabling unlimited expansion and differentiation into cells of all three germ layers ectoderm, mesoderm, and endoderm [21, 25, 32]. These distinctive characteristics make PSCs invaluable for studying early human development, modeling diseases, testing drugs, screening therapeutics, and advancing regenerative medicine. One of their most promising applications is the generation of insulin-producing SC- β cells. Through stepwise differentiation protocols that recapitulate embryonic pancreatic development, ESCs and iPSCs can be guided to produce functional SC- β cells capable of glucose-stimulated insulin secretion [8]. This scalable platform addresses the critical limitations associated with donor-derived β -cells and opens new avenues for innovative diabetes therapies.

4. Pluripotent stem cells directed differentiation toward β cells

The generation of functional pancreatic β -cells from hPSCs, including ESCs and iPSCs, relies on directed differentiation protocols that closely mimic the sequential

developmental stages of pancreas formation during embryogenesis. This approach, pioneered by researchers such as Melton and colleagues [8] has become a foundational strategy in the production of insulin-producing β -cells for use in diabetes research, drug screening, and potential cell replacement therapies. During the embryonic development process, the pancreas arises from the definitive endoderm, a germ layer formed early in gastrulation. To emulate this developmental trajectory *in vitro*, differentiation protocols employ stage-specific combinations of growth factors and small molecules that activate or inhibit key signaling pathways such as Nodal/Activin, BMP (Bone morphogenetic protein), FGF (Fibroblast growth factor), Notch, and retinoic acid (RA) to guide cells through precise lineage transitions. Each stage is marked by the expression of defined transcription factors that reflect cellular identity and maturation status. Based on current knowledge and widely adopted methods, the differentiation process typically involves a six-stage progression, designed to replicate the embryological sequence leading to β -cell development [8, 18, 20, 33, 34].

4.1 Stage 1: Definitive endoderm induction

The initial step in guiding pluripotent stem cells toward the pancreatic β -cell lineage involves the induction of definitive endoderm (DE). This embryonic germ layer gives rise to key organs such as the pancreas, liver, lungs, and gastrointestinal tract. *In vivo*, DE arises from the primitive streak during gastrulation, where cells undergo epithelial to mesenchymal transition (EMT) under the control of tightly regulated signaling pathways, particularly Nodal/TGF- β and Wnt. *In vitro*, this process is replicated by exposing pluripotent stem cells to cues that activate these same pathways. For example, Activin A a TGF- β family ligand activates the Smad2/3 signaling pathway, effectively mimicking Nodal function to initiate endodermal differentiation. Concurrent activation of the Wnt/ β -catenin pathway further enhances the efficiency of DE induction. The success of this stage is evaluated by the expression of key DE markers, most notably SRY-box transcription factor 17 (SOX17), a master regulator of endoderm fate, and Forkhead box protein A2 (FOXA2), which is essential for patterning and later organ-specific differentiation. Under optimized conditions, more than 80% of cells typically adopt a SOX17⁺/FOXA2⁺ phenotype within 2–3 days [8, 35–37]. Precise signal timing, concentration, and duration control are essential for consistent DE formation.

4.2 Stage 2: Primitive gut tube and posterior foregut formation

Following DE induction, the next key step in β -cell differentiation is directing the cells to form the primitive gut tube and progress into the posterior foregut, the developmental region that gives rise to the pancreas. This stage mimics the third and fourth weeks of human embryogenesis and requires precise modulation of key signaling pathways, particularly FGF, BMP, and retinoic acid (RA). Growth factors like FGF10 and FGF7 support the expansion and maintenance of foregut progenitors [37]. BMP signaling is carefully balanced, neither overly activated nor strongly inhibited, to prevent misdirection toward intestinal fates. Meanwhile, RA plays a vital role by repressing anterior endoderm identities (such as lung and thyroid) and promoting pancreatic gene programs. As cells undergo morphogenesis into a 3D tube-like structure, they begin expressing transcription factors such as hepatocyte nuclear factor-1 beta (HNF1B), hepatocyte nuclear factor-4 alpha (HNF4A), SOX9, and FOXA2, which mark successful posterior foregut patterning. This stage typically

spans 2–3 days and represents a developmental decision point. Proper signaling ensures that cells are primed to initiate PDX1 expression in the next stage, committing to the pancreatic lineage [8].

4.3 Stage 3: Pancreatic progenitor 1 (PP1) specification

During this stage, gut tube cells are directed toward an early pancreatic progenitor fate, marked by the upregulation of pancreatic duodenal homeobox 1 (PDX1). This transition is guided by a defined set of signaling cues, including retinoic acid (RA), which plays a key role in promoting posterior foregut identity and initiating pancreatic lineage specification. The culture conditions at this stage are optimized to suppress non-pancreatic fates and support the emergence of pancreatic-committed cells [38, 39].

4.4 Stage 4: Pancreatic progenitor 2 (PP2) maturation

Following the PP1 stage, cells continue to mature and begin to co-express PDX1 along with a subset expressing NKX homeobox 1 (NKX6.1), indicating progression toward a committed pancreatic progenitor state. Although the overall signaling conditions remain similar, the concentration of retinoic acid (RA) is reduced to fine-tune pathway activity and improve lineage specificity. This adjustment facilitates the expansion of a bipotent progenitor population that is more precisely directed toward endocrine lineages in the next stage [8].

4.5 Stage 5: Endocrine progenitor differentiation

Following the establishment of pancreatic progenitor identity, the next critical step involves directing these cells toward the endocrine lineage, which gives rise to hormone-producing islet cells, including insulin-secreting β -cells. This transition is initiated by the upregulation of Neurogenin-3 (NGN3), a Basic helix-loop-helix (bHLH) transcription factor that serves as the master regulator of endocrine differentiation. Transient expression of NGN3 is essential, as it triggers a cascade of gene expression changes leading to islet cell specification. The emergence of endocrine progenitors is confirmed by the co-expression of NGN3, Chromogranin A (CHGA), a general marker of neuroendocrine cells, alongside PDX1 and NKX6.1, which are retained from the pancreatic progenitor stage [40, 41].

Notch signaling is actively inhibited to promote this transition, commonly using γ -secretase inhibitors, since active Notch signaling maintains cells in an undifferentiated progenitor state. Inhibition of this pathway permits NGN3 upregulation and endocrine lineage commitment [42]. Precise control of timing and dosage of differentiation cues at this stage is critical; premature endocrine induction can compromise subsequent β -cell maturation and function. This stage lays the groundwork for the final maturation phase, where committed endocrine progenitors acquire the specialized features of glucose-responsive β -cells. This transition is driven by the transient upregulation of NGN3, a master regulator of endocrine specification [43, 44]. The induction of NGN3 initiates a transcriptional cascade that commits cells to the endocrine fate, with emerging progenitors typically co-expressing NGN3, CHGA, and retained markers PDX1 and NKX6.1. To promote this transition, Notch signaling is actively suppressed often through γ -secretase inhibition as sustained Notch activity preserves cells in an undifferentiated state [45–47]. The differentiation medium at this stage is supplemented with factors that support endocrine

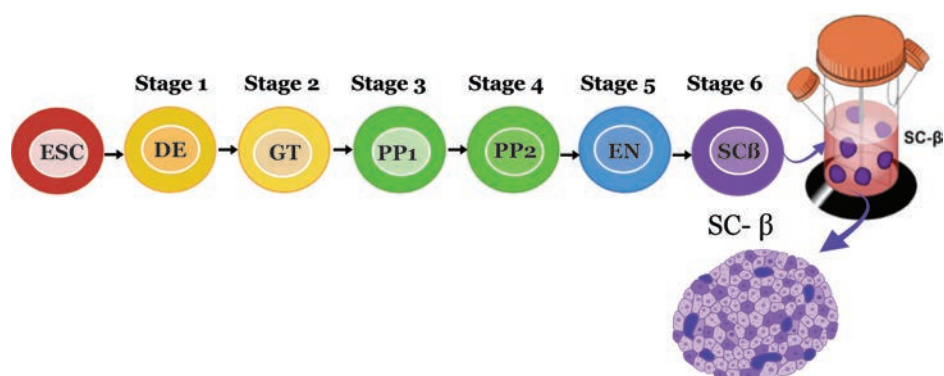


Figure 3. Directed differentiation of hPSCs into SC- β cells. Schematic representation of the six-stage protocol used to generate insulin-producing SC- β -cells from hPSCs. Each stage mimics sequential steps of pancreatic development, guiding cells through definitive endoderm (DE), gut tube (GT), early and late pancreatic progenitors (PP1 and PP2), and endocrine (EN) stages to produce functional SC- β cells.

induction, including modulators of TGF- β (Transforming growth factors), SHH (Sonic Hedgehog), and thyroid hormone pathways, along with reduced retinoic acid levels. The combination of these cues facilitates efficient NGN3 expression while maintaining lineage specificity [48].

4.6 Stage 6: SC- β cell maturation

The final stage of differentiation focuses on transforming endocrine progenitors into functionally mature, glucose-responsive β -cells that can secrete insulin in a regulated manner, mimicking the physiological behavior of native human islet β -cells. During this phase, cells begin to express key β -cell markers such as INS (insulin), C-peptide, NKX6.1, and most notably, MAF bZIP transcription factor A (MAFA), a transcription factor strongly associated with β -cell functional maturity and responsiveness to glucose stimulation. The co-expression of INS, MAFA, and NKX6.1, along with C-peptide, a byproduct of proinsulin processing, indicates successful acquisition of β -cell identity [8, 18]. At this stage, the derived β -cells acquire the ability to respond to GSIS, a critical functional hallmark of matured pancreatic β -cells (**Figure 3**).

5. Maturation of stem cell derived β -cells

The maturation of SC- β cells is a critical step in the development of functionally competent insulin-producing cells for use in regenerative therapies and diabetes research. While current differentiation protocols can generate cells expressing key β -cell markers such as INS, NKX6.1, and C-peptide, these cells often remain functionally immature. Specifically, they frequently exhibit impaired GSIS and lack the dynamic insulin release patterns characteristic of native adult β -cells. To enhance functional maturation, SC- β cells are typically transitioned from two-dimensional (2D) monolayer cultures to three-dimensional (3D) systems that more closely mimic the native islet microenvironment. Among these, 3D spinner flask cultures are widely used due to their scalability and ability to support the formation

of uniform islet-like clusters. These dynamic 3D environments improve nutrient diffusion, promote cell-cell and cell-matrix interactions, and provide mechanical cues that contribute to SC β -cell maturation. In addition to spinner flasks, spheroid plates, and other microwell-based platforms are also employed to facilitate reaggregation after stage 6 differentiation, allowing precise control over cluster size and density [8, 18, 19].

The 3D culture environment supports critical aspects of SC- β cell maturation, including the establishment of apical-basal polarity, enhanced mitochondrial activity, increased insulin granule density, and improved GSIS. Despite these advances, SC- β cell populations often remain heterogeneous, with subsets of cells displaying immature, fetal-like transcriptional profiles. To address this, ongoing studies are exploring strategies to further optimize the *in vitro* maturation process. These include modulating culture media composition by introducing dynamic glucose cycling, fatty acids, and thyroid hormone (e.g., T3), as well as adjusting oxygen tension to better replicate *in vivo* conditions. Co-culture with endothelial cells, mesenchymal stromal cells, or other islet cell types is also under investigation to provide supportive paracrine signals that enhance maturation [49–53]. *In vivo* transplantation remains the gold standard for evaluating the functional maturity of SC- β cells. Transplanted cells consistently exhibit enhanced maturation and insulin secretion compared to their *in vitro* counterparts, underscoring the critical influence of host-derived systemic and tissue-specific cues. Insights from these *in vivo* models are now being applied to optimize *in vitro* differentiation protocols, to produce uniformly mature, glucose-responsive SC- β cells suitable for both clinical and research applications.

6. Isolation and enrichment of β -cells

Given the heterogeneity that arises from *in vitro* differentiation, isolation and enrichment of functional β -cells is essential to enhance the purity, safety, and performance of these cells in both experimental and therapeutic settings. Post-differentiation, cultures often contain a mix of SC- β cells, other endocrine and non-endocrine cell types, and residual progenitors or off-target populations. To address this, several enrichment strategies have been developed. One widely used approach is surface marker-based sorting. For example, β -cell-specific markers such as CD49a (integrin $\alpha 1$) enable selective isolation using fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS) [54, 55]. In addition, functional selection techniques such as GSIS assays are employed to identify and retain cells with proper stimulus-secretion coupling, ensuring functional relevance [56, 57]. Reporter-based enrichment is another effective method, wherein gene reporters (e.g., GFP) driven by insulin promoters are introduced into pluripotent stem cells to visually track and sort insulin-producing cells [58]. Moreover, reaggregation of sorted cells into islet-like clusters has been shown to enhance cell-cell interactions, restore structural integrity, and improve functional properties such as insulin granule formation, synchronized calcium signaling, and glucose responsiveness features that closely mimic the native islet microenvironment. Implementing these enrichment strategies enhances the consistency and reliability of SC- β cell products, reduces the risk of undesired differentiation or teratoma formation, and improves the precision of disease modeling and drug screening [59–61]. These approaches are vital for advancing the clinical translation of SC- β cells and ensuring their therapeutic efficacy in diabetes treatment.

7. Functional validation of β -cells

The maturation of the SC- β cells must be evaluated to confirm their identity and functional maturity. Immunostaining and flowcytometry is typically performed to detect β cell-specific markers such as insulin, C-peptide, PDX1, NKX6.1, and MAFA, indicating proper lineage commitment and maturity. However, the expression of these transcription factors alone does not guarantee physiological function. Therefore, functional validation is critical, and GSIS remains the gold standard assay for assessing β cell activity. In a typical GSIS assay, β cells are sequentially exposed to low (e.g., 3.3 mM) and high (e.g., 16.7 mM) glucose concentrations, and insulin or C-peptide levels in the culture supernatant are measured, usually by human insulin ELISA [8]. Mature SC- β cells exhibit a low basal insulin release at low glucose, followed by a sharp increase in secretion when stimulated with high glucose [8, 62]. This biphasic insulin response reflects proper metabolic coupling, where glucose metabolism increases ATP production, closes ATP-sensitive potassium (K^+) channels and leads to membrane depolarization, calcium influx, and insulin granule exocytosis. In some cases, KCl or arginine may be used as depolarizing controls to test stimulus-secretion coupling independently of glucose metabolism (Figure 4A and B). To further assess SC- β cell potency, mitochondrial assays such as oxygen consumption rate (OCR) and ATP production are used to confirm that the cells have an intact oxidative metabolism, which is essential for ATP-sensitive insulin release [56]. In addition, calcium imaging or dynamic perfusion assays can be used to examine the kinetics and robustness of the insulin response, providing deeper insight into SC- β cell function at single-cell or population levels [63].

Moreover, the downregulation of progenitor markers like NGN3 and the upregulation of genes related to β -cell maturity (e.g., MAFA) further confirm functional differentiation. For translational relevance, *in vivo* validation, such as transplantation into streptozotocin (STZ)-induced diabetic mice, can demonstrate the ability of SC- β cells to restore normoglycemia and maintain glucose homeostasis over time [8, 18, 64]. Collectively, these multidimensional assessments evaluate whether SC- β

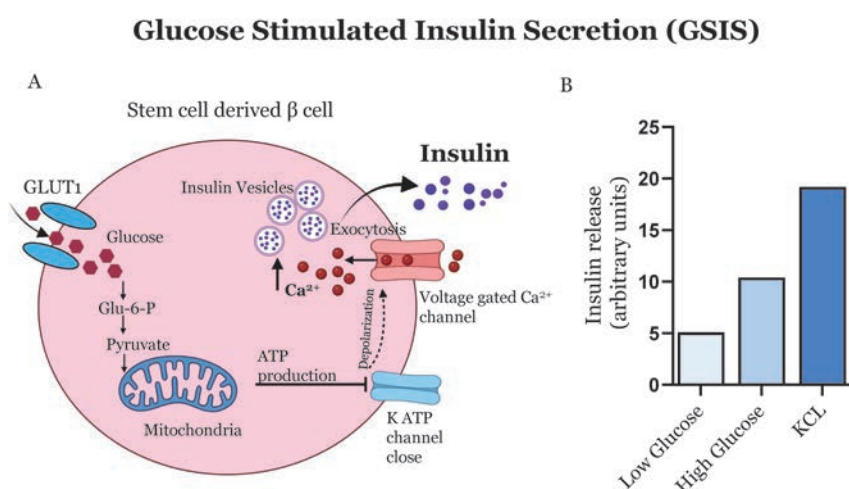


Figure 4. Schematic overview of GSIS in β cells. (A) High glucose levels are metabolized by β -cells, increasing ATP production, which closes KATP channels, triggers calcium influx, and stimulates insulin exocytosis. (B) A typical GSIS profile shows an increased insulin release at high glucose compared to basal levels.

cells are phenotypically authentic and metabolically competent. The main objective is to confirm the ability of the cell to perform GSIS, which is a hallmark of functional β -cells. Establishing this capability is critical for their use in drug discovery for disease and diabetes, and the development of future clinical therapies for diabetes.

8. Applications for SC- β cells in regenerative research

SC- β cells have broad applications in regenerative medicine, particularly in diabetes treatment and disease modeling. Their ability to restore insulin secretion through cell replacement therapy makes them a valuable tool in translational research. Building on this potential, SC- β cells are being used in disease modeling using genetic engineering, high-throughput drug screening, and in the development of immune evasion and encapsulation strategies to enhance graft survival and function.

8.1 Translational and therapeutic applications

SC- β cells have emerged as a powerful platform for both basic research and translational applications in diabetes. Using patient-specific iPSCs, researchers can generate SC- β cells that reflect the genetic and metabolic diversity observed in individuals with diabetes. These *in vitro* models provide critical insights into the molecular mechanisms of β -cell dysfunction and disease progression in a patient-relevant context [8, 18].

Several ongoing clinical trials (e.g., NCT03163511, NCT04786262) are evaluating the safety, efficacy, and long-term viability of SC- β cell transplantation. Many of these studies incorporate micro or macro encapsulation technologies to protect transplanted cells from immune rejection, with the goal of reducing or eliminating the need for lifelong immunosuppressive therapy. Recent advances in generating SC- β cells from stem cells have strengthened their potential to improve our understanding of diabetes, support the discovery of more effective therapies, and contribute to the development of regenerative treatments that may offer a long-term cure [8]. Beyond disease modeling, SC- β cells enable high-throughput drug screening to identify compounds that preserve β -cell identity, enhance insulin secretion, or promote regeneration. This capability also enables the design of personalized therapies based on individual disease characteristics. Creating functional, glucose-responsive SC- β cells is a promising step toward cell-based therapy for diabetes. These cells can help restore the body's ability to produce insulin and keep blood sugar levels in check, which may reduce or even eliminate the need for insulin injections.

8.2 Genetic engineering and disease modeling

Genetic engineering tools, especially CRISPR-Cas9, have transformed the ability to precisely modify the genome of hPSCs, allowing detailed studies of gene function and disease mechanisms in pancreatic β -cell biology. Through targeted correction of mutations in ESCs or iPSCs, researchers can effectively model both monogenic and polygenic forms of diabetes *in vitro*. This approach has proven especially powerful in studying conditions like neonatal diabetes, maturity-onset diabetes of the young (MODY), and syndromic forms of the disease that arise from single-gene defects [65].

Patient-derived iPSCs carrying disease-relevant mutations can be differentiated into SC- β cells, which then serve as platforms to examine how these mutations affect β -cell development, insulin secretion, and metabolic regulation. For example, iPSC

models of MODY caused by mutations in genes such as HNF1A, GCK (Glucokinase), and INS have revealed disease-specific phenotypes such as altered insulin gene expression, impaired glucose sensing, and increased susceptibility to cellular stress. Gene correction using CRISPR-Cas9 in these same lines has been shown to restore normal function, providing a valuable system to test personalized therapeutic approaches [66–68]. In addition to nuclear gene disorders, iPSC models are now being used to study mitochondrial diseases that affect β -cell function. One prominent example is MELAS (Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like episodes), a disorder commonly caused by the m.3243A > G mutation in mitochondrial tRNA^{Leu} (UUR). This mutation is strongly associated with mitochondrial diabetes. iPSCs derived from MELAS patients, which often harbor different levels of heteroplasmy, can be differentiated into SC- β cells to study how mitochondrial dysfunction impairs β -cell bioenergetics, insulin secretion, and stress responses. These models offer valuable insights into heteroplasmy-dependent phenotypes and allow for testing metabolic interventions, such as mitochondrial antioxidants, NAD⁺ (nicotinamide adenine dinucleotide) boosters, or even mitochondrial transfer strategies, to rescue β -cell function [69, 70]. Furthermore, engineered hPSCs have also been used to generate reporter cell lines, where fluorescent or luminescent reporters are placed under the control of SC- β cells specific promoters (e.g., INS or MAFA). These reporter lines facilitate real-time monitoring of differentiation efficiency, β -cell identity, and functional status and are especially useful in high-throughput drug screening and lineage tracing studies [71, 72]. The combination of genome editing and stem cell technologies provides a powerful tool for modeling diverse forms of diabetes like exploring β -cell pathophysiology, developing targeted and creating patient-specific therapies. These models bridge the gap between genotype and cellular phenotype, offering insights that are not easily obtainable from animal models or primary human tissue.

8.3 Immune evasion and encapsulation strategies

A major challenge in translating SC- β cells therapies into clinical treatments is immune rejection. Transplanted SC- β cells are recognized as foreign by the recipient's immune system, leading to graft destruction unless immunosuppressive drugs are administered. However, long-term use of immunosuppressive drugs carries significant risk, including infections, increased cancer risk, and organ damage [73, 74]. As a result, developing strategies to protect transplanted SC- β cells without systemic immunosuppression has become a central goal in regenerative medicine.

One promising approach is genetic engineering to generate hypoimmunogenic SC- β cells. Deletion of major histocompatibility complex (MHC) class I and II molecules reduce antigen presentation and limits recognition by cytotoxic and helper T cells. However, the absence of MHC class I molecules can trigger natural killer (NK) cell mediated cytotoxicity. To counteract this, immune-inhibitory ligands such as HLA-E or CD47 have been introduced to suppress NK cell activation. These combinatorial gene-editing strategies show considerable potential for enabling transplantation across MHC barriers with reduced risk of immune rejection [75, 76]. Another strategy to protect transplanted β -cells from immune attack is biomaterial-based encapsulation, which forms a physical barrier that prevents immune cell infiltration while allowing the diffusion of oxygen, nutrients, glucose, and insulin [64, 77]. Encapsulation technologies are generally categorized into microencapsulation and macroencapsulation. Microencapsulation involves embedding individual cells or small clusters within semi-permeable hydrogels, often alginate-based, which are

chemically modified to reduce fibrosis and immune activation. In contrast, macroencapsulation devices enclose larger cell aggregates within implantable and retrievable chambers made from advanced polymers or membranes engineered with defined porosity. These devices support molecular exchange while shielding the enclosed cells from immune surveillance [78, 79].

Several clinical trials are investigating encapsulation technologies. For example, ViaCyte has developed encapsulation devices such as the Encaptra™ system to protect stem cell-derived β -cells from immune rejection (NCT03163511), while Vertex Pharmaceuticals is evaluating VX-880 (NCT04786262), a stem cell-derived β -cell therapy, both with and without systemic immunosuppression. Additionally, emerging platforms are exploring co-encapsulation with immunomodulatory agents, such as cytokine blockers or immune checkpoint inhibitors, to locally suppress immune responses and reduce the need for systemic immunosuppressive drugs (NCT05210530). Despite promising progress, significant challenges remain. SC- β cell therapies still face major hurdles, including fibrotic overgrowth, hypoxia within encapsulation devices, and incomplete immune protection. Overcoming these barriers will be critical for achieving durable and widespread clinical success in diabetes therapy.

9. Challenges in clinical translation

SC- β cell technologies hold great promise for treating diabetes; several key challenges must be addressed before these therapies can be widely adopted in clinical practice. One of the foremost challenges is functional heterogeneity within differentiated cell populations. Despite significant improvements in directed differentiation protocols, SC- β cell preparations often contain a mixture of mature SC- β cells, other endocrine and non-endocrine cells, and residual progenitor off-target populations [8, 18]. This variability poses risks for inconsistent therapeutic outcomes and complicates product standardization.

Immature or non-functional cells within the graft can lead to failure of transplant or trigger adverse host responses. A major safety concern is the risk of tumorigenicity, as pluripotent stem cells have the potential for unlimited proliferation. If differentiation is incomplete, residual undifferentiated or misdirected cells may persist in the graft and form teratomas or other tumors [59–61, 80]. Minimizing this risk requires complete differentiation of pluripotent cells and the use of robust purification techniques, such as surface marker-based sorting, to eliminate undifferentiated or aberrant cells. At the same time, immune rejection remains a major challenge, especially in allogeneic settings. While strategies like encapsulation and immune-evasive genetic engineering are under active investigation, reliably achieving long-term graft survival without systemic immunosuppression is still difficult. In addition, for patients with type 1 diabetes, autoimmune responses may re-emerge and attack even autologous SC- β cells, underscoring the need for therapies that also address the root causes of immune dysregulation [64].

From a manufacturing and regulatory perspective, clinical-grade SC- β cells must be produced under good manufacturing practice (GMP) conditions, which require rigorous quality control, reproducibility, and compliance with safety standards [81, 82]. This includes the validation of reagents, scalability of production platforms (e.g., bioreactors, suspension culture), and the establishment of release criteria for clinical batches based on potency assays such as GSIS. Furthermore, complicated

translations are the challenges of long-term engraftment and vascularization. SC- β cells require a supportive microenvironment and stable vascular integration to function effectively *in vivo* as poor oxygenation, nutrient limitations, and inadequate innervation can impair graft performance over time. Ongoing efforts to optimize transplantation sites, co-deliver supporting cell types (e.g., endothelial cells, mesenchymal stromal cells), and engineer pro-vascular niches are critical to improving long-term outcomes [83]. Personalized autologous therapies, while offering patient-specific benefits, are hindered by high costs and prolonged manufacturing times. In contrast, universal allogeneic approaches offer broader applicability but encounter significant hurdles related to immune compatibility and regulatory approval. To ensure widespread accessibility, it is essential to develop platforms that are not only scalable and cost-effective but also ethically sustainable.

10. Ethical and societal considerations

As SC- β cell therapies move toward clinical use, it's important to think carefully about the ethical and social questions raised. These include the source of stem cells, how informed consent is handled, who gets access to treatment, concerns about long-term safety, the moral debate around embryo use, and the risk of conflicts of interest in both research and commercial development. While human ESCs have been instrumental in SC- β research, their derivation from early-stage embryos raises ethical concerns for many who view embryos as possessing moral status. In contrast, iPSCs, generated by reprogramming adult somatic cells, avoid embryo-related concerns. However, iPSCs introduce their own challenges, including donor consent, ownership and use of biological material, variability in differentiation efficiency, and the ethical implications of genetic modifications [84, 85]. Additional concerns include donor anonymity, the long-term traceability of transplanted cells, and the protection of patient data. The high cost of SC- β therapies may also limit accessibility due to affordability and the potential to exacerbate existing health disparities. Ensuring that clinical trials and future therapeutic access reflect population diversity will be critical for equity and inclusion [86].

Safety remains a major priority. Risks such as tumorigenicity, immune rejection, off-target effects from gene editing, and unintended cellular behavior must be carefully assessed through long-term preclinical and clinical evaluation. Regulatory frameworks must also evolve to address advancements in encapsulation technologies, genome editing, and engineered transplant platforms. Public perception is equally important. Misleading claims, lack of poor communication can erode trust and delay the adoption of otherwise transformative therapies. Fostering public confidence will require clear, accurate, and inclusive dialog among researchers, clinicians, policymakers, and the broader community [87, 88]. Strong ethical oversight and responsible communication are essential to ensure the safe, effective, and equitable integration of SC- β therapies into clinical practice.

11. Future directions and emerging technologies

As SC- β cell therapies continue to evolve, a new generation of technologies is emerging to address current limitations and drive clinical translation forward. A significant area of focus is improving differentiation protocols to increase the yield, purity, and functional maturation of SC- β cells. Researchers are refining these

processes by mimicking *in vivo* developmental cues through dynamic signaling pathways, the precise timing of transcription factor activation, and advanced culture systems. Integration of single-cell transcriptomics and spatial multi-omics has enabled the identification of rare subpopulations, maturation bottlenecks, and new biomarkers that can be targeted to enhance SC- β cell function [89].

Artificial intelligence (AI) and machine learning are also being used to analyze large-scale omics data, predict differentiation outcomes, and optimize protocols with greater efficiency and consistency [90, 91]. In parallel, organoid and islet-on-a-chip platforms are offering more physiologically relevant *in vitro* models that replicate the 3D architecture, cellular diversity, and microvascular features of native human islets. These systems facilitate real-time analysis of insulin secretion, immune interactions, and drug responses under dynamic glucose conditions [92]. Bioprinting and tissue engineering are advancing the generation of vascularized, transplantable islet-like constructs by integrating SC- β cells with endothelial and stromal support cells in controlled architectures. Genetic engineering enables the design of SC- β cells with enhanced glucose responsiveness, immune evasion capabilities, and safety features such as programmable gene circuits and biosensors [93, 94]. Meanwhile, personalized regenerative therapies using patient-derived iPSCs are making it possible to tailor SC- β cell therapies to individual patients, particularly for monogenic and select forms of type 1 and type 2 diabetes. Future approaches may also involve integrating SC- β cells with implantable biosensors or closed-loop insulin delivery systems to improve therapeutic precision and real-time metabolic control. Collectively, these advances underscore the integration of stem cell science, engineering innovations, artificial intelligence, and personalized medicine paving the way for SC- β cell therapy to emerge as a scalable and potentially transformative treatment for diabetes.

12. Conclusions

The generation of functional pancreatic SC- β cells from pluripotent stem cells marks a major milestone in both diabetes research and therapy. Over the past two decades, substantial progress has been achieved in mimicking embryonic pancreatic development using stepwise differentiation protocols. These advances have enabled the production of insulin-secreting β -like cells that closely resemble *in vivo* counterparts in phenotype and, to a growing extent, in function. Despite these achievements, several challenges remain. Current SC- β cell populations often exhibit functional immaturity and cellular heterogeneity, which can limit their utility in both translational research and clinical applications. Efforts to address these issues through 3D culture systems, co-culture strategies, dynamic nutrient conditions, and *in vivo* validation continue to improve the physiological performance of SC- β cells. Furthermore, the integration of emerging technologies such as CRISPR-based disease modeling, biomaterial-driven encapsulation systems, and patient-specific iPSC platforms is expanding the applications of SC- β cells in disease modeling, drug screening, and personalized cell therapy. At the same time, ethical, immunological, and regulatory considerations must be carefully addressed to enable safe and equitable clinical translation. As differentiation protocols continue to improve and become more standardized, SC- β cells are increasingly positioned to become a foundational element of regenerative medicine for diabetes, with the ability to restore endogenous insulin production. These cells hold the promise to significantly reshape diabetes treatment and represent one of the most promising frontiers in biomedical research today.

Conflict of interest

QPP is holds intellectual property licensed by Vertex Pharmaceuticals and serves on the Scientific Advisory Board of Mellicell.

Author details

Zenith Khashim and Quinn P. Peterson*

Department of Physiology and Biomedical Engineering, Mayo Clinic, Rochester, MN, United States of America

*Address all correspondence to: peterson.quinn@mayo.edu

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References

- [1] Campbell JE, Newgard CB. Mechanisms controlling pancreatic islet cell function in insulin secretion. *Nature Reviews. Molecular Cell Biology*. 2021;**22**(2):142-158
- [2] Röder PV et al. Pancreatic regulation of glucose homeostasis. *Experimental & Molecular Medicine*. 2016;**48**(3):e219
- [3] Pisania A et al. Quantitative analysis of cell composition and purity of human pancreatic islet preparations. *Laboratory Investigation*. 2010;**90**(11):1661-1675
- [4] Bosco D et al. Unique arrangement of alpha- and beta-cells in human islets of Langerhans. *Diabetes*. 2010;**59**(5):1202-1210
- [5] Segerstolpe Å et al. Single-cell transcriptome profiling of human pancreatic islets in health and type 2 diabetes. *Cell Metabolism*. 2016;**24**(4):593-607
- [6] Högberg NJ, Ishahak M, Millman JR. Developments in stem cell-derived islet replacement therapy for treating type 1 diabetes. *Cell Stem Cell*. 2023;**30**(5):530-548
- [7] Wang S et al. Transplantation of chemically induced pluripotent stem-cell-derived islets under abdominal anterior rectus sheath in a type 1 diabetes patient. *Cell*. 2024;**187**(22):6152-6164.e18
- [8] Pagliuca FW et al. Generation of functional human pancreatic β cells *in vitro*. *Cell*. 2014;**159**(2):428-439
- [9] Ricordi C et al. Automated method for isolation of human pancreatic islets. *Diabetes*. 1988;**37**(4):413-420
- [10] Lehmann R et al. Superiority of small islets in human islet transplantation. *Diabetes*. 2007;**56**(3):594-603
- [11] Lakey JR, Mirbolooki M, Shapiro AM. Current status of clinical islet cell transplantation. *Methods in Molecular Biology*. 2006;**333**:47-104
- [12] Kin T et al. Enhancing the success of human islet isolation through optimization and characterization of pancreas dissociation enzyme. *American Journal of Transplantation*. 2007;**7**(5):1233-1241
- [13] Ichii H et al. Rescue purification maximizes the use of human islet preparations for transplantation. *American Journal of Transplantation*. 2005;**5**(1):21-30
- [14] Toso C et al. Factors affecting human islet of Langerhans isolation yields. *Transplantation Proceedings*. 2002;**34**(3):826-827
- [15] Shapiro AM, Pokrywczynska M, Ricordi C. Clinical pancreatic islet transplantation. *Nature Reviews. Endocrinology*. 2017;**13**(5):268-277
- [16] Lemos JRN, Skyler JS. Challenges in beta cell replacement for type 1 diabetes. *Hormone Research in Paediatrics*. 2025;**98**:435-449
- [17] Matsumoto S. Clinical allogeneic and autologous islet cell transplantation: Update. *Diabetes and Metabolism Journal*. 2011;**35**(3):199-206
- [18] Rezania A et al. Reversal of diabetes with insulin-producing cells derived *in vitro* from human pluripotent stem cells. *Nature Biotechnology*. 2014;**32**(11):1121-1133
- [19] Nair GG et al. Recapitulating endocrine cell clustering in culture promotes maturation of human

- stem-cell-derived β cells. *Nature Cell Biology*. 2019;**21**(2):263-274
- [20] Jin W, Jiang W. Stepwise differentiation of functional pancreatic β cells from human pluripotent stem cells. *Cell Regeneration*. 2022;**11**(1):24
- [21] Thomson JA et al. Embryonic stem cell lines derived from human blastocysts. *Science*. 1998;**282**(5391):1145-1147
- [22] Rossant J. Stem cells and early lineage development. *Cell*. 2008;**132**(4):527-531
- [23] Murry CE, Keller G. Differentiation of embryonic stem cells to clinically relevant populations: Lessons from embryonic development. *Cell*. 2008;**132**(4):661-680
- [24] Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. *Nature*. 1981;**292**(5819):154-156
- [25] Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006;**126**(4):663-676
- [26] Takahashi K et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. 2007;**131**(5):861-872
- [27] Maehr R et al. Generation of pluripotent stem cells from patients with type 1 diabetes. *Proceedings of the National Academy of Sciences of the United States of America*. 2009;**106**(37):15768-15773
- [28] Millman JR et al. Generation of stem cell-derived β -cells from patients with type 1 diabetes. *Nature Communications*. 2016;**7**:11463
- [29] Yamada M et al. Human oocytes reprogram adult somatic nuclei of a type 1 diabetic to diploid pluripotent stem cells. *Nature*. 2014;**510**(7506):533-536
- [30] Schwank G et al. Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. *Cell Stem Cell*. 2013;**13**(6):653-658
- [31] Lian Q et al. Functional mesenchymal stem cells derived from human induced pluripotent stem cells attenuate limb ischemia in mice. *Circulation*. 2010;**121**(9):1113-1123
- [32] Yu J et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science*. 2007;**318**(5858):1917-1920
- [33] Kumar SS et al. Recent developments in β -cell differentiation of pluripotent stem cells induced by small and large molecules. *International Journal of Molecular Sciences*. 2014;**15**(12):23418-23447
- [34] Jiang W et al. *In vitro* derivation of functional insulin-producing cells from human embryonic stem cells. *Cell Research*. 2007;**17**(4):333-344
- [35] Loh KM et al. Efficient endoderm induction from human pluripotent stem cells by logically directing signals controlling lineage bifurcations. *Cell Stem Cell*. 2014;**14**(2):237-252
- [36] D'Amour KA et al. Efficient differentiation of human embryonic stem cells to definitive endoderm. *Nature Biotechnology*. 2005;**23**(12):1534-1541
- [37] Zorn AM, Wells JM. Vertebrate endoderm development and organ formation. *Annual Review of Cell and Developmental Biology*. 2009;**25**:221-251
- [38] Arroyave F, Uscátegui Y, Lizcano F. From iPSCs to pancreatic β cells: Unveiling molecular pathways and enhancements with vitamin C

and retinoic acid in diabetes research. *International Journal of Molecular Sciences*. 2024;**25**(17):9654

[39] Johannesson M et al. FGF4 and retinoic acid direct differentiation of hESCs into PDX1-expressing foregut endoderm in a time- and concentration-dependent manner. *PLoS One*. 2009;**4**(3):e4794

[40] Gu G, Dubauskaite J, Melton DA. Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. *Development*. 2002;**129**(10):2447-2457

[41] Jennings RE et al. Human pancreas development. *Development*. 2015;**142**(18):3126-3137

[42] Shih HP et al. A Notch-dependent molecular circuitry initiates pancreatic endocrine and ductal cell differentiation. *Development*. 2012;**139**(14):2488-2499

[43] Scavuzzo MA et al. Endocrine lineage biases arise in temporally distinct endocrine progenitors during pancreatic morphogenesis. *Nature Communications*. 2018;**9**(1):3356

[44] Cai Q et al. Prospectively isolated NGN3-expressing progenitors from human embryonic stem cells give rise to pancreatic endocrine cells. *Stem Cells Translational Medicine*. 2014;**3**(4):489-499

[45] Murtaugh LC et al. Notch signaling controls multiple steps of pancreatic differentiation. *Proceedings of the National Academy of Sciences of the United States of America*. 2003;**100**(25):14920-14925

[46] Silva IBB et al. Stem cells differentiation into insulin-producing cells (IPCs): Recent advances and current challenges. *Stem Cell Research & Therapy*. 2022;**13**(1):309

[47] Memon B et al. Enhanced differentiation of human pluripotent stem cells into pancreatic progenitors co-expressing PDX1 and NKX6.1. *Stem Cell Research & Therapy*. 2018;**9**(1):15

[48] Sali S et al. A perfect islet: Reviewing recent protocol developments and proposing strategies for stem cell derived functional pancreatic islets. *Stem Cell Research & Therapy*. 2025;**16**(1):160

[49] Velazco-Cruz L et al. Acquisition of dynamic function in human stem cell-derived β cells. *Stem Cell Reports*. 2019;**12**(2):351-365

[50] Talavera-Adame D et al. Endothelial cells in co-culture enhance embryonic stem cell differentiation to pancreatic progenitors and insulin-producing cells through BMP signaling. *Stem Cell Reviews and Reports*. 2011;**7**(3):532-543

[51] Barsby T, Otonkoski T. Maturation of beta cells: Lessons from *in vivo* and *in vitro* models. *Diabetologia*. 2022;**65**(6):917-930

[52] Feyen DAM et al. Metabolic maturation media improve physiological function of human iPSC-derived cardiomyocytes. *Cell Reports*. 2020;**32**(3):107925

[53] Aguayo-Mazzucato C et al. Thyroid hormone promotes postnatal rat pancreatic β -cell development and glucose-responsive insulin secretion through MAFA. *Diabetes*. 2013;**62**(5):1569-1580

[54] Molakandov K et al. Selection for CD26(–) and CD49A(+) cells from pluripotent stem cells-derived islet-like clusters improves therapeutic activity in diabetic mice. *Frontiers in Endocrinology (Lausanne)*. 2021;**12**:635405

[55] Parent AV et al. Development of a scalable method to isolate subsets of stem

cell-derived pancreatic islet cells. *Stem Cell Reports*. 2022;**17**(4):979-992

[56] Davis JC et al. Glucose response by stem cell-derived β cells *in vitro* is inhibited by a bottleneck in glycolysis. *Cell Reports*. 2020;**31**(6):107623

[57] Jang D et al. Single cell glucose-stimulated insulin secretion assay using nanowell-in-microwell plates. *Lab on a Chip*. 2024;**24**(18):4232-4241

[58] Zanfrini E et al. Generation and application of novel hES cell reporter lines for the differentiation and maturation of hPS cell-derived islet-like clusters. *Scientific Reports*. 2024;**14**(1):19863

[59] Shilleh AH, Beard S, Russ HA. Enrichment of stem cell-derived pancreatic beta-like cells and controlled graft size through pharmacological removal of proliferating cells. *Stem Cell Reports*. 2023;**18**(6):1284-1294

[60] Barra JM et al. Cryopreservation of stem cell-derived β -like cells enriches for insulin-producing cells with improved function. *Diabetes*. 2024;**73**(10):1687-1696

[61] King AJ et al. Normal relationship of beta- and non-beta-cells not needed for successful islet transplantation. *Diabetes*. 2007;**56**(9):2312-2318

[62] Peterson QP et al. A method for the generation of human stem cell-derived alpha cells. *Nature Communications*. 2020;**11**(1):2241

[63] Jun Y et al. Engineered vasculature induces functional maturation of pluripotent stem cell-derived islet organoids. *Developmental Cell*. 20 May 2025:S1534-5807(25)00262-X. DOI: 10.1016/j.devcel.2025.04.024

[64] Vegas AJ et al. Long-term glycemic control using polymer-encapsulated human stem cell-derived beta cells in immune-competent mice. *Nature Medicine*. 2016;**22**(3):306-311

[65] Balboa D et al. Insulin mutations impair beta-cell development in a patient-derived iPSC model of neonatal diabetes. *eLife*. 2018;**7**:e38519

[66] Hua H et al. iPSC-derived β cells model diabetes due to glucokinase deficiency. *The Journal of Clinical Investigation*. 2017;**127**(3):1115

[67] Cujba AM et al. An HNF1 α truncation associated with maturity-onset diabetes of the young impairs pancreatic progenitor differentiation by antagonizing HNF1 β function. *Cell Reports*. 2022;**38**(9):110425

[68] Kachamakova-Trojanowska N, Stepniowski J, Dulak J. Human iPSCs-derived endothelial cells with mutation in HNF1A as a model of maturity-onset diabetes of the young. *Cells*. 2019;**8**(11):1440

[69] Ma H et al. Metabolic rescue in pluripotent cells from patients with mtDNA disease. *Nature*. 2015;**524**(7564):234-238

[70] Lorenz C et al. Human iPSC-derived neural progenitors are an effective drug discovery model for neurological mtDNA disorders. *Cell Stem Cell*. 2017;**20**(5):659-674.e9

[71] Lee NS et al. A novel dual-color reporter for identifying insulin-producing beta-cells and classifying heterogeneity of insulinoma cell lines. *PLoS One*. 2012;**7**(4):e35521

[72] Bayly CL et al. An INSULIN and IAPP dual reporter enables tracking of functional maturation of stem

cell-derived insulin producing cells. *Molecular Metabolism*. 2024;**89**:102017

[73] Fishman JA. Infection in organ transplantation. *American Journal of Transplantation*. 2017;**17**(4):856-879

[74] Wang X et al. Advancements and challenges in immune protection strategies for islet transplantation. *Journal of Diabetes*. 2025;**17**(1):e70048

[75] Deuse T et al. Hypoimmunogenic derivatives of induced pluripotent stem cells evade immune rejection in fully immunocompetent allogeneic recipients. *Nature Biotechnology*. 2019;**37**(3):252-258

[76] Han X et al. Generation of hypoimmunogenic human pluripotent stem cells. *Proceedings of the National Academy of Sciences of the United States of America*. 2019;**116**(21):10441-10446

[77] Scharp DW, Marchetti P. Encapsulated islets for diabetes therapy: History, current progress, and critical issues requiring solution. *Advanced Drug Delivery Reviews*. 2014;**67-68**:35-73

[78] Purpura KA et al. Systematic engineering of 3D pluripotent stem cell niches to guide blood development. *Biomaterials*. 2012;**33**(5):1271-1280

[79] Kirk K et al. Human embryonic stem cell derived islet progenitors mature inside an encapsulation device without evidence of increased biomass or cell escape. *Stem Cell Research*. 2014;**12**(3):807-814

[80] D'Amour KA et al. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. *Nature Biotechnology*. 2006;**24**(11):1392-1401

[81] Marfil-Garza BA, Shapiro AMJ, Kin T. Clinical islet transplantation:

Current progress and new frontiers. *Journal of Hepato-Biliary-Pancreatic Sciences*. 2021;**28**(3):243-254

[82] Shapiro AM et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *The New England Journal of Medicine*. 2000;**343**(4):230-238

[83] Tomei AA et al. Device design and materials optimization of conformal coating for islets of Langerhans. *Proceedings of the National Academy of Sciences of the United States of America*. 2014;**111**(29):10514-10519

[84] Lo B, Parham L. Ethical issues in stem cell research. *Endocrine Reviews*. 2009;**30**(3):204-213

[85] Hyun I. The bioethics of stem cell research and therapy. *The Journal of Clinical Investigation*. 2010;**120**(1):71-75

[86] Sugarman J. Human stem cell ethics: Beyond the embryo. *Cell Stem Cell*. 2008;**2**(6):529-533

[87] Volarevic V et al. Ethical and safety issues of stem cell-based therapy. *International Journal of Medical Sciences*. 2018;**15**(1):36-45

[88] Sipp D et al. Marketing of unproven stem cell-based interventions: A call to action. *Science Translational Medicine*. 2017;**9**(397):eaag0426

[89] Veres A et al. Charting cellular identity during human *in vitro* β -cell differentiation. *Nature*. 2019;**569**(7756):368-373

[90] Angermueller C et al. Deep learning for computational biology. *Molecular Systems Biology*. 2016;**12**(7):878

[91] Nosrati H, Nosrati M. Artificial intelligence in regenerative medicine:

Applications and implications.
Biomimetics (Basel). 2023;**8**(5):442

[92] Grassi L et al. Organoids as a new model for improving regenerative medicine and cancer personalized therapy in renal diseases. *Cell Death & Disease*. 2019;**10**(3):201

[93] Templer S. Closed-loop insulin delivery systems: Past, present, and future directions. *Frontiers in Endocrinology* (Lausanne). 2022;**13**:919942

[94] Millman JR, Pagliuca FW. Autologous pluripotent stem cell-derived β -like cells for diabetes cellular therapy. *Diabetes*. 2017;**66**(5):1111-1120

Tumor-on-a-Chip: From Biomimetic Tumor-Immune Microenvironments to Therapeutic Insights

Cetin Yuksel

Abstract

Cancer remains a leading cause of mortality worldwide, despite significant advancements in understanding its biology and developing therapeutic strategies. A major obstacle in cancer research lies in replicating a physiologically relevant tumor microenvironment (TME) *in-vitro* and identifying effective methods for anticancer drug screening. Tumor-on-a-chip (Toc) models are a rapidly emerging class of microfluidic platforms that replicate key aspects of the TME, offering unprecedented control over cellular interactions and enabling more accurate *in-vitro* simulations of cancer progression. These models integrate both tumor cells and various components of the TME, including stromal cells, endothelial cells, and immune cells, providing a more physiologically relevant system for studying tumor biology and therapeutic responses. The TME plays a critical role in regulating tumor growth, metastasis, and response to immunotherapy, yet traditional 2D and animal models often fail to fully capture the dynamic and complex interplay between tumor cells and immune components. This chapter highlights recent advancements in the development of Toc systems, focusing on the integration of the tumor-immune microenvironment and its implications for cancer research and personalized medicine. The challenges and future directions in optimizing these models to enhance their predictive power for clinical outcomes include improving the complexity of tumor-immune interactions, better mimicking the heterogeneity of patient-specific tumors, refining the integration of immune cells and stromal components, enhancing the scalability and reproducibility of the models, and addressing the limitations in long-term culture and physiological relevance for drug testing and personalized medicine applications.

Keywords: tumor-on-a-chip, tumor microenvironment, microfluidic devices, 3D organoids, cancer research

1. Introduction

The substantial rates of cancer-related mortality and morbidity continue to pose a serious global health challenge, emphasizing the urgent demand for more efficient

and targeted anticancer treatments. Although numerous promising therapeutic agents have been discovered over the past five decades, the clinical success rate remains regrettably low. More than 80% of anticancer drug candidates fail in clinical trials, primarily due to insufficient therapeutic efficacy or adverse side effects [1, 2]. Furthermore, the cost associated with bringing a new drug to market has risen significantly, making the preclinical evaluation of efficacy and toxicity increasingly expensive and time-consuming [3, 4]. Even after regulatory approval, certain drugs may later be recalled due to previously undetected adverse effects, such as severe damage to the heart, liver, or kidneys—posing significant health risks to patients [5]. A major contributor to these challenges is the limited predictive power of current pre-clinical models. Due to fundamental species differences, results from animal studies often fail to accurately forecast human responses, including toxicity and side effects [6, 7]. These limitations underscore the need for more predictive and physiologically relevant models that can closely mimic human tumor biology [7].

In response to this need, organ-on-a-chip technologies have emerged as powerful tools capable of replicating the three-dimensional structure and functional dynamics of human organs [7–11]. These microengineered platforms merge the advantages of microfluidics and 3D cell culture to mimic the complexity and characteristics native tissue environments more precisely than traditional models [12, 13]. Organ-on-a-chip systems have good performance, including improved physiological relevance, reduced costs, enhanced experimental reproducibility, and fewer ethical concerns, while also enabling high-throughput testing [14, 15]. Through intricate microfluidic architecture, these devices can mimic dynamic *in-vivo* tissue conditions such as fluid shear stress, nutrient gradients, and drug exposure in a controlled environment. This level of biomimicry supports realistic cell culture conditions, allowing researchers to study organ-specific physiology or evaluate tissue responses *in-vitro* [16–18]. Furthermore, by linking multiple organ-on-a-chip modules, researchers can begin to simulate inter-organ interactions and study drug pharmacokinetics and metabolism at a systemic level [12, 19].

Within this context, Toc platforms have garnered significant attention as a promising approach for investigating cancer development and therapeutic strategies. These systems utilize microfluidic designs and advanced cell culture methods to replicate key features of the TME, including the extracellular matrix (ECM), irregular vasculature, infiltrating immune and stromal cells, cancer stem cells, and nutrient diffusion limitations [20]. Such complex components of the TME are known to influence cancer cell proliferation, invasion, and metastasis through both biochemical signals and mechanical forces [21, 22]. To better understand cancer biology, it is essential to move beyond the study of isolated tumor cells and instead consider the intricate interactions between tumor cells, surrounding stromal and immune cells, and ECM remodeling. Tumor-on-a-chip devices are capable of replicating many of these core elements of the TME, offering an innovative and powerful tool for studying tumor progression, drug responses, and personalized medicine strategies [8, 23–25]. Recent research has explored various applications of Toc platforms. *Ex-vivo* Toc models were reported for analyzing intercellular communication within the TME [24]. Toc was specifically designed for evaluating nanoparticle-based cancer treatments [26]. Significant advancements have been reviewed in mimicking the mechanical and chemical properties of the TME, along with proposals for future directions in the field [27]. Despite these advances, much of the literature has primarily focused on the applications of Toc systems, with relatively little attention given to the technological aspects of their fabrication.

This review aims to bridge that gap by not only discussing recent innovations in microfluidic tumor models for cancer research and drug screening but also by highlighting the fabrication techniques, biomaterials, and tissue engineering strategies used to recreate physiologically relevant tumor environments. The design and material considerations involved in constructing Toc platforms were outlined. Next, various organ-specific tumor chips, such as those modeling lung, breast, brain, and melanoma tumors, as well as metastasis, to illustrate how these systems replicate the 3D architecture and function of human tissues, were discussed. Following this, their applications in drug discovery, with particular emphasis on multiorgan chip systems that integrate complex cell co-cultures to assess therapeutic efficacy and toxicity, were explored. Last, the current limitations, remaining challenges, and emerging opportunities for advancing Toc technologies were discussed. This review provides a comprehensive overview of the evolving field of Toc systems, emphasizing their potential to revolutionize cancer research and therapy development through biomimetic modeling and precision medicine.

2. Tumor-on-a-chip

Tumor-on-a-chip platforms offer the ability to replicate various stages of tumor development, including tumor growth, angiogenesis, and progression from early neoplastic changes to advanced malignancies. These stages often involve processes such as epithelial-mesenchymal transition (EMT), cancer cell invasion, and metastasis [28]. Several fabrication techniques have been utilized to construct Toc devices. Among the most common employed technologies are photolithography, replica molding, soft lithography, microcontact printing, and bioprinting [25, 29]. In devices fabricated using soft lithography, the process typically begins with the creation of a microchannel mold on a silicon wafer *via* photolithography. A liquid polymer, most often polydimethylsiloxane (PDMS) is then cast over the mold to produce a transparent, flexible stamp with embedded microstructures. These stamps are used to transfer the pattern onto various polymer surfaces, resulting in intricate 3D microchannel system [30, 31].

More recently, bioprinting based on layer-by-layer deposition has gained traction in constructing Toc production [32, 33]. This technique allows for the simultaneous printing of different cell types and biofunctional materials onto biocompatible substrates, enabling the creation of complex 3D constructs with high spatial precision and reproducibility [34, 35]. Bioprinting encompasses multiple methods, including fused deposition modeling (FDM), stereolithography (SLA), inkjet, and laser-assisted bioprinting. The advantage of bioprinting technology used to produce Toc is to facilitate the replication of the heterogeneous TME and intricate 3D ECM like architectures, which have been difficult to reproduce using traditional approaches [36]. Using bioinks, researchers can fabricate multicellular structures that include cancer-associated fibroblasts, immune cells, and endothelial cells, allowing the formation of vascular networks [37, 38]. Additionally, bioprinting enables the spatially controlled deposition of biologically active molecules, such as proteins and growth factors, which are critical for regulating tumor cell behavior, including signaling, proliferation, and migration [39]. Another important feature of bioprinting is its ability to directly pattern and integrate living cells within microfluidic channels, which is essential for modeling vascular structures and physiological barriers [40, 41]. Vascularization is vital for maintaining tissue viability and supporting nutrient

exchange, and it also plays a key role in segregating different tissue compartments. In tumors, the vascular network is markedly different from blood vessels, exhibiting heterogeneity, high permeability, multidirectional flow of blood, and disordered organization [42]. Achieving functional vascularization remains one of the major challenges in designing *in-vitro* tumor models [43]. Therefore, bioprinting holds great promise for the development of miniaturized and biomimetic multiorgan tumor models, which can accelerate biomedical research and broaden the scope of applications in clinical and pharmaceutical fields.

2.1 Biomaterials for tumor-on-a-chip

Microfluidic Toc platforms have found extensive applications in pharmaceutical research and toxicity testing. Among the materials for fabricating Toc devices, polydimethylsiloxane (PDMS) is the most commonly used one due to its several advantages, such as optical transparency, gas permeability, biocompatibility, and mechanical flexibility, which collectively support real-time imaging and continuous monitoring of cellular behaviors and therapeutic responses within the device. Compared to traditional materials like glass or rigid plastics, PDMS provides a mechanical environment more akin to soft tissues, featuring a porous and less rigid structure that supports cell viability and function. However, one of the limitations of PDMS lies in its inherent hydrophobic nature, which can result in the absorption or nonspecific binding of hydrophobic compounds. This property restricts its effectiveness in chemical and drug screening applications [44]. To address this issue, an alternative microfluidic platform was developed using poly(methyl methacrylate) (PMMA), which is chemically bonded to a porous, orbitally etched polyethylene terephthalate (PETE) membrane [45]. This design prevents the diffusion of small lipophilic compounds and enhances drug screening accuracy. Their studies showed that human lung adenocarcinoma cells cultured in PMMA-based microfluidic devices provided more consistent and reliable data on the cytotoxic effects of vincristine compared to those cultured in PDMS devices. The PMMA setup also allowed the formation of 3D microstructures that better reflect *in-vivo* organ-level conditions. Another widely used biomaterial in Toc systems is gelatin, a naturally derived polymer from collagen. Due to its excellent cell adhesion properties and capacity to deliver bioactive compounds, gelatin is frequently employed in drug delivery models and tissue engineering applications [46]. It supports not only cell proliferation but also complex formation with proteins, nucleotides, polysaccharides, and other charged molecules, enabling targeted or sustained release of therapeutic agents. Furthermore, photocrosslinkable gelatin methacrylate (GelMA) hydrogels have been effectively utilized in various regenerative applications, including corneal repair, nerve regeneration, and cartilage engineering [47]. An additional emerging material is bacterial cellulose paper, which combines nanostructured cellulose properties with the benefits of natural origin, offering excellent biocompatibility and cost-effectiveness for bioengineering applications. Basement membrane extract (BME), also known as Matrigel, is another important biomaterial used in tumor modeling. Mimicking the structure of the native basement membrane, BME is composed primarily of laminin-111, collagen IV, entactin, and heparan sulfate proteoglycan [48]. These components contribute to both the structural framework and signaling environment needed for tissue organization. Tumor cells grown in BME often exhibit high proliferative capacity and invasive behavior, making it a useful tool for studying tumor aggressiveness and evaluating potential treatment strategies [48].

2.2 Fabrication technology for tumor-on-a-chip

Advancements in materials science and fabrication technologies, such as microfluidics and microprinting, in conjunction with stem cell research, have led to the development of three-dimensional, tissue-like models known as organoids and spheroids [49]. These 3D models are powerful tools for investigating human development, disease mechanisms, and therapeutic responses, and they also hold promise for advancing personalized medicine (**Figure 1**). Organoids are typically derived from various types of stem cells, including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and adult stem cells [50]. When incorporated into Toc platforms, organoids enhance the physiological relevance of the model by more accurately replicating organ-specific architecture and gene expression profiles found *in-vivo*, achieving better biomimicry than either system alone [51]. Among 3D culture

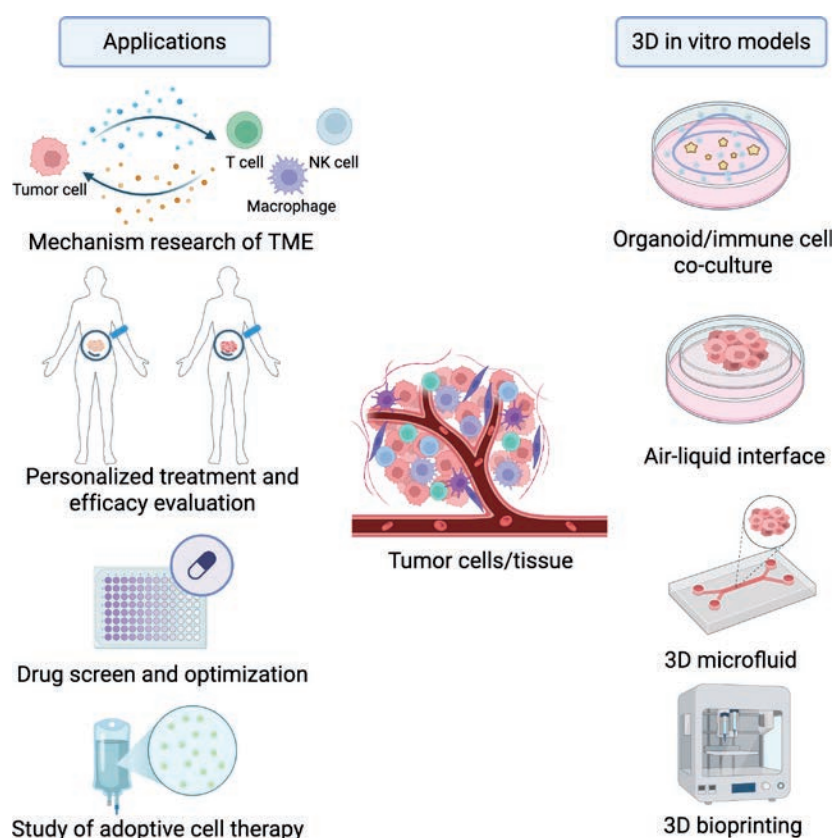


Figure 1.

A schematic summary of 3D in-vitro models and their applications in tumor immunology research. With the continuous development and innovation of in-vitro culture techniques, more and more 3D in-vitro tumor models containing more immune cells are being designed. The direct addition of exogenous immune cells to a conventional organoid culture system can mimic the interaction between tumor cells and a specific kind of immune cells. The air liquid interface (ALI) culture method can preserve the original immune cells in tumor tissues. 3D microfluidic technology can also preserve tumor-infiltrating lymphocytes while adding exogenous immune cells, with the advantage of high-throughput integration. 3D bioprinting uses tissue engineering techniques to reconstruct the tumor-immune microenvironment more flexibly. These models have important applications in the study of tumor microenvironment-related mechanisms, prediction of patient immunotherapy efficacy, screening of novel immunotherapeutic agents, and the study of adoptive cell therapy. (BioRender).

models, tumor spheroids are one of the most widely studied and characterized for improving predictive reliability in preclinical drug testing. For instance, a microfluidic chip was used to perform drug screening and flow cytometry analysis on a large population of uniformly sized tumor spheroids, demonstrating the effectiveness of 3D spheroid models in evaluating drug responses (**Figure 1**) [52].

The microvascular network plays a critical role in human physiology, facilitating nutrient delivery and waste removal throughout the circulatory system [53]. Leveraging organ-on-a-chip technologies, researchers have engineered microvascular systems that replicate the selective permeability and barrier functions of natural vasculature. These models more accurately mimic the microarchitecture and physiological behaviors of human organs, enabling more precise characterization of organ-level functions [53]. Traditional Toc systems often involve cells from a single organ source, which limits their ability to reflect systemic physiological interactions. To overcome this limitation, researchers have developed multiorgan-on-a-chip platforms to evaluate the therapeutic efficacy and off-target toxicity of anticancer agents in a more integrated biological context. These platforms are constructed by interconnecting mature organ-on-a-chip modules, each optimized for a specific function, into a cohesive system [54]. For example, the absorption, metabolism, and toxicity of the bioactive compound ginsenoside compound K were investigated using a multiorgan chip system composed of vascular, intestinal, kidney, and liver models [55]. Similarly, a pumpless multiorgan-on-a-chip platform incorporating a pharmacokinetic-pharmacodynamic (PK–PD) mathematical model was created to study drug mechanisms involving inter-organ interactions [56].

2.3 Different types of tumor-on-a-chip models

A diverse range of human-derived *in-vitro* Toc models has been established to investigate critical aspects of tumor development, including proliferation, angiogenesis, metastatic behavior, and therapeutic response. These models integrate state-of-the-art microfabrication methods, such as photolithography, soft lithography, and bioprinting with microfluidic systems and tissue engineering technologies to recreate essential features of the TME. These include biochemical gradients, microenvironmental cues, dynamic interactions between cells and the ECM, and multicellular architectures involving both tumor and stromal components. In this section, Toc models designed for cancers with particularly high prevalence and mortality, such as those mimicking the lung, liver, brain, colorectal, breast, and pancreatic tumors were emphasized (**Table 1**).

Metastasis remains one of the most critical obstacles in cancer therapy today. It significantly contributes to cancer lethality, accounting for more than 90% of cancer-related deaths [73]. Although most existing Toc systems are designed to model primary (*in-situ*) tumors, our understanding of the processes underlying cancer metastasis, particularly the early events that initiate metastatic behavior, such as specific signaling pathways and microenvironmental influences, remains limited. As a result, there is a pressing need for experimental systems capable of replicating and characterizing the metastatic microenvironment in detail [23, 74]. A notable example addressing this need is the metastasis-on-a-chip (MOC) platform developed [75]. In this model, organoid cultures are integrated into a microfluidic system, allowing simulated circulating flow between tissues. Tumor cells originating from colorectal cancer (CRC) organoids were observed to detach, enter the simulated circulation, and subsequently colonize liver organoids downstream. This setup was among the first

Tumor-on-a-chip models	Cell types	Applications	Ref.
Lung tumor chip	Human non-small cell lung cancer (NSCLC) cell line	Establish robust experimental models to investigate the underlying causes of therapy resistance commonly observed in lung cancer patients	[57]
	Human alveolar epithelial cells and human pulmonary microvascular endothelial cells	Recreate the essential alveolar-capillary interface of the human lung <i>in-vitro</i> to study host responses to microbial agents and inflammatory cytokines delivered to the alveolar compartment	[29]
Liver tumor chip	HepG2/C3A, MEG-01, MES-SA, MES-SA/DX-5	Propose a dual-action therapeutic approach that selectively inhibits the proliferation of MESSA/DX-5 cells, offering potential <i>in-vivo</i> benefits by targeting multidrug-resistant (MDR) cancers with low adverse effects	[58]
	iPS C2a, Functional endothelial cells, cardiomyocytes, and hepatocytes	Optimize engineered microtissues and modular multiorgan platforms to explore pharmacological responses in interconnected cardiac, hepatic, and vascular systems	[59]
Brain tumor chip	Brain tumor stem-like cells	Examine the function of primary patient-derived BTSCs	[60]
	Glioblastoma cells (U87)	Develop a 3D brain cancer chip for drug screening	[61]
	C6 glioma cells	Study the cellular response to different concentrations of colchicine	[62]
Colorectal-tumor chip	CRC cell line HCT-116	Evaluate precision nanomedicine delivery	[63]
Breast tumor chip	MDA-MB-453, MDA-MB-231, and HCC1937	Develop a microfluidic device for a 3D breast cancer screening platform	[64]
Pancreatic tumor chip	MIA PaCa-2, BxPC3, HT-29	Replicate cellular morphologies and reflect the death of endothelial cells during the metastasis process	[65]
	HepG2, MSC, HUVEC	Provide a suitable platform for pancreatic cancer cell growth for studying the metastasis of pancreatic cancer	[66]
	Patient-derived PDAC organoids, PSCs, and macrophages	Anti-stromal compounds enhance PDAC chemotherapy response in a tumor chip	[67]
Renal tumor chip	RCC cell lines A498 and RCC4, CAR-T cells directed to ROR1	Increased stiffness of the collagen matrix limits CAR-T-cell infiltration	[68]
Ovarian tumor chip	HUVECs, platelets, A2780, HOMECS, OVCAR3	Influence on vascular luminal microenvironment and platelet extravasation	[69]
	HOMECs, platelets, A2780/OVCAR3	Interaction through GPVI and galectin-3, impacting metastatic potential	[70]
Prostate tumor chip	Benign human prostate stromal cells, EMP or C4-2 or 22Rv1 tumor cells, primary human prostate basal epithelial cells	Prostate tumor cells drive the conversion of normal fibroblasts into CAFs, which promote tumor invasion <i>via</i> the secretion of TGF- β	[71]
	HUVECs, osteoblasts, PC3 spheroids	Co-culture of PC3, osteoblasts, and endothelial cells turned PC3-based spheroids toward a more quiescent state	[72]

Table 1.
 Different types of tumor-on-a-chip.

in-vitro models to replicate the full process of metastasis from a three-dimensional primary tumor site to a three-dimensional target organ site, effectively mimicking physiological patterns of cancer spread. In further research, a multisite metastasis-on-a-chip system was designed to evaluate the organ-specific preferences of metastatic cancer cells [23]. Utilizing 3D photopatterning, they engineered a chip with a central CRC organoid chamber connected to multiple downstream chambers containing liver, lung, and endothelial structures. Under continuous fluid flow, fluorescent imaging revealed that HCT-116 CRC cells preferentially migrated to the liver and lung compartments, organs that are also primary metastatic targets in CRC patients. This model not only provides insights into the mechanisms of organotropism in metastasis but also offers a promising platform for identifying potential therapeutic targets [23]. Similarly, another multiorgan-on-a-chip system was developed to study lung cancer metastasis [76]. Their device included one upstream lung module and three downstream modules representing the brain, bone, and liver. The model successfully mimicked the metastasis mechanism of lung cancer cells from the primary lung site to these secondary organs, reflecting clinically observed metastatic routes. Such systems represent a major advancement in understanding tumor spread and assessing organ-specific metastatic behavior under controlled conditions.

2.3.1 Lung tumor-on-a-chip

Lung cancer remains one of the most prevalent and lethal forms of cancer worldwide [77]. Developing a lung model is therefore essential for elucidating disease mechanisms and advancing treatment strategies [57, 77]. A typical lung-on-a-chip device features two microfluidic channels separated by a porous ECM membrane. One side of the membrane is lined with human lung epithelial cells, while the opposite side contains pulmonary microvascular endothelial cells, allowing the device to replicate key physiological functions of the lung. By introducing lung tumor cells into the epithelial layer, researchers transform this system into a lung tumor chip. Following electroplating, the culture medium is withdrawn from the upper channel to form a gas-liquid interface, while nutrients are delivered *via* the microvascular channel. This setup enables optical monitoring of tumor growth and invasion. Using this model, it was discovered that simulated breathing motions restricted tumor cell expansion, while the absence of mechanical movement allowed the cells to spread more freely [29]. Later, they developed a microfluidic platform that mimicked orthotopic non-small-cell lung cancer, enabling *in-vitro* assessment of tumor progression, therapeutic response, and tumor dormancy [57]. Their findings indicated that tumor growth compromises alveolar movement, forming a feedback loop that promotes further tumor expansion. Co-culture studies also revealed that interactions between tumor and alveolar epithelial cells increase cellular communication, while endothelial cells act to suppress these effects [57].

A lung-on-a-chip was designed using human bronchial epithelial cells at the air-liquid interface, which they integrated with a liver-on-a-chip module composed of hepatocytes, forming a dual-organ platform. This configuration allowed for the evaluation of cellular viability through adenosine 5'-triphosphate (ATP) quantification and assessment of apoptotic and necrotic cell populations [77].

In early efforts, the alveolar-capillary interface was recreated using a bioinspired microdevice, enabling the study of lung responses to pathogens and inflammatory cytokines [29]. Similarly, a nanofiber-based alveolar model was developed using

polylactic acid-glycolic acid (PLGA) membranes with favorable porosity and biocompatibility, mimicking the alveolar respiratory membrane [78]. These lung tumor models often utilize non-small-cell lung cancer cells (A549), lung fibroblasts (HFL1), and targeted epidermal growth factor receptor (EGFR)-targeted treatments to explore resistance mechanisms. In co-culture with HUVECs, A549 cells were shown to induce endothelial apoptosis, facilitating tumor invasion. These lung-on-a-chip platforms offer accessible and effective tools for personalized medicine and therapeutic screening.

2.3.2 Liver tumor-on-a-chip

As the primary metabolic organ, the liver performs critical functions such as glucose and ammonia regulation, hormone synthesis, and detoxification [79]. Its complex structure comprises mainly hepatocytes (parenchymal cells), which represent around 80% of total liver cells [80]. Liver cancer affects nearly 850,000 individuals annually, making liver tumor chips vital for investigating tumor behavior and drug metabolism [79].

Hepatocytes play a central role in drug metabolism studies on-chip, particularly in identifying liver toxicity. For example, the prodrug thymopeptide TF5 is converted into 5-fluorouracil (5-FU), which can cause hepatotoxicity; similarly, doxorubicin metabolism has been linked to cardio- and myelotoxicity [80]. Therefore, hepatocytes are integrated into microscale cell culture analogs (μ CCAs) to simulate drug metabolism in cancer therapy. A multiorgan chip model was developed incorporating liver, intestine, and breast tissue to evaluate combination drug therapies for potential efficacy in treating multidrug-resistant cancers [58]. A cardiac-liver-vascular microphysiological system was created using tissues derived from human pluripotent stem cells for drug safety testing [59]. Other platforms combine liver with breast, cervical [81], prostate, kidney [82], and lung-fat [83] systems. Overall, liver-integrated μ CCA models are promising tools for studying cancer metastasis and therapeutic resistance.

2.3.3 Brain tumor-on-a-chip

Glioma is the most prevalent and lethal form of brain tumor [84]. A widely used method for studying cellular migration and interactions in glioma research is the transwell-based co-culture system, which supports cell cultivation in minimal fluid volumes [84]. To more accurately replicate *in-vivo* conditions, researchers have utilized microfluidic platforms to recreate the perivascular niche (PVN), which is an essential microenvironment for brain tumor stem-like cells (BTSCs) [60]. These systems demonstrated that BTSCs tend to localize in PVNs, and that microvascular channels can act as conduits for tumor cell dissemination. Such models offer valuable insight into the dynamic behavior and heterogeneity of tumor cells, providing a robust framework for studying glioma biology [60]. A 3D brain TCC system was developed using polyethylene glycol diacrylate (PEGDA) hydrogel, enabling both drug delivery and biological evaluation [61]. By introducing mepitastatin and irinotecan into the model, they validated its use for glioma drug screening and controlled drug release testing. Similarly, a microfluidic platform was engineered consisting of four parallel chambers to analyze the responses of rat C6 glioma cells to colchicine [62]. Their observations revealed

significant morphological alterations and increased cell death with higher drug concentrations or prolonged exposure. This result may aid in the development of novel glioma therapies and glial-cell-based biosensors for glioma diagnostics [62].

2.3.4 Colorectal tumor-on-a-chip

Colorectal cancer (CRC) ranks as the second-deadliest cancer, primarily due to metastasis and limited chemotherapy efficacy [85]. Endothelial cells lining colonic microvasculature (HCoMECs) are critical for maintaining vascular integrity and are often included in microfluidic CRC models [85]. Drugs like gemcitabine (GEM), although commonly used, suffer from rapid degradation and poor tumor targeting [86–88]. Key factors, like MMP-1, involved in ECM degradation, and VEGF, a driver of angiogenesis, are important markers in CRC progression and have been modeled on-chip [89–91]. A 3D microfluidic model was designed with a central hydrogel chamber containing HCT-116 cells and two perfusion channels on either side [63]. VEGF-induced sprouting and targeted drug delivery could be observed through fluorescence imaging [63]. Building on this concept, a two-chamber 3D microscale perfusion tissue culture model (3D-mPTC) was created to evaluate liver metabolism-mediated cytotoxicity of anticancer compounds [92]. This approach was further advanced by designing a dual-organ chip that included liver and tumor compartments [93]. They showed that capecitabine (CAP) and its metabolite, 5-fluorouracil (5-FU), could suppress the proliferation of HCT-116 colorectal cancer cells, and they extended this analysis to other drug precursors, like tegafur, across various organ systems.

2.3.5 Breast tumor-on-a-chip

Breast tumor cells display significantly different morphologies in 2D versus 3D cultures. While nonmalignant and malignant cells appear similar in 2D, 3D cultures reveal polarized structures in nonmalignant cells and spherical masses in malignant ones [64]. Researchers observed that tumor cells in 3D cultures responded less effectively to anticancer drugs like paclitaxel, olaparib, and cisplatin [64, 94], indicating that 3D breast tumor chips could improve drug response predictions. Given that approximately 70% of breast cancer patients develop bone metastases, modeling bone colonization is also essential [95]. A 3D bone chip featuring mineralized collagen was used to create a breast cancer bone metastasis model, allowing analysis of interactions between tumor cells and osteoblasts [95]. A key requirement for effective anticancer drug discovery is the ability of Toc models to faithfully replicate *in-vivo* tumor characteristics. In 2014, a breast-on-a-chip model was developed in which a monolayer of luminal breast epithelial cells was cultured on a semicircular acrylic scaffold, mimicking the architecture of mammary ducts [96]. Tumor cells grown within the microchannels of this platform exhibited distinct morphologies and varied drug sensitivities compared to those in standard 2D monolayer cultures. These differences underline the potential of 3D microenvironments for more accurate drug screening. Researchers have also developed TME-on-a-chip models tailored for breast cancer, incorporating either healthy or cancerous liver tissue to investigate nanoparticle transport and molecular diffusion. Compared to normal liver vasculature, breast cancer cells in this system exhibited elevated vascular porosity and increased permeability within TME [96]. Such platforms enable detailed exploration of how different

tissues and tumors influence drug/nanoparticle behavior, specifically their transport, effectiveness, and selectivity, and support high-throughput screening for optimized therapeutic regimens.

2.3.6 Pancreatic tumor-on-a-chip

Pancreatic tumors are particularly challenging due to their invasive nature, poor vascularization, and dense fibrotic stroma [97]. Developing Tumor-on-a-Chip platforms helps bridge preclinical and clinical drug testing by better replicating the native TME. Compared to 2D monolayer cultures, spheroid-based 3D models of pancreatic cancer display higher drug resistance and mimic *in-vivo* conditions more closely [98]. A vascularized pancreatic tumor chip was developed to examine cellular morphologies and endothelial cell death during metastasis [65]. Later, a layer-by-layer 3D modeling technique was introduced to reconstruct the TME and study metastatic mechanisms [66]. This system simulated the hematogenous spread of MIA PaCa-2, human pancreatic carcinomas and cell lines and the lymphatic dissemination of BxPC3 pancreatic cells. Two organ systems were cultured within a vascular biomimetic environment to study pancreatic cancer cell growth, establishing a platform supportive of metastasis and drug screening [66].

Pancreatic Ductal Adenocarcinoma (PDAC) is anticipated to become the second most common cause of cancer-related mortality by 2030, marked by its aggressive nature, limited treatment success, and poor prognosis. Median survival is less than 6 months, and the five-year survival rate remains under 4% [99]. The TME plays a pivotal role in PDAC progression, particularly in its immune evasion and resistance to therapy [100]. To better understand PDAC's complex biology, researchers have developed humanized microfluidic models that replicate key elements of the disease environment. One such model utilized a co-culture of pancreatic stellate cells (PSCs) and ductal adenocarcinoma cells within collagen I and hyaluronic acid matrixes, enabling real-time visualization of cellular interactions with the ECM [101]. This system proved effective in investigating the intricate relationships between cancer cells and the stroma, as well as in evaluating therapeutic interventions. In another approach, a multi-compartmental PDAC-on-a-chip device was created to culture PDAC cells, PSCs, and fibroblasts separately [102]. Results indicated that fibroblasts adopted a myofibroblast-like phenotype and secreted elevated levels of IL-6 in the presence of cancer cells, providing a model that reflects early PDAC pathogenesis [102]. Vascularization remains a critical component of accurate tumor modeling, especially in PDAC, where high interstitial fluid pressure leads to vessel collapse and inadequate perfusion. A perfusable 3D organoid-on-a-chip system was developed for co-culturing patient-derived PDAC organoids with stromal fibroblasts. This model effectively replicated desmoplasia and reduced responsiveness to standard chemotherapy, highlighting its utility for drug screening and personalized therapy development [103]. Similarly, a biomimetic 3D organotypic model was developed that revealed how pancreatic tumor cells induce endothelial damage, leading to hypoxic conditions. The study identified activin-ALK7 signaling as a driver of endothelial ablation, suggesting it as a potential therapeutic target [104].

Despite the advances in modeling stromal dynamics, most PDAC-on-a-chip systems have yet to fully capture the complexity of immune interactions within the TME. However, recent progress in immuno-oncology has led to the creation of platforms designed to mimic these immune-tumor dynamics and biophysical properties more precisely [105]. One example is a polyethylene glycol (PEG)-based hydrogel that

recapitulates the desmoplastic stroma of PDAC. This platform revealed that laminin-integrin $\alpha 3/\alpha 6$ signaling is essential for pancreatic organoid viability, particularly when primary fibroblasts and macrophages are incorporated to simulate a more realistic TME [105]. This field was further advanced by developing a dual-chamber microfluidic chip with continuous perfusion, integrating patient-derived organoids along with human PSCs and macrophages. Their findings highlighted mutual interactions between tumor organoids and stromal cells that facilitated tumor progression. Notably, the chip allowed assessment of combined chemotherapy and anti-stromal therapies, which showed greater efficacy than chemotherapy alone. This underscores the promise of Toc systems for identifying synergistic treatment strategies that target both cancer cells and their surrounding microenvironment in PDAC [67].

2.3.7 Renal tumor-on-a-chip

Kidney cancer is the eighth most prevalent malignancy in the United States, with a notably poor prognosis for metastatic cases, less than 12% of patients survive beyond 5 years post-diagnosis [106]. The vast majority (85–90%) of these cancers originate from the epithelial cells of the proximal tubules in the renal cortex and are classified as renal cell carcinoma (RCC). Among RCC subtypes, clear cell renal cell carcinoma (ccRCC) is the most common, comprising roughly 75% of cases. It is characterized histologically by clusters of malignant epithelial cells with transparent cytoplasm, surrounded by a complex and branching vascular network [106].

Microfluidic platforms have emerged as valuable tools in mimicking the highly vascularized and structurally abnormal tumor environment found in RCC. These systems effectively replicate critical features, such as disorganized vasculature, excessive angiogenic activity, and enhanced vascular permeability. In an advanced study, a three-dimensional *in-vitro* co-culture model was established using vascular endothelial cells derived from induced pluripotent stem cells (iECs), alongside ccRCC epithelial cell lines (A498 or Caki-2). This model accurately recreated the TME of ccRCC, including hypoxic conditions, angiogenesis, and metabolic alterations, facilitating investigation of tumor-vascular dynamics. As a proof of concept, the model demonstrated the efficacy of cabozantinib in suppressing tumor proliferation and metastasis, highlighting its potential for personalized therapy, particularly in screening antiangiogenic and immune-modulating drugs using patient-specific iPSCs [107].

Another innovative approach involved the integration of primary human ccRCC cells from six donors with renal cortical cells and human endothelial cells, HUVECs, in a perfused, vascularized 3D system named “ccRCC-on-a-chip.” This co-culture setup effectively reproduced angiogenic features typical of ccRCC tumors, including vascular network formation and endothelial sprouting. Elevated levels of angiogenesis-related factors, VEGFA, PGF, and ANGPTL4, were observed in ccRCC tumors. Targeting ANGPTL4 using a neutralizing antibody led to reduced angiogenesis, suggesting its functional role in tumor vascularization. The model is a promising tool for studying tumor-vascular interactions, drug screening, and future investigations into immune responses within the RCC microenvironment [108].

In a follow-up study, a simplified, non-vascularized version of the chip was developed to evaluate tumor migration, invasion, and responses to conventional and cell-based therapies. The chip consists of three interconnected regions: A central matrix compartment for collagen injection, flanked by two lateral channels for perfusion of drugs or immune cells, such as T lymphocytes [68]. This 3D system allowed real-time monitoring of spheroid behavior, including migration and cytotoxic response

to drugs in a collagen ECM. Importantly, gene expression analysis revealed that RCC cells cultured in 3D conditions more closely resembled primary tumor profiles compared to 2D cultures. Gene set enrichment analysis identified pathways related to hypoxia and tumor progression. Among the upregulated genes in 3D culture, VEGFA, ANGPTL4, and PGF were consistently elevated, supporting their role in angiogenesis. Furthermore, bortezomib, a chemotherapeutic agent, showed increased cytotoxicity at lower concentrations in 3D models. The study also demonstrated the challenges of T-cell infiltration within dense extracellular matrixes, a phenomenon not captured by conventional 2D cytotoxicity assays, underscoring the added value of the 3D model in evaluating immunotherapeutic strategies [68].

2.3.8 Ovarian tumor-on-a-chip

Ovarian cancer (OC) is recognized as the most lethal gynecologic malignancy globally and stands as the fifth leading cause of cancer-related deaths among women in the United States [109]. According to recent projections by the American Cancer Society, approximately 20,000 women are expected to be newly diagnosed with OC in 2024, with over 12,000 anticipated deaths attributed to the disease [110]. The persistently high mortality rate is largely due to the lack of effective early detection methods, the late-stage diagnosis in most patients, and the diverse histological subtypes that complicate treatment strategies [111]. Beyond tumor biology, platelets have emerged as active players in cancer progression [112]. They interact with both primary and circulating tumor cells, enhancing metastatic spread. In ovarian cancer, platelets are capable of migrating into tumor tissue, where they contribute to cancer cell proliferation and resistance to chemotherapy [112]. However, the precise mechanisms governing platelet infiltration into tumors remain unclear. To address this, a microfluidic chip was developed to simulate platelet extravasation. Their platform included a tissue compartment lined with A2780 ovarian cancer cells and connected to a three-dimensional endothelial lumen, which was perfused with human platelets. The model successfully replicated *in-vivo* phenomena, showing platelet adhesion to tumor-exposed endothelial cells, subsequent migration into the tumor tissue and colonization [69].

Further investigation revealed that cytokines secreted by ovarian cancer cells such as IL-6, IL-8, MCP-1, and TNF- α contributed to the disruption of endothelial junctions, formation of intercellular gaps, and impaired endothelial barrier integrity. These changes were associated with the activation of the Src/ERK/FAK signaling cascade and decreased expression of key vascular barrier regulators, including VE-cadherin and VE-PTP [69]. Notably, treatment with atorvastatin reversed these effects by suppressing cytokine overexpression, normalizing pathway activity, and enhancing endothelial barrier stability. These results were validated across five high-grade serous ovarian cancer (HGSOC) patient samples, supporting atorvastatin's potential as a vasculature-stabilizing agent that may impede platelet infiltration and reduce metastasis risk. In a follow-up study, their chip was refined by integrating a more physiologically relevant ECM and evaluating the role of glycoprotein VI (GPVI), a key platelet receptor, in mediating platelet-tumor interactions. Their findings demonstrated that GPVI interacts with galectin-3, a tumor-associated protein overexpressed in advanced ovarian cancer, facilitating platelet extravasation. Inhibition of GPVI not only reduced platelet infiltration but also led to decreased tumor cell proliferation, invasiveness, and chemotherapy resistance. This underscores the therapeutic potential of targeting platelet-tumor interactions in OC and provides a strong rationale for further exploration of platelet-modulating strategies in the TME [70].

2.3.9 Prostate tumor-on-a-chip

Prostate cancer (PCa) is the second most common cause of cancer-related mortality among men in the United States, with over 35,000 projected deaths in 2024 [110]. The incidence and death rates increase significantly with age, with approximately 60% of diagnoses occurring in individuals over the age of 65 [110]. Early PCa development and progression are largely dependent on androgen signaling, particularly the activation of the androgen receptor (AR) by testosterone, which governs the expression of numerous genes involved in cellular proliferation and survival [113]. Although androgen deprivation therapy (ADT) is the primary treatment modality, nearly 10–20% of patients develop castration-resistant prostate cancer (CRPC), which no longer responds to ADT and is associated with a poor prognosis, with median survival ranging from 9 to 13 months in metastatic cases [113]. Alterations within the TME are central to PCa progression and therapy resistance [114]. To address these challenges, a simplified microfluidic model of prostate cancer was designed that replicates key features such as epithelial characteristics and secretion of prostate-specific antigen (PSA) and miRNAs. The system consists of two culture chambers subjected to pulsatile flow, causing cyclical ECM hydrogel contraction. This environment enabled the formation of androgen-sensitive human prostate adenocarcinoma cell line (LNCaP) spheroids (androgen-sensitive) and supported the proliferation of PC3 cells (androgen-insensitive). Under dynamic flow, both cell types exhibited enhanced polarity and increased expression of epidermal growth factor receptor (EGFR) compared to traditional 2D cultures. Additionally, LNCaP cells demonstrated upregulation of adhesion-related molecules such as epithelial cell adhesion molecule (EPCAM) and cytokeratins (CK5 and CK19), which may help withstand mechanical stress [115]. The microfluidic model also revealed altered expression of specific microRNAs such as miR-3687 and miR-205, which have been proposed as potential CRPC biomarkers [116, 117]. These findings highlight how biomechanical stimuli, like flow, can significantly influence prostate cancer cell behavior and gene expression. However, future improvements are necessary to incorporate more elements of the TME to better understand the role of microRNAs in early disease detection and progression.

Seeking to replicate the complex stromal-epithelial interactions of the prostate, the benign human prostate epithelial (BHPRE) and the stromal (BHPRS) cells were embedded into a two-channel vertical microfluidic chip, separated by a porous polyester membrane. This configuration supported long-term co-culture, enabling both paracrine and endocrine signaling, which induced basal epithelial cell differentiation into luminal secretory cells [118]. Building on this model, the team later developed a prostate cancer-on-a-chip by co-culturing human PCa cell lines (EMP, C4-2, or 22Rv1) with fibroblasts on opposite sides of the membrane under flow conditions [71]. Tumor cells were shown to convert normal fibroblasts into cancer-associated fibroblasts (CAFs), marked by increased α SMA and COL1A1 expression, reduced AR levels, and elongated morphology. Additionally, a modified chip design allowed assessment of tumor cell invasion across the membrane. Both PCa cells and CAFs migrated into adjacent compartments, suggesting a TGF- β -mediated role of the stroma in promoting tumor invasiveness. While this model provides valuable insights into tumor-stroma crosstalk and PCa progression, it lacks immune system components and does not explore how stromal interactions impact chemotherapeutic response or resistance. To investigate PCa interactions with bone and vasculature, a tri-cellular microfluidic chip was engineered to co-culture PC3 prostate cancer cells with osteoblasts and endothelial cells (ECs). This configuration

led to reduced proliferation of PC3 spheroids compared to traditional 2D triculture and induced a more quiescent cell state, though viability remained intact, indicating the chip closely mimics *in-vivo* metastatic PCa behavior [72]. Future work should aim to dissect the distinct roles of osteoblast and EC signaling in shaping PCa cell fate, to identify potential therapeutic targets that might mitigate bone or systemic metastasis. Moreover, since the prostate TME is populated with immune cells, such as macrophages and mast cells, there is a critical need to develop advanced models that integrate these immune components to better understand their influence on disease progression and treatment outcomes.

3. Conclusions and future perspectives

This review has explored the current landscape of Toc technologies and their emerging roles in advancing cancer research and therapeutic development. Compared to traditional two-dimensional *in-vitro* cultures and *in-vivo* animal models, Toc platforms offer the ability to replicate the complex architecture and dynamic features of tumor tissues found within the human body. These microengineered systems provide a more physiologically relevant environment, making them valuable tools for the development and evaluation of anticancer treatments. To enhance scalability and functionality, microfluidic technologies have been integrated into these systems, enabling modular and high-throughput designs that better recapitulate *in-vivo* conditions. Toc devices are capable of mimicking key characteristics of the TME, offering a more precise and realistic model for studying tumor progression, invasion, therapeutic response, and drug-induced toxicity. As a result, these platforms have garnered increasing attention as potential alternatives to conventional preclinical models, leading to significant scientific advancements. Numerous Toc models have been successfully engineered to simulate tumors in various organs, including the lung, liver, breast, brain, colorectal, ovarian, and prostate. These systems are widely employed for drug screening and for gaining mechanistic insights into cancer metastasis and tumor biology. As a complement or substitute to standard preclinical testing models, they hold promise for enhancing basic research, improving drug development pipelines, and ultimately contributing to more effective cancer therapies.

This review provides a comprehensive examination of the microfabrication techniques, classification, and applications of Toc systems, offering a more detailed overview to support wider implementation of this technology. Despite notable progress, several obstacles remain before these platforms can be fully adopted in pharmaceutical and clinical settings: i) Modeling complex signaling pathways: Although Toc devices can replicate key aspects of the TME and be integrated into multiorgan systems (e.g., body-on-a-chip models), it remains difficult to emulate the intricate signaling cascades involving distant organs—particularly those governed by the endocrine and immune systems. ii) Scalable fabrication and standardization: These systems rely heavily on advanced microfabrication processes, presenting challenges in consistent manufacturing and data reproducibility. Many researchers may lack the specialized skills needed to operate or design such devices. Therefore, the development of user-friendly interfaces and standardized protocols is critical to ensure that nonexpert users can effectively utilize these platforms and generate reliable, translatable data for clinical translation. iii) Material limitations: Polydimethylsiloxane (PDMS) is the most commonly used material in the fabrication of Toc devices. However, its tendency to absorb hydrophobic molecules—including drugs and

proteins—can compromise experimental accuracy by altering drug availability and activity. While alternative materials are under development, they must meet specific criteria: Optical transparency, biocompatibility, structural flexibility, chemical inertness, and cost-effectiveness—all of which limit material choices.

In conclusion, although Toc technology is still evolving and faces technical and translational challenges, it offers great potential to revolutionize cancer research and therapy. Achieving this will require strong interdisciplinary collaboration among experts in materials science, biomedical engineering, cancer biology, biophysics, and clinical oncology. By working together, researchers can refine and optimize Toc systems, paving the way for their integration into personalized medicine and clinical practice.

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Conflict of interest

The authors declare no conflict of interest.

Author details

Cetin Yuksel

The Scientific and Technological Research Council of Turkey (TUBITAK), Marmara Research Center (MAM), Life Sciences, Biotechnology Unit, Gebze-Kocaeli, Turkey

*Address all correspondence to: yuksel.cetin@tubitak.gov.tr

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References

- [1] Smietana K, Siatkowski M, Moller M. Trends in clinical success rates. *Nature Reviews Drug Discovery*. 2016;**15**:379-380. DOI: 10.1038/nrd.2016.85
- [2] Hay M, Thomas DW, Craighead JL, Economides C, Rosenthal J. Clinical development success rates for investigational drugs. *Nature Biotechnology*. 2014;**32**:40-51. DOI: 10.1038/nbt.2786
- [3] Prasad V, Mailankody S. Research and development spending to bring a single cancer drug to market and revenues after approval. *JAMA Internal Medicine*. 2017;**177**:1569-1575. DOI: 10.1001/jamainternmed.2017.3601
- [4] Satoh T, Sugiura S, Shin K, Oniki-Nagasaki R, Ishida S, Kikuchi K, et al. A multi-throughput multi-organ-on-a-chip system on a plate formatted pneumatic pressure-driven medium circulation platform. *Lab on a Chip*. 2018;**18**:115-125. DOI: 10.1039/C7LC00952F
- [5] Shehab N, Lovegrove MC, Geller A, Rose K, Weidle NJ, Budnitz DS. US emergency department visits for outpatient adverse drug events, 2013-2014. *JAMA*. 2016;**316**:2115-2125. DOI: 10.1001/jama.2016.16201
- [6] Sertkaya A, Wong HH, Jessup A, Beleche T. Key cost drivers of pharmaceutical clinical trials in the United States. *Clinical Trials*. 2016;**13**:117-126. DOI: 10.1177/1740774515625964
- [7] Hachey SJ, Hughes CCW. Applications of tumor chip technology. *Lab on a Chip*. 2018;**18**:2893-2912. DOI: 10.1039/c8lc00330k
- [8] Tsai HF, Trubelja A, Shen AQ, Bao G. Tumour-on-a-chip: Microfluidic models of tumour morphology, growth and microenvironment. *Journal of The Royal Society Interface*. 2017;**14**:20170137. DOI: 10.1098/rsif.2017.0137
- [9] Sosa-Hernández JE, Villalba-Rodríguez AM, Romero-Castillo KD, Aguilar-Aguila-Isaías MA, García-Reyes IE, Hernández-Antonio A, et al. Organs-on-a-chip module: A review from the development and applications perspective. *Micromachines*. 2018;**9**:536. DOI: 10.3390/mi9100536
- [10] Vivas A, van den Berg A, Passier R, Odijk M, van der Meer AD. Fluidic circuit board with modular sensor and valves enables stand-alone, tubeless microfluidic flow control in organs-on-chips. *Lab on a Chip*. 2022;**22**(6):1231-1243. DOI: 10.1039/d1lc00999k
- [11] Bovard D, Iskandar A, Luettich K, Hoeng J, Peitsch MC. Organs-on-a-chip: A new paradigm for toxicological assessment and preclinical drug development. *Toxicology Research and Application*. 2017;**1**:1-16. DOI: 10.1177/2397847317726351
- [12] Ronaldson-Bouchard K, Vunjak-Novakovic G. Organs-on-a-chip: A fast track for engineered human tissues in drug development. *Cell Stem Cell*. 2018;**22**:310-324. DOI: 10.1016/j.stem.2018.02.011
- [13] Wu Q, Liu J, Wang X, Feng L, Wu J, Zhu X, et al. Organ-on-a-chip: Recent breakthroughs and future prospects. *Biomedical Engineering Online*. 2020;**19**:9. DOI: 10.1186/s12938-020-0752-0
- [14] Park SE, Georgescu A, Huh D. Organoids-on-a-chip. *Science*.

- 2019;**364**:960-965. DOI: 10.1126/science.aaw7894
- [15] Zhang B, Korolj A, Lai BFL, Radisic M. Advances in organ-on-a-chip engineering. *Nature Reviews Materials*. 2018;**3**:257-278. DOI: 10.1038/s41578-018-0034-7
- [16] Ma YHV, Middleton K, You L, Sun Y. A review of microfluidic approaches for investigating cancer extravasation during metastasis. *Microsystems & Nanoengineering*. 2018;**4**:1-13. DOI: 10.1038/micronano.2017.104
- [17] Chen MB, Whisler JA, Fröse J, Yu C, Shin Y, Kamm RD. On-chip human microvasculature assay for visualization and quantification of tumor cell extravasation dynamics. *Nature Protocols*. 2017;**12**(5):865-880. DOI: 10.1038/nprot.2017.018
- [18] Rothbauer M, Zirath H, Ertl P. Recent advances in microfluidic technologies for cell-to-cell interaction studies. *Lab on a Chip*. 2018;**18**:249-270. DOI: 10.1039/c7lc00815e
- [19] Skardal A, Murphy SV, Devarasetty M, Mead I, Kang HW, Seol YJ. Multi-tissue interactions in an integrated three-tissue organ-on-a-chip platform. *Scientific Reports*. 2017;**7**:8837. DOI: 10.1038/s41598-017-08879-x
- [20] Portillo-Lara R, Annabi N. Microengineered cancer-on-a-chip platforms to study the metastatic microenvironment. *Lab on a Chip*. 2016;**16**:4063-4081. DOI: 10.1039/C6LC00718J
- [21] Swartz MA, Noriho I, Roberts EW, Sangaletti S, Wong MH, Yull FE, et al. Tumor microenvironment complexity: Emerging roles in cancer therapy. *Cancer Research*. 2012;**72**(10):2473-2480. DOI: 10.1158/0008-5472.CAN-12-0122
- [22] Romero-López M, Trinh AL, Sobrino A, Hatch MMS, Keating MT, Fimbres C, et al. Recapitulating the human tumor microenvironment: Colon tumor-derived extracellular matrix promotes angiogenesis and tumor cell growth. *Biomaterials*. 2017;**116**:118-129. DOI: 10.1016/j.biomaterials.2016.11.034
- [23] Aleman J, Skardal A. A multi-site metastasis-on-a-chip microphysiological system for assessing metastatic preference of cancer cells. *Biotechnology and Bioengineering*. 2019;**116**:936-944. DOI: 10.1002/bit.26871
- [24] Kumar V, Varghese S. Ex vivo tumor-on-a-chip platforms to study intercellular interactions within the tumor microenvironment. *Advanced Healthcare Materials*. 2019;**8**:e1801198. DOI: 10.1002/adhm.201801198
- [25] Shirure VS, Bi Y, Curtis MB, Lezia A, Goedegebuure MM, Goedegebuure SP, et al. Tumor-on-a-chip platform to investigate progression and drug sensitivity in cell lines and patient-derived organoids. *Lab on a Chip*. 2018;**18**:3687-3702. DOI: 10.1039/c8lc00596f
- [26] Wang Y, Cuzzucoli F, Escobar A, Lu S, Liang L, Wang S. Tumor-on-a-chip platforms for assessing nanoparticle-based cancer therapy. *Nanotechnology*. 2018;**29**(33):332001. DOI: 10.1088/1361-6528/aac7a4
- [27] Wan L, Neumann CA, LeDuc PR. Tumor-on-a-chip for integrating a 3D tumor microenvironment: Chemical and mechanical factors. *Lab on a Chip*. 2020;**20**:873-888. DOI: 10.1039/c9lc00550a
- [28] Sontheimer-Phelps A, Hassell BA, Ingber DE. Modelling cancer in microfluidic human organs-on-chips. *Nature Reviews Cancer*. 2019;**19**:65-81. DOI: 10.1038/s41568-018-0104-6

- [29] Huh D, Matthews BD, Mammoto A, Montoya-Zavala M, Hsin HY, Ingber DE. Reconstituting organ-level lung functions on a chip. *Science*. 2010;**328**(5986):1662-1668. DOI: 10.1126/science.1188302
- [30] Ma Y, Thiele J, Abdelmohsen L, Xu J, Huck WTS. Biocompatible macro-initiators controlling radical retention in microfluidic on-chip photo-polymerization of water-in-oil emulsions. *Chemical Communications*. 2014;**50**:112-114. DOI: 10.1039/c3cc46733c
- [31] Kang E, Shin S, Lee KH, Lee SH. Novel PDMS cylindrical channels that generate coaxial flow, and application to fabrication of microfibers and particles. *Lab on a Chip*. 2010;**10**:1856-1861. DOI: 10.1039/c002695f
- [32] Wang Y, Shi W, Kuss M, Mirza S, Qi D, Krasnoslobodtsev A, et al. 3D bioprinting of breast cancer models for drug resistance study. *ACS Biomaterials Science & Engineering*. 2018;**4**(12):4401-4411. DOI: 10.1021/acsbiomaterials.8b01277
- [33] Matai I, Kaur G, Seyedsalehi A, McClinton A, Laurencin CT. Progress in 3D bioprinting technology for tissue/organ regenerative engineering. *Biomaterials*. 2020;**226**:119536. DOI: 10.1016/j.biomaterials.2019.119536
- [34] Moroni L, Burdick JA, Highley C, Lee SJ, Morimoto Y, Takeuchi S, et al. Biofabrication strategies for 3D *in-vitro* models and regenerative medicine. *Nature Reviews Materials*. 2018;**3**(5):21-37. DOI: 10.1038/s41578-018-0006-y
- [35] Costantini M, Idaszek J, Szöke K, Jaroszewicz J, Dentini M, Barbetta A, et al. 3D bioprinting of BM-MSCs-loaded ECM biomimetic hydrogels for *in-vitro* neocartilage formation. *Biofabrication*. 2016;**8**(3):035002. DOI: 10.1088/1758-5090/8/3/035002
- [36] Colosi C, Shin SR, Manoharan V, Massa S, Costantini M, Barbetta A, et al. Microfluidic bioprinting of heterogeneous 3D tissue constructs using low-viscosity bioink. *Advanced Materials*. 2016;**28**:677-684. DOI: 10.1002/adma.201503310
- [37] Junttila MR, de Sauvage FJ. Influence of tumour micro-environment heterogeneity on therapeutic response. *Nature*. 2013;**501**:346-354. DOI: 10.1038/nature12626
- [38] Xu F, Celli J, Rizvi I, Moon S, Hasan T, Demirci U. A three-dimensional *in-vitro* ovarian cancer coculture model using a high-throughput cell patterning platform. *Biotechnology Journal*. 2011;**6**(2):204-212. DOI: 10.1002/biot.201000340
- [39] Keenan TM, Folch A. Biomolecular gradients in cell culture systems. *Lab on a Chip*. 2008;**8**:34-57. DOI: 10.1039/b711887b
- [40] Yu F, Choudhury D. Microfluidic bioprinting for organ-on-a-chip models. *Drug Discovery Today*. 2019;**24**:1248-1257. DOI: 10.1016/j.drudis.2019.03.025
- [41] Knowlton S, Joshi A, Yenilmez B, Ozbolat IT, Chua CK, Khademhosseini A, et al. Advancing cancer research using bioprinting for tumor-on-a-chip platforms. *International Journal of Bioprinting*. 2016;**2**(2):3-8. DOI: 10.18063/IJB.2016.02.003
- [42] Nagy JA, Chang SH, Dvorak AM, Dvorak HF. Why are tumour blood vessels abnormal and why is it important to know? *British Journal of Cancer*. 2009;**100**:865-869. DOI: 10.1038/sj.bjc.6604929

- [43] Rouwkema J, Khademhosseini A. Vascularization and angiogenesis in tissue engineering: Beyond creating static networks. *Trends in Biotechnology*. 2016;**34**:733-745. DOI: 10.1016/j.tibtech.2016.03.002
- [44] Auner AW, Tasneem KM, Markov DA, McCawley LJ, Hutson MS. Chemical-PDMS binding kinetics and implications for bioavailability in microfluidic devices. *Lab on a Chip*. 2019;**19**:864-874. DOI: 10.1039/c8lc00796a
- [45] Nguyen T et al. Robust chemical bonding of PMMA microfluidic devices to porous PETE membranes for reliable cytotoxicity testing of drugs. *Lab on a Chip*. 2019;**19**(21):3706-3713. DOI: 10.1039/C9LC00338J
- [46] Hoch E, Hirth T, Tovar GEM, Borchers K. Chemical tailoring of gelatin to adjust its chemical and physical properties for functional bioprinting. *Journal of Materials Chemistry B*. 2013;**1**:5675-5685. DOI: 10.1039/C3TB20745E
- [47] Echave MC et al. Recent advances in gelatin-based therapeutics. *Expert Opinion on Biological Therapy*. 2019;**19**:773-779. DOI: 10.1080/14712598.2019.1610383
- [48] Benton G, Arnaoutova I, George J, Kleinman HK, Koblinski J. Matrigel: From discovery and ECM mimicry to assays and models for cancer research. *Advanced Drug Delivery Reviews*. 2014;**79-80**:3-18. DOI: 10.1016/j.addr.2014.06.005
- [49] Breslin S, O'Driscoll L. Three-dimensional cell culture: The missing link in drug discovery. *Drug Discovery Today*. 2013;**18**:240-249. DOI: 10.1016/j.drudis.2012.10.003
- [50] Velasco V, Shariati SA, Esfandyarpour R. Microtechnology-based methods for organoid models. *Microsystems & Nanoengineering*. 2020;**6**:76. DOI: 10.1038/s41378-020-00185-3
- [51] Kasendra M et al. Development of a primary human small intestine-on-a-chip using biopsy-derived organoids. *Scientific Reports*. 2018;**8**:2871. DOI: 10.1038/s41598-018-21201-7
- [52] Patra B, Peng C-C, Liao W-H, Lee C-H, Tung Y-C. Drug testing and flow cytometry analysis on a large number of uniform sized tumor spheroids using a microfluidic device. *Scientific Reports*. 2016;**6**:21061. DOI: 10.1038/srep21061
- [53] Wang X, Sun Q, Pei J. Microfluidic-based 3D engineered microvascular networks and their applications in vascularized microtumor models. *Micromachines*. 2018;**9**:493. DOI: 10.3390/mi9100493
- [54] Cong Y et al. Drug toxicity evaluation based on organ-on-a-chip technology: A review. *Micromachines*. 2020;**11**:381. DOI: 10.3390/mi11040381
- [55] Liu D et al. Investigation of absorption, metabolism and toxicity of ginsenosides compound K based on human organ chips. *International Journal of Pharmaceutics*. 2020;**587**:119669. DOI: 10.1016/j.ijpharm.2020.119669
- [56] Lee H et al. A pumpless multi-organ-on-a-chip (MOC) combined with a pharmacokinetic–pharmacodynamic (PK–PD) model. *Biotechnology and Bioengineering*. 2017;**114**:432-443. DOI: 10.1002/bit.26087
- [57] Hassell BA et al. Human organ chip models recapitulate orthotopic lung cancer growth, therapeutic responses, and tumor dormancy *in-vitro*. *Cell*

Reports. 2017;**21**:508-516. DOI: 10.1016/j.celrep.2017.09.043

[58] Tatosian DA, Shuler ML. A novel system for evaluation of drug mixtures for potential efficacy in treating multidrug resistant cancers. *Biotechnology and Bioengineering*. 2009;**103**:187-198. DOI: 10.1002/bit.22219

[59] Vunjak-Novakovic G, Bhatia S, Chen C, Hirschi K. HeLiVa platform: Integrated heart-liver-vascular systems for drug testing in human health and disease. *Stem Cell Research & Therapy*. 2013;**4**:S8. DOI: 10.1186/scrt369

[60] Xiao Y et al. Ex vivo dynamics of human glioblastoma cells in a microvasculature-on-a-chip system correlates with tumor heterogeneity and subtypes. *Advancement of Science*. 2019;**6**:1801531. DOI: 10.1002/adv.201801531

[61] Fan Y, Nguyen DT, Akay Y, Xu F, Akay M. Engineering a brain cancer chip for high-throughput drug screening. *Scientific Reports*. 2016;**6**:25062. DOI: 10.1038/srep25062

[62] Liu W et al. Assay of glioma cell responses to an anticancer drug in a cell based microfluidic device. *Microfluidics and Nanofluidics*. 2010;**9**:717-725. DOI: 10.1007/s10404-010-0584-5

[63] Carvalho MR et al. Colorectal tumor-on-a-chip system: A 3D tool for precision onco-nanomedicine. *Science Advances*. 2019;**5**:eaaw1317. DOI: 10.1126/sciadv.aaw1317

[64] Lanz HL, Saleh A, Kramer B, Cairns J, Ng CP, Yu J, et al. Therapy response testing of breast cancer in a 3D highthroughput perfused microfluidic platform. *BMC Cancer*. 2017;**17**:709. DOI: 10.1186/s12885-017-3709-3

[65] Nishiguchi A, Matsusaki M, Kano MR, Nishihara H, Okano D, Asano Y, et al. *In-vitro* 3D blood/lymph-vascularized human stromal tissues for preclinical assays of cancer metastasis. *Biomaterials*. 2018;**179**:144-155. DOI: 10.1016/j.biomaterials.2018.06.019

[66] Mao M, Mao M, Bei HP, Lam CH, Chen P, Wang S, et al. Human-on-leaf-chip: A biomimetic vascular system integrated with chamber-specific organs. *Small*. 2020;**16**:2000546. DOI: 10.1002/smll.202000546

[67] Haque MR, Wessel CR, Leary DD, Wang C, Bhushan A, Bishehsari F. Patient-derived pancreatic cancer-on-a-chip recapitulates the tumor microenvironment. *Microsystems & Nanoengineering*. 2022;**8**:36. DOI: 10.1038/s41378-022-00370-6

[68] Miller CP, Fung M, Jaeger-Ruckstuhl CA, Xu Y, Warren EH, Akilesh S. Therapeutic targeting of tumor spheroids in a 3D microphysiological renal cell carcinoma-on-a-chip system. *Neoplasia*. 2023;**46**:100948. DOI: 10.1016/j.neo.2023.100948

[69] Saha B, Mathur T, Handley KF, Hu W, Afshar-Kharghan V, Sood AK. OvCa-Chip microsystem recreates vascular endothelium-mediated platelet extravasation in ovarian cancer. *Blood Advances*. 2020;**4**:3329-3342. DOI: 10.1182/bloodadvances.2020001632

[70] Saha B, Mathur T, Tronolone JJ, Chokshi M, Lokhande GK, Selahi A. Human tumor microenvironment chip evaluates the consequences of platelet extravasation and combinatorial antitumor-antiplatelet therapy in ovarian cancer. *Science Advances*. 2021;**7**:eabg5283. DOI: 10.1126/sciadv.abg5283

- [71] Jiang L, Khawaja H, Tahsin S, Clarkson TA, Miranti CK, Zohar Y. Microfluidic-based human prostate-cancer-on-chip. *Frontiers in Bioengineering and Biotechnology*. 2024;**12**:1302223. DOI: 10.3389/fbioe.2024.1302223
- [72] Hsiao AY, Torisawa Y, Tung YC, Sud S, Taichman RS, Pienta KJ. Microfluidic system for formation of PC-3 prostate cancer co-culture spheroids. *Biomaterials*. 2009;**30**:3020-3027. DOI: 10.1016/j.biomaterials.2009.02.047
- [73] Lei Q et al. Multifunctional mesoporous silica nanoparticles with thermal responsive gatekeeper for NIR light-triggered chemo/photo thermal-therapy. *Small*. 2016;**12**:4286-4298. DOI: 10.1002/smll.201601137
- [74] Han S et al. Hydrophobic patterning-based 3D microfluidic cell culture assay. *Advanced Healthcare Materials*. 2018;**7**:1800122. DOI: 10.1002/adhm.201800122
- [75] Skardal A, Devarasetty M, Forsythe S, Atala A, Soker S. A reductionist metastasis-on-a-chip platform for *in-vitro* tumor progression modeling and drug screening. *Biotechnology and Bioengineering*. 2016;**113**:2020-2032. DOI: 10.1002/bit.25950
- [76] Xu Z et al. Design and construction of a multi-organ microfluidic chip mimicking the in-vivo microenvironment of lung cancer metastasis. *ACS Applied Materials & Interfaces*. 2016;**8**:25840-25847. DOI: 10.1021/acsami.6b08746
- [77] Bovard D et al. A lung/liver-on-a-chip platform for acute and chronic toxicity studies. *Lab on a Chip*. 2018;**18**:3814-3829. DOI: 10.1039/c8lc01029c
- [78] Yang X et al. Nanofiber membrane supported lung-on-a-chip microdevice for anti-cancer drug testing. *Lab on a Chip*. 2018;**18**:486-495. DOI: 10.1039/C7LC01224A
- [79] Wiśniewski JR, Vildhede A, Norén A, Artursson P. In-depth quantitative analysis and comparison of the human hepatocyte and hepatoma cell line HepG2 proteomes. *Journal of Proteomics*. 2016;**136**:234-247. DOI: 10.1016/j.jprot.2016.01.016
- [80] Kashaninejad N et al. Organ-tumor-on-a-chip for chemosensitivity assay: A critical review. *Micromachines*. 2016;**7**:130. DOI: 10.3390/mi7080130
- [81] Chaw KC, Manimaran M, Tay EH, Swaminathan S. Multi-step microfluidic device for studying cancer metastasis. *Lab on a Chip*. 2007;**7**:1041-1047. DOI: 10.1039/B707399M
- [82] Drewitz M et al. Towards automated production and drug sensitivity testing using scaffold-free spherical tumor microtissues. *Biotechnology Journal*. 2011;**6**:1488-1496. DOI: 10.1002/biot.201100290
- [83] Zhang C, Zhao Z, Abdul Rahim NA, van Noort D, Yu H. Towards a human-on-chip: Culturing multiple cell types on a chip with compartmentalized microenvironments. *Lab on a Chip*. 2009;**9**:3185-3192. DOI: 10.1039/b915147h
- [84] Saliba J et al. Development of microplatforms to mimic the *in-vivo* architecture of CNS and PNS physiology and their diseases. *Genes*. 2018;**9**:285. DOI: 10.3390/genes9060285
- [85] Shangguan W et al. Endothelium originated from colorectal cancer stem cells constitute cancer blood vessels. *Cancer Science*. 2017;**108**:1357-1367. DOI: 10.1111/cas.13262

- [86] Jiménez-Fonseca P et al. Gemcitabine plus capecitabine (Gem-Cape) biweekly in chemorefractory metastatic colorectal cancer. *Clinical & Translational Oncology*. 2015;**17**:384-392. DOI: 10.1007/s12094-014-1243-1
- [87] Saif MW et al. The efficacy of gemcitabine as salvage treatment in patients with refractory advanced colorectal cancer (CRC): A single institution experience. *Anticancer Research*. 2011;**31**:2971-2974
- [88] Yu X et al. An *in-vitro* and *in-vivo* study of gemcitabine-loaded albumin nanoparticles in a pancreatic cancer cell line. *International Journal of Nanomedicine*. 2015;**10**:6825-6834. DOI: 10.2147/IJN.S93835
- [89] Shen CJ, Kuo YL, Chen CC, Chen MJ, Cheng Y. M. MMP1 expression is activated by slug and enhances multi-drug resistance (MDR) in breast cancer. *PLoS One*. 2017;**12**:e0174487. DOI: 10.1371/journal.pone.0174487
- [90] Foley CJ et al. Matrix metalloprotease-1a promotes tumorigenesis and metastasis. *The Journal of Biological Chemistry*. 2012;**287**:24330-24338. DOI: 10.1074/jbc.M112.356303
- [91] Musah S et al. Mature induced-pluripotent-stem-cell-derived human podocytes reconstitute kidney glomerular-capillary-wall function on a chip. *Nature Biomedical Engineering*. 2017;**1**:1-12. DOI: 10.1038/s41551-017-0069
- [92] Ma L, Barker J, Zhou C, Li W, Zhang J, Lin B, et al. Towards personalized medicine with a three-dimensional microscale perfusion-based two-chamber tissue model system. *Biomaterials*. 2012;**33**:4353-4361. DOI: 10.1016/j.biomaterials.2012.02.054
- [93] Imura Y, Sato K, Yoshimura E. Micro total bioassay system for ingested substances: Assessment of intestinal absorption, hepatic metabolism, and bioactivity. *Analytical Chemistry*. 2010;**82**:9983-9988. DOI: 10.1021/ac100806x
- [94] Boyle P. Triple-negative breast cancer: Epidemiological considerations and recommendations. *Annals of Oncology*. 2012;**23**:vi7-vi12. DOI: 10.1093/annonc/mds187
- [95] Hao S, Ha L, Cheng G, Wan Y, Xia Y, Sosnoski DM, et al. A spontaneous 3D bone-on-a-chip for bone metastasis study of breast cancer cells. *Small*. 2018;**14**:e1702787. DOI: 10.1002/smll.201702787
- [96] Vidi PA, Maleki T, Ochoa M, Wang L, Clark SM, Leary JF, et al. Disease-on-a-chip: Mimicry of tumor growth in mammary ducts. *Lab on a Chip*. 2014;**14**:172-177. DOI: 10.1039/c3lc50819f
- [97] Jiang H, Hegde S, Knolhoff BL, Zhu Y, Herndon JM, Meyer MA, et al. Targeting focal adhesion kinase renders pancreatic cancers responsive to checkpoint immunotherapy. *Nature Medicine*. 2016;**22**:851-860. DOI: 10.1038/nm.4123
- [98] Wen Z, Liao Q, Hu Y, You L, Zhou L, Zhao Y. A spheroid-based 3-D culture model for pancreatic cancer drug testing, using the acid phosphatase assay. *Brazilian Journal of Medical and Biological Research*. 2013;**46**:634-642. DOI: 10.1590/1414-431X20132647
- [99] Haque MR, Rempert TH, Al-Hilal TA, Wang C, Bhushan A, Bishehsari F. Organ-chip models: Opportunities for precision medicine in pancreatic cancer. *Cancers*. 2021;**13**:4487. DOI: 10.3390/cancers13174487

- [100] Balachandran VP, Łuksza M, Zhao JN, Makarov V, Moral JA, Remark R. Identification of unique neoantigen qualities in long-term survivors of pancreatic cancer. *Nature*. 2017;**551**:512-516. DOI: 10.1038/nature24462
- [101] Drifka CR, Eliceiri KW, Weber SM, Kao WJ. A bioengineered heterotypic stroma–cancer microenvironment model to study pancreatic ductal adenocarcinoma. *Lab on a Chip*. 2013;**13**:3965-3975. DOI: 10.1039/c3lc50487e
- [102] Sgarminato V, Marasso SL, Cocuzza M, Scordo G, Ballesio A, Ciardelli G. PDAC-on-chip for *in-vitro* modeling of stromal and pancreatic cancer cell crosstalk. *Biomaterials Science*. 2023;**11**:208-224. DOI: 10.1039/d2bm00881e
- [103] Lai BFL, Lu RXZ, Hu Y, Davenport Huyer L, Dou W, Wang EY. Recapitulating pancreatic tumor microenvironment through synergistic use of patient organoids and organ-on-a-chip vasculature. *Advanced Functional Materials*. 2020;**30**:2000545. DOI: 10.1002/adfm.202000545
- [104] Nguyen DHT, Lee E, Alimperti S, Norgard RJ, Wong A, Lee JJK. A biomimetic pancreatic cancer on-chip reveals endothelial ablation via ALK7 signaling. *Science Advances*. 2019;**5**:eaav6789. DOI: 10.1126/sciadv.aav6789
- [105] Below CR, Kelly J, Brown A, Humphries JD, Hutton C, Xu J. A microenvironment-inspired synthetic three-dimensional model for pancreatic ductal adenocarcinoma organoids. *Nature Materials*. 2022;**21**:110-119. DOI: 10.1038/s41563-021-01085-1
- [106] Heidegger I, Pircher A, Pichler R. Targeting the tumor microenvironment in renal cell cancer biology and therapy. *Frontiers in Oncology*. 2019;**9**:1-11. DOI: 10.3389/fonc.2019.00490
- [107] Virumbrales-Muñoz M, Ayuso JM, Loken JR, Denecke KM, Rehman S, Skala MC. Microphysiological model of renal cell carcinoma to inform anti-angiogenic therapy. *Biomaterials*. 2022;**283**:121454. DOI: 10.1016/j.biomaterials.2022.121454
- [108] Miller CP, Shin W, Ahn EH, Kim HJ, Kim DH. Engineering microphysiological immune system responses on chips. *Trends in Biotechnology*. 2020;**38**:857-872. DOI: 10.1016/j.tibtech.2020.01.003
- [109] Garcia A, Singh H. Bevacizumab and ovarian cancer. *Therapeutic Advances in Medical Oncology*. 2013;**5**:133-141. DOI: 10.1177/1758834012467661
- [110] Siegel RL, Giaquinto AN, Jemal A. Cancer statistics. *CA: A Cancer Journal for Clinicians*. 2024;**74**:12-49. DOI: 10.3322/caac.21820
- [111] Christie EL, Bowtell DDL. Acquired chemotherapy resistance in ovarian cancer. *Annals of Oncology: Official Journal of the European Society for Medical Oncology*. 2017;**28**:viii13-viii15. DOI: 10.1093/annonc/mdx446
- [112] Cho MS, Bottsford-Miller J, Vasquez HG, Stone R, Zand B, Kroll MH. Platelets increase the proliferation of ovarian cancer cells. *Blood*. 2012;**120**:4869-4872. DOI: 10.1182/blood-2012-06-438598
- [113] Kirby M, Hirst C, Crawford ED. Characterising the castration-resistant prostate cancer population: A systematic review. *International Journal of Clinical Practice*. 2011;**65**:1180-1192. DOI: 10.1111/j.1742-1241.2011.02799.x

[114] Ge R, Wang Z, Cheng L. Tumor microenvironment heterogeneity an important mediator of prostate cancer progression and therapeutic resistance. *npj Precision Oncology*. 2022;**6**:31. DOI: 10.1038/s41698-022-00272-w

[115] Padmyastuti A, Sarmiento MG, Dib M, Ehrhardt J, Schoon J, Somova M. Microfluidic-based prostate cancer model for investigating the secretion of prostate-specific antigen and microRNAs *in-vitro*. *Scientific Reports*. 2023;**13**:11623. DOI: 10.1038/s41598-023-38834-y

[116] Arrighetti N, Beretta GL. miRNAs as therapeutic tools and biomarkers for prostate cancer. *Pharmaceutics*. 2021;**13**:380. DOI: 10.3390/pharmaceutics13030380

[117] Rönnau CGH, Fussek S, Smit FP, Aalders TW, van Hooij O, Pinto PMC. Upregulation of miR-3195, miR-3687 and miR-4417 is associated with castration-resistant prostate cancer. *World Journal of Urology*. 2021;**39**:3789-3797. DOI: 10.1007/s00345-021-03723-4

[118] Jiang L, Ivich F, Tahsin S, Tran M, Frank SB, Miranti CK. Human stroma and epithelium co-culture in a microfluidic model of a human prostate gland. *Biomicrofluidics*. 2019;**13**:064116. DOI: 10.1063/1.5126714

Isolation and Regeneration of Wheat Mesophyll Protoplasts: Using a Short Protocol

*Beata Dedičová, Niklas Olsson, Emelie Ivarson
and Per Hofvander*

Abstract

Plant protoplasts serve as valuable tools for studying the fundamental properties and functions of cells. For example, protoplasts have been used to analyze cell organelles, the cell cycle, and cell wall deposition. Another application involves protoplast fusion to create somaclonal hybrids and facilitate genetic modifications. Additionally, plant protoplasts are successfully utilized in experiments aimed at mitigating heavy metal toxicity, plant stress, and perception responses, as well as robust expression screening systems for recalcitrant species. Protoplasts from various plant species have become essential in genome editing technologies, with their application established in numerous laboratories today. Here, we present a reproducible and concise method for isolating wheat mesophyll protoplasts from two spring varieties, Canon and Alderon, derived from *in vitro* seedlings. A cell counter and fluorescent staining were employed to determine the concentration and viability of freshly isolated wheat protoplasts. A modified culture medium initiated the first cell divisions of one wheat genotype within 8 days after the protoplasts were placed in the culture.

Keywords: *in vitro* seedlings, isolation, purification, potato dextrose broth, fluorescent staining, cell division

1. Introduction

Genome editing technology has been established for various monocotyledonous species, such as rice, wheat, and maize [1–4]. Recently, several advanced technologies have emerged that can alter genetic material in plants without using DNA [5, 6]; these technologies are referred to as RNPs (ribonucleoproteins), in which a complex of the Cas9 protein and guide RNAs is delivered to the cells or protoplast cultures, resulting in no integration of recombinant DNA into the genome [7]. These techniques are revolutionizing plant research by enabling gene manipulation or stacking and will accelerate plant breeding and germplasm development [8] in ways previously thought impossible. Many genome editing protocols utilize plant protoplasts [9, 10]. Therefore, a simple and reproducible protocol for isolation and regeneration, independent of genotype, is critical for successful experimentation.

Even though protoplast cultures have been the focus of the scientific community for over 50 years [11–13], the problem of having a functional isolation and regeneration protocol available remains relevant.

Cereals, such as wheat and oats, along with the most commercially essential genotypes, still primarily face the challenge of plant regeneration from protoplast cultures (personal observation). Often, the protocols are not reproducible for other varieties or may fail in different laboratories. Small details frequently determine the success or failure of these experiments, as the protoplast isolation process involves numerous steps and parameters that must be carefully executed and considered [14, 15]. Enzymatic solutions, purification steps, and culture media can sometimes be quite complex [16]. These intricate protocols and procedures must be continuously modified and fine-tuned in-house for the specific plant species, even for the particular variety of interest [17]. Several critical factors must be considered, including the cellular dedifferentiation of somatic cells and epigenetic modifications, which begin with the use of different sources of plant material for protoplast isolation and persist throughout their growth conditions [18].

Successful plant regeneration from protoplast cultures requires the *de novo* synthesis of cell walls, allowing cell division to occur. The removal of cell walls can be defined as a stress condition, where the mechanical properties of the cells are altered, leading to stress-related expression of various genes [19] and transcription factors [20–23], which occurs alongside structural changes in plant chromatin [18, 24].

Our research aimed to simplify the protocol for isolating and culturing hexaploid spring wheat protoplasts, using in vitro seedlings as the starting material and a suitable culture medium. This method enables the resynthesis of cell walls and promotes cell division.

2. Materials and methods

2.1 Plant material

Seeds of Spring hexaploid wheat cv. Canon (Lantmannen, Svalöv, Sweden) and Alderon (KWS, Einbeck, Germany) were surface-sterilized with 70% ethanol for 30 seconds and then treated with 100% commercial bleach, Klorine Original (containing 2.7% active chlorine, Colgate – Palmolive AB, Sweden), along with 20 μl of Tween-20 (Thermo Fisher Sci., Stockholm, Sweden), while shaking at 30 rpm for 20 minutes on a table shaker. Afterward, three rinses with sterile, deionized, distilled water were performed. Seeds were then placed on MS-20 medium (MS complete medium as described by Murashige and Skoog in 1962 [25], supplemented with 2% sucrose and 3% gelrite) in a 9 cm deep Petri dish. The seeds were incubated in a Percival growth chamber (CLF Plant Climatics, Wertlingem, Germany) at 23°C in darkness for 2 weeks, followed by third week with seedlings growing under a light intensity of 40 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and a 16-hour photoperiod. All plant material manipulation was conducted in a sterile flow cabinet.

We also utilized leaves from in vitro tobacco plants (*Nicotiana benthamiana* L.) growing on hormone-free MS medium supplemented with 20 g/L sucrose and 3 g/L gelrite. The pH was adjusted to a range of 5.6–5.8. Tobacco in vitro plants were cultivated at 23°C with a 16-hour photoperiod and a light intensity of 60 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ in a Percival growth cabinet (CLF Plant Climatics, Germany). Experiments with tobacco leaves served as a control for all wheat protoplast isolation and purification steps.

2.2 Protoplast isolation and purification

The middle part of green wheat seedling leaves (approximately 5–7 cm long, 1.5 g of fresh weight) was cut into small pieces (2–5 mm) using sterile scissors, directly into 10 ml of plasmolysis solution (0.5 M D-sorbitol stored at room temperature; 523 mOsm/L) in a deep Petri dish. The original solution was removed and replaced with a fresh one. The osmolality of all solutions was measured using a freeze-point automatic micro osmometer (Type 13/12DR auto col., Roebeling, Germany). The cut leaf tissue was incubated in a flow cabinet for 30 minutes at room temperature, covered with aluminum foil to prevent exposure to light. The plasmolysis solution was pipetted off and replaced with 15–20 ml of enzyme solution (1.5% cellulase R10, 0.75% macerozyme R10, 0.6 M D-mannitol, 10 mM MES monohydrate, 10 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 0.1% bovine serum albumin, Sigma-Aldrich, Stockholm, Sweden). Before use, the pH of the enzyme solution was adjusted to 5.7 with KOH and then filter-sterilized. The solution was incubated for 10 minutes at 55°C in a water bath, after which 10 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was added upon cooling to room temperature. A 0.45 μm syringe filter was used for filter sterilization, and the osmolality of the enzyme solution ranged between 730 and 750 mOsm/L. The Petri dish was covered with aluminum foil and incubated at 23°C overnight without shaking.

All tools were sterilized through autoclaving, and all solutions and culture media used during the protoplast isolation procedure and cultivation were sterilized by filtration (using a 0.2 μm syringe filter) and maintained on ice.

The next day, the Petri dish (containing the enzyme solution with leaf tissues) was removed from the incubator and gently agitated for 15–30 minutes at 5 rpm on a small table shaker at room temperature, still covered with aluminum foil. Gentle shaking (5 rpm) facilitated the release of mesophyll protoplasts into the enzymatic solution. To stop tissue maceration, we added the same volume of cold, sterile W5 washing solution (154 mM NaCl, 125 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 5 mM KCl, and 2 mM MES monohydrate), supplemented with 5 mM L-glucose [26] and 10 mM L-arginine [27] (pH 5.7; 650 mOsm/L), before filtering the mixture. Then, 10 ml of cold W5 washing solution containing 5 mM L-glucose was added to each of four empty, autoclaved glass round-bottom tubes. Another 10 to 15 ml of protoplast filtrate was directly filtered into each tube through a double strainer (70 and 40 μm ; Sarstedt, Stockholm, Sweden) or a conical 50 ml Falcon tube (Sarstedt, Stockholm, Sweden). The tubes were sealed, and the protoplasts were spun in a precooled centrifuge at 7°C and 50 g for 6–7 minutes.

The supernatant containing tissue debris was removed by gentle pipetting, and the pellet contained isolated mesophyll protoplasts, which were visible at the bottom of the round glass tubes or a 50 mL Falcon tube. This pellet of freshly isolated mesophyll protoplasts was directly resuspended in a culture medium or another solution, depending on the experiment's purpose and continuation.

2.3 Protoplast culture media

We used both plant species immediately after protoplast isolation in a liquid culture medium composed of Kao and Michayluk's macro- and microelements [16], supplemented with 2.5 mg/L 2,4-D (dichlorophenoxyacetic acid), 6 mM $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 20 g/L sucrose, 10 g/L maltose, 0.5 M D-mannitol, and 10 mM L-arginine. The pH of the medium was adjusted to 5.7 with 0.1 M NaOH, resulting in an osmolality of 720 mOsm/L. Protoplast cultures were grown in the dark at 23 or 25°C. Several wheat experiments used a modified culture medium with 1% potato dextrose broth

(PDB; Sigma-Aldrich, Stockholm, Sweden) added to the original formula. All components for seed germination medium, tobacco *in vitro* cultures, protoplast washing solution, culture medium, and enzymes were sourced from Duchefa, the Netherlands.

2.4 Fluorescent staining of wheat and tobacco protoplasts

The concentration and viability of isolated wheat and tobacco protoplasts were measured using a DeNovix Cell Counter, DropFL (DeNovix, Wilmington, Texas, USA). The fluorescein diacetate (FDA stock solution 0.5 mg/ml, dissolved in acetone) stained live protoplasts, while propidium iodide (PI stock solution 100 mg/ml, dissolved in 0.4 M D-mannitol) stained dead protoplasts (both dyes from Sigma-Aldrich, Stockholm, Sweden). About 10 microliters of each dye from the stock solution were mixed separately with 0.5 mL of 0.4 M D-mannitol. We added 5 μ l of freshly isolated mesophyll protoplasts, resuspended in a washing solution (W5 supplemented with 5 mM L-glucose), to each dye sample. Subsequently, 10 μ l of each stained protoplast mixture was loaded into the cell counter and analyzed.

3. Results

The technology for isolating mesophilic protoplasts involves several consecutive steps, as illustrated in **Figure 1**. Each stage involves several key experimental parameters that must be tested to develop a reproducible protocol for a specific plant species.

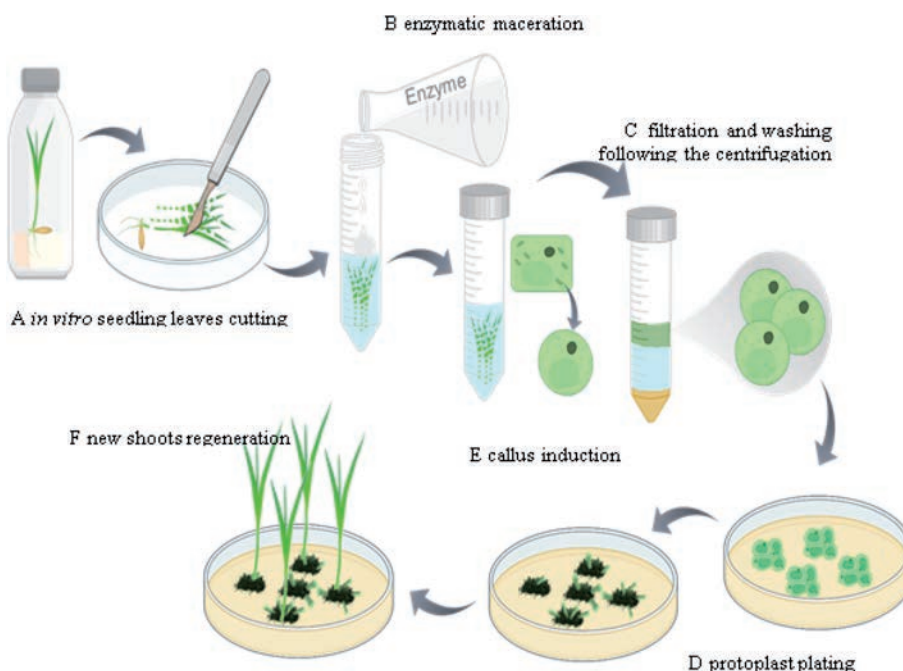


Figure 1.

A schematic procedure for protoplast isolation and regeneration, comprising the following steps: cutting in vitro seedling leaves, enzymatic maceration, filtration, washing following centrifugation, protoplast plating, callus formation, and regeneration of a new shoot.

We trimmed the central part of the wheat leaves with scissors (**Figure 2a**), and the tissue was sliced into smaller pieces in a deep Petri dish filled with 15 ml of 0.5 M D-sorbitol (**Figure 2b**). An overnight enzymatic inoculation (**Figure 2c**), followed by slow shaking (5 rpm) at room temperature the next morning, facilitated the release of protoplasts into the solution (**Figure 2d**).

Filtration of the protoplast suspension using two filters with pore sizes of 70 and 40 μm removed most of the tissue debris. Following washing in a large volume of cold [28] W5 supplemented with 5 mM L-glucose, low-speed centrifugation (50 rpm) for 5–7 min at 7°C, the protoplasts were sedimented in the pellet. At the same time, the remaining tissue debris tended to float in the supernatant solution. Additional sucrose gradient cleaning was unnecessary, and we also omitted the following washing step needed after the sucrose gradient.

Using a glass round-bottom tube instead of plastic conical Falcon tubes enhanced the quality of the protoplasts in the pellet after centrifugation (**Figure 2e**). In round-bottom tubes, the pellet was looser and much easier to resuspend in the subsequent solution or culture medium, which can be crucial for protoplast viability.

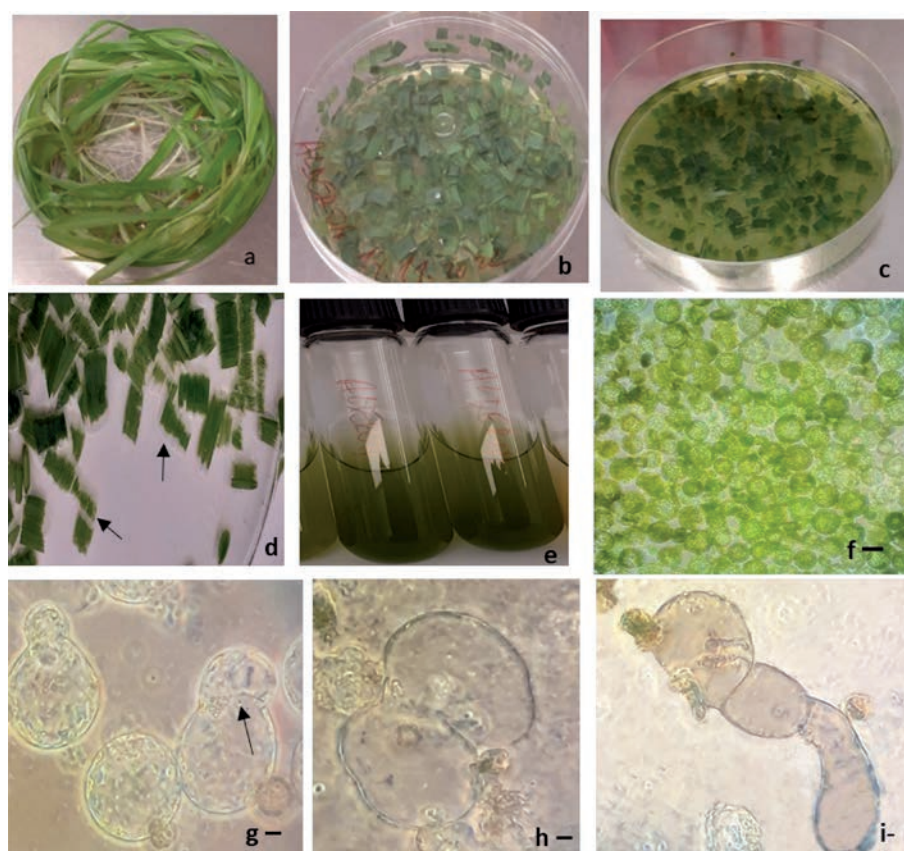


Figure 2. Wheat protoplast isolation: (a) in vitro seedlings of cv. Canon, (b) cv. Alderon in D-sorbitol for tissue plasmolysis, (c) cv. Alderon is undergoing enzymatic digestion overnight, (d) tissue remains after brief, slow shaking (5 rpm), with arrows indicating empty tissues, (e) filtered protoplasts in glass round-bottom tubes, (f) freshly isolated protoplasts of cv. Canon at 20x magnification, (g) the first cell division of cv. Canon, marked by the arrow, (h) cv. Canon in culture medium with PDB showing the first cell division, (i) cv. Canon in culture medium with PDB demonstrating the second cell division at 40x magnification, size bar 10 mm.

For both plant species, protoplasts were cultivated directly after washing and spinning in a liquid culture medium containing Kao and Michayluk macro and micro-elements [16] supplemented with 2.5 mg/L 2,4-D, dichlorophenoxyacetic acid, 6 mM $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, 2% sucrose, 1% maltose, 0.5 M D-mannitol, and 10 mM L-arginine. The medium pH was adjusted to 5.7 with 0.1 M NaOH, and its osmolarity was 720 mOsm/L.

Our experimentation and results with wheat showed that several key factors affect the successful isolation of viable wheat protoplasts. Some of them are handling the experiments under dark conditions whenever possible, using cold washing solutions and pre-cooled centrifugation. The serum bovine albumin in the enzymatic solution and L-arginine in the culture medium are essential, as they cause less movement of the chloroplasts toward the plasma membrane and help stabilize the isolated protoplasts [27]. Additionally, the brief protocol described here omits the purification step using a sucrose gradient [9], followed by another washing and centrifugation step, which can be detrimental to the vitality of protoplasts from specific cereal genotypes (data not shown).

Protoplast cultures were cultivated in the dark at 23°C or 25°C (**Figure 2f** and **3d**). The first cell division of wheat cells was observed 20 days after isolation, unlike in tobacco protoplast cultures, where the first cell division occurred after 10 days (**Figure 3e**). Multicellular structures were formed 2 weeks after the protoplasts were isolated (**Figure 3f, g**). Experiments with tobacco cultures were conducted as controls to test different handling steps in the protoplast isolation process.

Several wheat experiments were performed using a modified culture medium, where 1% potato Dextrose broth (PDB) was added to the original formula. This modification was significantly beneficial. It shortened the time to the first cell divisions,

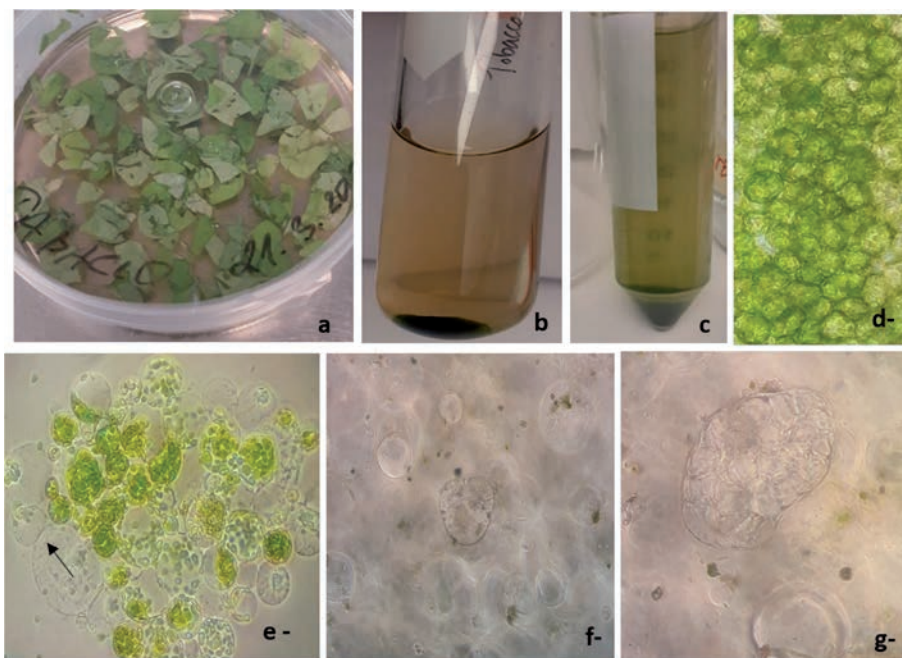


Figure 3.

Isolation of tobacco protoplasts: (a) enzymatic tissue maceration; (b) protoplast pellet in a round glass tube; (c) protoplast pellet in a conical tube; (d) freshly isolated protoplasts at 20x magnification; (e) the first cell division at 20x magnification; (f) the second cell division; (g) multicellular structure at 40x magnification, with a scale bar of 10 mm.

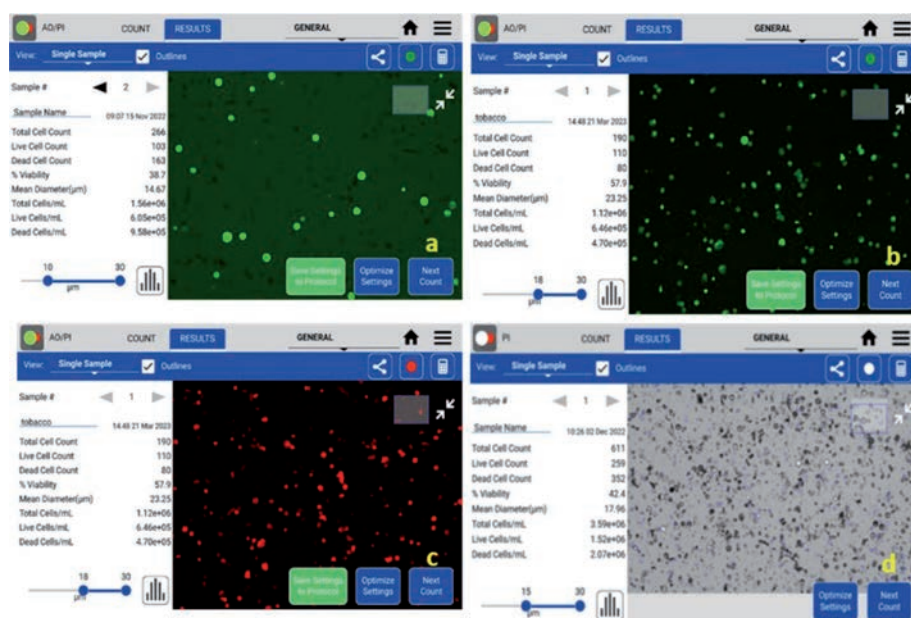


Figure 4. Freshly isolated wheat (a, b) and tobacco (c) protoplasts stained with FDA (a,b) and PI (c). Light picture of isolated wheat protoplasts (d).

when wheat protoplasts started to divide after 8 days in culture (Figure 2h), and continued to divide (Figure 2i). All these small technical steps, as tested and described here, improved the wheat protoplast isolation and cultivation protocol.

3.1 Staining of PRP

Fluorescein diacetate is a monopolar ester that passes through the protoplast membrane and is hydrolyzed in the cytoplasm by intracellular esterases. During this reaction, fluorescein is released, accumulating in the protoplast's cytoplasm as a green fluorescent stain. This reaction occurs only in living cells or protoplasts [28, 29]. In contrast, propidium iodide passes through damaged membranes, reacts with DNA or RNA, and forms a bright red complex that emits red fluorescence in the nucleus of dead cells or protoplasts [30].

In our experiments, the viability of isolated wheat and tobacco protoplasts was assessed by fluorescent staining with a 5 mg/ml dye mixture of Fluorescein diacetate and Propidium iodide using the DeNovix CellDrop Counter. In several independent experiments, the percentage of viable mesophyll protoplasts from wheat and tobacco fluctuated between 30% and 80% in both plant species (Figure 4). Generally, leaf segments from 7 to 10 *in vitro* wheat seedlings can produce approximately 6.105 protoplasts in a 1 ml volume, a suitable concentration for further culturing experiments or protoplast transfection [31].

4. Discussion

Although the technology of isolating and regenerating plant protoplasts has been known for several decades [32, 33], reliable and reproducible protocols remain elusive

for particular plant species. Cereals like oats and wheat are still problematic species, and a fast, consistent isolation and regeneration protocol must be established [9, 34, 35].

This study developed a robust and user-friendly method for isolating mesophyll protoplasts. The technique was found to work well for wheat and tobacco and to be genotype-independent in the respective crop, which is very valuable for further experimentation.

Cereal protoplast cultures are typically isolated from cell suspension cultures [36–39]. The advantage of using this starting material for protoplast isolation is that these cultures are already aseptic and conditioned for growth in culture. The protoplast yield from this material is generally high. However, maintaining suspension cultures over the long term is time-consuming and labor-intensive, presenting challenges regarding genetic stability [40, 41]. Additionally, they often lose their regeneration capacity over time [42], making it necessary to establish a new cell line regularly.

Unlike suspension cultures, the protoplast yield from the meristematic cells is lower. They are also fusogenic and sometimes challenging to handle, given the new insights into plant cell regeneration [21, 43] and the broad study of *de novo* cell reprogramming triggers [18, 44–48], which play a vital role in understanding this phenomenon.

The composition of culture media for protoplast cultures resembles that commonly used in tissue cultures. Since protoplasts lack a cell wall, they efficiently absorb nutrients from the liquid medium during the initial stages of culturing. As a result, their nutrient levels are typically adjusted to lower the concentration of inorganic substances, such as ammonium [49].

Our initial experiments tested several plant tissue culture media widely used for *in vitro* cultures, including Murashige and Skoog, NLN medium, and Imbrie-Milligan medium [25, 50, 51], as well as their various modifications. We observed no early stages of *de novo* synthesis of the cell walls in any of these media tested (data not shown), as indicated by the increased size of the protoplasts and the formation of cytoplasmic strands, as described by Vasil and Vasil [52].

Kao and Michayluk developed and described a highly complex medium for protoplast cultures of *Vicia hajastan* [16]. Their medium includes standard inorganic components such as fourteen vitamins, various organic acids, ten sugars and sugar alcohols, twenty-one amino acids, six nucleic acid bases, three growth regulators, casein hydrolysate, and coconut water.

In our hands, only the liquid medium containing macro- and microelements from Kao and Michayluk demonstrated *de novo* cell wall synthesis and cell division (**Figure 2g**), typically after 20 days of dark culture at 23°C with wheat protoplasts. When we supplemented this culture medium with potato dextrose broth, we observed the first cell divisions of the wheat protoplasts 8 days after isolation (**Figure 2h and i**). This modification of the medium significantly reduced the lag phase of wheat protoplasts. The potato dextrose broth is not a typical component of plant tissue culture media; however, it is more commonly used in microbiology for growing fungi and bacteria [53]. It remains unclear whether the presence of dextrose, potato, or the combination of both compounds results in the early cell divisions of wheat protoplasts.

5. Conclusions

This study developed a robust, user-friendly method for isolating wheat mesophyll protoplasts. The technique was effective for both wheat and oats (data not

shown). It demonstrated genotype independence within these crops, which is highly valuable for further experimentation, particularly in the context of genome editing and breeding. The percentage of viable mesophyll protoplasts from wheat and tobacco fluctuated between 30 and 80% for both plant species. Protoplasts from the wheat genotype Canon began dividing 8 days after isolation when the modified Kao and Michayluk medium was supplemented with potato dextrose broth. This is the first reference to using this compound in a culture medium for cereal protoplast cultures.

Authors contributions

Ph and BD conceptualized the study; BD designed and planned experiments. BD, NO, and EI conducted and evaluated experiments. BD wrote the manuscript. PH and REI edited and revised it. All authors read and approved the final manuscript.

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Data sharing statement

All experiment data obtained are presented here.

Author details

Beata Dedičová*, Niklas Olsson, Emelie Ivarson and Per Hofvander
Department of Plant Breeding, SLU Alnarp, Swedish University of Agricultural Sciences (SLU), SE, Alnarp, Sweden

*Address all correspondence to: beata.dedicova@slu.se

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References

- [1] Zafar K et al. Genome editing technologies for rice improvement: Progress, prospects, and safety concerns. *Frontiers in Genome Editing*. 2020;**2**:1-16. DOI: 10.3389/fgeed.2020.00005
- [2] Brandt KM et al. A streamlined protocol for wheat (*Triticum aestivum*) protoplast isolation and transformation with CRISPR-Cas ribonucleoprotein complexes. *Frontiers in Plant Science*. 2020;**11**:1-14. DOI: 10.3389/fpls.2020.00769
- [3] Aesaert S et al. Optimized transformation and gene editing of the B104 public maize inbred using improved tissue culture and morphogenic regulators. *Frontiers in Plant Science*. 2020;**13**:1-15. DOI: 10.3389/fpls.2022.883847
- [4] Wang JY, Doudna JA. CRISPR technology: A decade of genome editing is only the beginning. *Science*. 2023;**379**(1):12. DOI: 10.1126/science.add8643
- [5] Demirer GS et al. Carbon nanotube-mediated DNA delivery without transgene integration in intact plants. *Nature Protocols*. 2019;**14**(10):2954-2971. DOI: 10.1038/s41596-019-0208-9
- [6] Li J, Li Y, Ma L. Recent advances in CRISPR/Cas9 and applications for wheat functional genomics and breeding. *aBIOTECH*. 2021;**2**:375-385. DOI: 10.1007/s42994-021-00042-5
- [7] Zhang H et al. Nanoparticle cellular internalization is not required for RNA delivery to mature plant leaves. *Nature Nanotechnology*. 2022;**17**(2):197-205. DOI: 10.1038/s41565-021-01018-8
- [8] Zhang S, Shen JSL, Cheng Y. Strategies in delivering Cas9 ribonucleoprotein for CRISPR/Cas9 genome editing. *Theranostics*. 2021;**11**(2):614-648. DOI: 10.7150/thno.47007
- [9] Luo G, Li B, Gao C. Protoplast isolation and transfection in wheat. In: Wang K, Zhang F, editors. *Protoplast Technology*. Media, LLC: Springer Sci. and Business; 2022. p. 261
- [10] Wang W et al. Transgenerational CRISPR/Cas9 activity facilitates multiplex gene editing in allopolyploid wheat. *The CRISPR Journal*. 2018;**1**:65-74. DOI: 10.1089/crispr.2017.0010
- [11] Vasil V, Redway F, Vasil IK. Regeneration of plants from embryogenic suspension culture protoplasts of wheat (*Triticum aestivum* L.). *Nature Biotechnology*. 1990;**8**:429-434. DOI: 10.1038/nbt0590-429
- [12] Datta SK, Datta K, Potrykus I. Fertile Indica rice plants regenerated from protoplasts isolated from microspore-derived cell suspensions. *Plant Cell Reports*. 1990;**9**:253-256. DOI: 10.1007/bf00232295
- [13] Jähne A, Lazzeri AP, Lörz H. Regeneration of fertile plants from protoplasts derived from embryogenic cell suspensions of barley (*Hordeum vulgare* L.). *Plant Cell Reports*. 1991;**10**:1-6. DOI: 10.1007/BF00233022
- [14] Evans DE, Coleman JOD, Kearns A, editors. *Plant Cell Culture: The Basics*, Galrland Science. London, UK: Taylor & Francis; 2020. eBook. ISBN9781003076940. DOI: 10.1201/9781003076940
- [15] Wang K, Zhang F, editors. *Protoplast technology*. In: *Methods and Protocols*. Methods in Molecular Biology. Vol. 2464.

Hertfordshire, UK: Humanna PRESS;
2022

[16] Kao KN, Michayluk MR. Nutritional requirements for growing *Vicia hajastana* cells and protoplasts in liquid media at very low population density. *Planta*. 1975;**126**:105-110. DOI: 10.1007/BF00380613

[17] Reed KM, Bargmann BOR. Protoplast regeneration and its use in new plant breeding technologies. *Frontiers in Genome Editing*. 2021;**3**:1-25. DOI: 10.3389/fgeed.2021.734951

[18] Lee K, Seo PJ. Dynamic epigenetic changes during plant regeneration. *Trends in Plant Science*. 2018;**23**(3):235-247. DOI: 10.1016/j.tplants.2017.11.009

[19] Liu X et al. Uncovering the transcriptional regulatory network involved in boosting wheat regeneration and transformation. *Nature Plants*. 2023;**9**:908-925. DOI: 10.1038/s41477-023-01406-z

[20] Reyna-Lorens I et al. Plant protoplasts in the age of synthetic biology. *Journal of Experimental Botany*. 2023;**74**:3821-3832. DOI: 10.1093/jxb/erad172

[21] Sugimoto K, Temman H, Kadokura S, Matsunaga S. To regenerate or not to regenerate: Factors that drive plant regeneration. *Current Opinion in Plant Biology*. 2019;**47**:138-150. DOI: 10.1016/J.PBI.2018.12.002

[22] Xu M, Du Q, Tian C, Wang Y, Jiao Y. Stochastic gene expression drives mesophyll protoplast regeneration. *Science Advances*. 2021;**7**:8466-8477. DOI: 10.1126/SCIADV.ABG8466

[23] Fruleux A, Verger S, Boudaoud A. Feeling stressed or strained? A biophysical model for cell wall

mechanosensing in plants. *Frontiers in Plant Science*. 2019;**10**:1-12. DOI: 10.3389/FPLS.2019.00757

[24] Probst AV, Mittelsten SO. Stress-induced structural changes in plant chromatin. *Current Opinion in Plant Biology*. 2015;**27**:8-16. DOI: 10.1016/J.PBI.2015.05.011

[25] Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*. 1962;**15**:473-497. DOI: 10.1111/j.1399-3054.1962.tb08052.x

[26] Menczel L, Nagy F, Kiss Zs R, Maliga P. Streptomycin resistance and sensitive somatic hybrids of *Nicotiana tabacum* + *Nicotiana knightiana* correlation to resistance to *N. Tabacum* plastids. *Theoretical and Applied Genetics*. 1981;**59**:191-195. DOI: 10.1007/BF00264975

[27] Altman A, Kaur-Sawhney R, Galston AW. Stabilization of oat leaf protoplast through polyamine-mediated inhibitors of senescence. *Plant Physiology*. 1977;**60**:570-574. DOI: 10.1104/pp.60.4.570

[28] Puite K, Broeke WT, Scharf J. Inhibition of cell wall synthesis improves flow cytometric sorting of potato heterofusions resulting in hybrid plants. *Plant Science*. 1988;**56**:61-68. DOI: 10.1016/0168-9452(88)90186-0

[29] Jones KH, Senft JA. A method to determine cell viability was improved by simultaneous staining with fluorescein diacetate and propidium iodide. *The Journal of Histochemistry and Cytochemistry*. 1985;**33**(1):77-79. DOI: 10.1177/33.1.2578146

[30] Huang C-N, Cornejo MJ, Bush DS, Jones RL. Estimating the viability of plant protoplasts using double and single

staining. Protoplasma. 1986;**135**:80-87.
DOI: 10.1007/BF01277001

[31] Charlot F, Goudounet G, Nogue F, Perroud P-F. *Physcomitrum patens* protoplasts and protoplast transfection. In: Wang K, Zhang F, editors. Protoplast Technology. London, UK: Springer Sci. and Business Media, LLC; 2022. p. 261, pp. 3-19

[32] Cocking EC. A method for the isolation of plant protoplasts and vacuoles. Nature. 1960;**10**:963-963.
DOI: 10.1038/187962a0

[33] Cocking EC. Plant cell protoplasts-isolation and development. Annual Review of Plant Biology. 1972;**23**:29-50. DOI: 10.1146/annurev. pp.23.060172.000333

[34] Gasparis S. Oats, Methods, and Protocols. Methods in Molecular Biology. Vol. 1536. Springer Protocols, Humana Press; 2017. p. 269

[35] Pahti KM, Sprink T. From petri dish to field: Plant tissue culture and genetic engineering of oats for improved agricultural outcomes. Plants. 2023;**12**:1-20. DOI: 10.3390/plants12213782

[36] Maddock SE. Suspension and protoplast culture of hexaploid wheat (*Triticum aestivum* L.). Plant Cell Reports. 1987;**6**:23-26. DOI: 10.1007/BF00269731

[37] Li Z-Y, Xia G-M, Chen H-M, Guo G-O. Plant regeneration from protoplasts derived from embryogenesis suspension cultures of wheat (*Triticum aestivum* L.). Journal of Plant Physiology. 1992;**139**:714-718. DOI: 10.1016/S0176-1617(11)81716-5

[38] Data K, Data SK. Transformation of rice via PEG-mediated DNA uptake into protoplasts. In: Hall RD, editor. Plant Cell

Culture Protocols. Vol. 111. Totowa, NJ: Humana Press Inc; 1999

[39] Rakatondrafara A, Jackson JR, Kneller EP, Miller WA. Preparation and electroporation of oat protoplasts from suspension cultures. Current Protocols in Microbiology. 2007;**16**(1):191-219. DOI: 10.1002/9780471729259.mc16d03s05

[40] Winfield M, Davey MR, Karp A. A comparison of chromosome instability in diploid, tetraploid, and hexaploid wheat cell suspensions. Heredity. 1993;**70**:187-194. DOI: 10.1007/BF00224100

[41] Karp et al. Chromosome instability in bread wheat (*Triticum aestivum*) cell suspensions and their dividing protoplasts. In: Bajaj YPS, editor. Biotechnology in Agriculture and Forestry, Vol. 13. Wheat. Berlin: Springer-Verlag; 1990. p. 687. pp. 481-493

[42] Morinaka H, Sakamoto Y, Sugimoto AK. How do plants reprogram the fate of differentiated cells? Current Opinion in Plant Biology. 2023;**74**:1-7. DOI: 10.1016/j.pbi.2023.102377

[43] Lazzeri PA, Shewry PR. Biotechnology of cereals. Biotechnology and Genetic Engineering Reviews. 1993;**11**(1):79-146. DOI: 10.1080/02648725.1993.10647899

[44] Durgaprasad K et al. Gradient expression of transcription factor imposes a boundary on organ regeneration potential in plants. Cell Reports. 2019;**29**(2):4553-4463. DOI: 10.1016/j.celrep.2019.08.099

[45] Su YH, Tang LP, Zhao XY, Zhang XS. Plant cell totipotency: Insights into cellular reprogramming. Journal of Integrative Plant Biology. 2021;**63**(1):228-240. DOI: 10.1111/jipb.12972

[46] Morinaka H, Coleman D, Sakamoto Y, Iwase A, Keiko Sugimoto K. Molecular mechanisms of plant regeneration from differentiated cells: Approaches from historical tissue culture systems. *Plant and Cell Physiology*. 2023;**64**(3):297-304. DOI: 10.1093/pcp/pcac172

[47] Pasternak T et al. From single cell to plants: Mesophyll protoplasts as a versatile system for investigating plant cell reprogramming. *International Journal of Molecular Sciences*. 2020;**21**(12):1-14. DOI: 10.3390/ijms21124195

[48] Xu M et al. Stochastic gene expression drives the regeneration of mesophyll protoplasts. *Science Advances*. 2021;**7** eabg8466, 1-14

[49] Bokelmann GS, Roest S. Plant regeneration from potato (*Solanum tuberosum* cv Bintje). *Zeitschrift für Pflanzenphysiologie*. 1983;**109**:259-265. DOI: 10.1016/S0044-328X(83)80228-1

[50] Lichter R. Induction of haploid plants from isolated pollen of *Brassica napus*. *Zeitschrift für Pflanzenphysiologie*. 1982:427-434. DOI: 10.1016/S0044-328X(82)80040-8

[51] Imbrie-Milligan CW, Hodges TK. Microcallus formation from maize protoplasts prepared from embryogenic callus. *Planta*. 1986;**168**:395-401. DOI: 10.1007/BF00392367

[52] Vasil IK, Vasil V. Isolation and culture of protoplasts. *International Review of Cytology*. 1980;**11B**:1-19. DOI: 10.1016/S0074-7696(08)60323-X

[53] Zhao S, Shamoun SF. Effects of potato dextrose broth and gelatin on germination and efficacy of *Phoma exigua*, a potential biocontrol agent for salal (*Gaultheria shallon*). *Canadian Journal of Plant Pathology*. 2005;**27**:234-244. DOI: 10.1080/07060660509507221

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This book covers cell culture technologies, providing an overview of isolating cells from their native tissue and cultivating them in a controlled, artificial laboratory environment. This process is a fundamental technique in red and green biotechnology and life science research. Essential considerations regarding cryopreservation, recovery, and mycoplasma contamination, along with how to prevent and treat them in primary cell cultures, are discussed. This book presents updated research findings on primary cell isolation from tissues, the generation of differentiated cells from pluripotent stem cells, their in vitro growth in 2-D or 3-D, and their analysis using modern technologies. Furthermore, the use of organoids in a complex tumour-on-a-chip model is discussed. In addition to the medical application of cell cultures, plant cell culture-specific points are also addressed. This book is a valuable resource for anyone studying or researching primary cell culture applications and techniques, offering invaluable insights into emerging trends and techniques.

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