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Original Article

Advancing Pharmacognostic Assessment through Perfused 3D Tumor Co-Cultures: Critical Insights into Drug Transport, Immune Modulation, and Resistance

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ABSTRACT

Background: Perfused Three-dimensional (3D) tumor co-culture systems are increasingly recognized as valuable platforms for pharmacognostic assessment, providing microenvironmental fidelity that enhances evaluation of natural product bioactivity, drug transport, and resistance development. Conventional Two-dimensional (2D) assays lack the architectural complexity and spatial gradients that govern compound penetration, immune cell access, and microenvironment-driven therapeutic outcomes. In contrast, perfused 3D constructs integrate vascular-mimetic flow, multicellular co-cultures, and high-content imaging to model drug transport barriers, immune-tumor interactions, and clonal evolution under treatment pressure with improved physiological relevance. **Materials and Methods:** Recent engineering advances, including tunable hydrogels, microfluidic perfusion, bioprinting, and embedded biosensing enable depth-resolved oxygen mapping, real-time viability assessment, and multimodal profiling of phenotypic and molecular responses. **Results:** These innovations support more accurate prediction of drug penetration, metabolic stress, immune localization, and emergent resistance phenotypes, offering translational value for both natural product research and oncology drug development. **Conclusion:** This critical review outlines key technological approaches, validation strategies, and quality-control considerations required to integrate perfused 3D systems into pharmacognostic workflows, highlighting their potential to bridge mechanistic biology with clinically relevant therapeutic response modeling.

Keywords: Biosensing, Clonal evolution, Drug penetration, Immune-tumor interactions, *In situ* three-dimensional co-culture, Longitudinal imaging, Perfused tumor models, Pharmacognostic assessment, Spatial microenvironment, Standardized reporting, Translational oncology.

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INTRODUCTION

High attrition rates in oncology drug development continue to underscore the limitations of conventional preclinical systems in predicting therapeutic response, resistance mechanisms, and the pharmacological behaviour of both synthetic agents and natural products. Traditional 2D monolayer cultures lack the architectural complexity, spatial gradients, and multicellular interactions that critically influence drug penetration, immune modulation, and microenvironment-dependent resistance, factors central to pharmacognostic evaluation.

Perfused 3D tumor co-culture systems have therefore emerged as advanced platforms capable of integrating drug transport dynamics, immune cell behaviour, and clonal selection within physiologically relevant microenvironments. These systems conceptually align with clonal expansion principles described by Peng *et al.*,[1] and offer a mechanistic bridge between pharmacognosy and translational oncology. Although animal models provide systemic insight, interspecies differences in genetics, immune composition, and pharmacokinetics limit their utility for evaluating complex natural product mixtures and phytochemical interactions.[2]

In situ 3D co-culture systems enable multicellular constructs to form directly within biomimetic matrices or perfused microenvironments, preserving native cell-cell and cell-matrix interactions essential for mechanistic pharmacognostic studies. These platforms support longitudinal interrogation of tumor microenvironment dynamics, including how extracellular matrix composition, stromal remodeling, and spatial gradients modulate the activity of phytochemicals, plant-derived extracts, and synthetic analogues.[3,4]

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Recent engineering advances, including tunable hydrogels, microfluidic perfusion, organ-on-chip systems, and 3D bioprinting have expanded the applicability of these platforms to pharmacognostic research.[5] Tunable hydrogels allow precise control over stiffness and ligand presentation, enabling investigation of mechanotransduction and matrix-dependent modulation of drug and natural product responses. Microfluidic systems provide defined perfusion, shear stress, and endothelial interfaces that mimic vascular delivery and immune cell trafficking. Bioprinting supports spatial patterning of tumor, stromal, and vascular compartments, facilitating standardized comparative assays. Complementary innovations in label-free imaging, embedded biosensors, and spatial multi-omics now enable multimodal, time-resolved readouts linking compound transport dynamics to phenotypic and molecular responses.[6,7]

Despite these advances, reproducibility and regulatory readiness remain constrained by variability in biomaterials, cell sourcing, device geometry, and analytic endpoints; inconsistent reporting of key parameters; and the absence of community reference standards or ring trials for cross-site benchmarking.[8,9] For pharmacognosy and oncology alike, harmonized reporting, standardized quality-control metrics, and rigorous validation against *in vivo* and clinical outcomes are essential for broader adoption.

This critical review highlights foundational engineering approaches, representative validation studies, and translational metrics relevant to integrating perfused 3D systems into pharmacognostic workflows. Specifically, we compare core platform technologies and their primary readouts, summarize validation examples linking *in situ* 3D metrics such as drug penetration, immune infiltration, and clonal dynamics to *in vivo* or clinical outcomes, and propose a minimum reporting dataset, quality-control benchmarks, and a translational roadmap including reference constructs, ring trials, and prospective clinical pilots to support regulatory engagement and clinical adoption.

MATERIALS AND METHODS

Hydrogel Platforms and Extracellular Matrix Engineering

Hydrogels form the foundational scaffold for *in situ* 3D co-culture systems, providing tunable mechanical and biochemical properties essential for modeling microenvironmental influences on drug and natural product responses. Synthetic polymers such as polyethylene glycol (PEG) allow precise modulation of stiffness, ligand density, and degradability, enabling controlled investigation of mechanotransduction and matrix-dependent modulation of phytochemical activity. Natural matrices, including collagen, fibrin, and Matrigel offer native binding sites and enzymatic degradability that support physiologic remodeling and cell-directed architecture, features critical for evaluating how extracellular matrix composition shapes compound penetration and cellular response.[10]

Even modest changes in hydrogel stiffness or crosslink density can induce mesenchymal transition and upregulate drug efflux pathways, significantly altering chemosensitivity in otherwise identical constructs.[11,12] For pharmacognostic reproducibility, serum-free formulations or well-characterized extracellular matrix substitutes should be used, with explicit reporting of polymer concentration, crosslinking conditions, ligand density, and mechanical properties such as Young's modulus.[13]

A representative example is the tunable hyaluronic acid-collagen hybrid hydrogel that recapitulated the desmoplastic stroma of pancreatic cancer and reproduced drug penetration patterns observed in patient tissue explants, demonstrating how matrix composition directly influences translational readouts relevant to both natural products and synthetic agents.[14]

Microfluidics and Tumor-on-Chip Systems

Microfluidic platforms enable precise control over perfusion, shear stress, nutrient delivery, and oxygen gradients, whilst supporting immune cell trafficking and endothelial interfaces essential for modeling vascular-like compound transport. When integrated with hydrogel chambers, tumor-on-chip systems facilitate both convective and diffusive transport and allow longitudinal sampling of effluent for biomarker analysis and pharmacokinetic profiling capabilities highly relevant for evaluating natural product mixtures and phytochemical diffusion.[15] Figure 1 illustrates a perfusion-enabled microfluidic tumor-on-chip architecture, showing dedicated media and drug inlets, an imaging window for real-time monitoring, and waste outlets that together sustain spheroid viability and generate reproducible oxygen and nutrient gradients essential for longitudinal assessment of drug penetration and metabolic stress.

Early perfusion studies demonstrated that oxygen gradients generate hypoxic cores with altered DNA repair capacity and radiosensitivity, establishing a mechanistic link between microenvironmental oxygenation and treatment response.[16] Best practices include quantitative characterization of flow rates, shear stress, and oxygen tension using embedded sensors, alongside validation against computational diffusion models.[17]

Clinically relevant applications are emerging. A tumor-immune chip co-culturing patient-derived tumor fragments with autologous peripheral blood mononuclear cells successfully predicted checkpoint inhibitor responsiveness in retrospective cohorts when cytotoxicity and cytokine release were used as endpoints.[18]

Three-Dimensional Bioprinting and Spatial Patterning

3D bioprinting enables programmable placement of diverse cell types and extracellular matrix components to recreate tissue architecture, stromal niches, and perfusable vascular channels. Extrusion-based printing of cell-laden bioinks remains widely used for tumor constructs due to its balance between print fidelity and cell viability. Advanced strategies incorporate sacrificial inks to generate perfusable microvessels and gradient generators to model intratumoral heterogeneity within a single construct.[19] Figure 2 illustrates how perfused 3D co-culture systems integrate multicellular architecture, vascular-mimetic perfusion, and spatial gradients to model drug transport, immune-tumor interactions, and clonal evolution within physiologically relevant microenvironments.

Depth-resolved oxygen heatmaps, confocal live/dead imaging, and oxygen diffusion profiles highlight how construct geometry and vascularization shape viability and microenvironmental gradients.[20] Transparent documentation of bioink rheology, nozzle dimensions, printing pressures, crosslinking conditions, and post-print culture timelines is essential for reproducibility.[21] Figure 3 illustrates how spatial patterning and oxygen distribution emerge within bioprinted 3D tumor constructs, with depth-resolved oxygen heatmaps, live/dead imaging, and diffusion profiles revealing hypoxic cores and gradient-driven viability changes that underscore the importance of vascular integration and standardized bioink parameters.

Acoustically assisted bioprinting of patient-derived tumor microtissues has reproduced invasive fronts and predicted metastatic behaviour observed in matched clinical samples, demonstrating its utility for modeling invasion pathways and evaluating natural product-mediated anti-invasive effects.[22]

Vascularization Strategies and Perfusable Constructs

Recreating perfusable vasculature is essential for accurately modeling drug delivery kinetics, immune cell trafficking, and compound penetration. Strategies include self-assembly of capillary networks from endothelial and perivascular cells, templating of microchannels using sacrificial materials, and endothelialization of 3D-printed conduits followed by perfusion conditioning to establish mature barrier properties.[23]

These gradients demonstrate how vascular architecture influences immune infiltration patterns and effector cell balance, critical determinants of immunotherapeutic efficacy and natural product-mediated immune modulation. Figure 4 depicts immune cell localization and infiltration dynamics within perfused 3D tumor constructs, showing spatial gradients of tumor-associated neutrophils and T cells, schematic distribution patterns, and TAN: T-cell ratios that highlight how vascular architecture shapes immune access and effector balance.

Functional validation of vascularized constructs typically involves permeability assays, transendothelial electrical resistance (TEER), and profiling of flow-responsive gene expression. Constructs with mature vasculature more accurately recapitulate drug distribution and clearance kinetics observed *in vivo*, reinforcing their translational utility for pharmacokinetic and pharmacognostic investigations.[24]

Integrated Biosensing and Longitudinal Imaging

Label-free, longitudinal readouts are essential for non-destructive tracking of treatment responses in *in situ* systems. Imaging modalities such as optical coherence tomography, two-photon microscopy, and light-sheet imaging provide deep visualization in optically transparent matrices. Embedded impedance sensors and oxygen and pH microsensors enable continuous monitoring of physiological conditions within microfluidic chips.[25]

Multiplexed approaches, including live-cell imaging, effluent cytokine profiling, and single-cell sequencing offer multimodal perspectives on phenotypic evolution under therapy, including responses to natural products and complex extracts.[26] A practical demonstration employed label-free tomographic imaging to monitor apoptotic progression and recovery in glioblastoma spheroids treated with chemotherapy, revealing dosing regimens that produced transient versus durable responses.[27]

Practical Protocols and Quality Control Checklist

A robust *in situ* 3D co-culture protocol for translational drug and natural product testing should include clearly defined cell sourcing, including catalogue numbers, passage limits, and donor metadata; validated hydrogel formulations with mechanical characterization; specified seeding densities and spatial arrangements; controlled perfusion with oxygen regulation; calibrated sensor and imaging setups; and endpoint assays integrating viability, functional measures, and molecular profiling.

Quality control should track construct-to-construct variability, aiming for a coefficient of variation below 20% for primary readouts, and incorporate both positive and negative controls. Parallel testing in matched 2D, static 3D, and perfused 3D formats strengthens claims of translational relevance and clarifies the added value of the *in situ* approach.[28]

Applications in Translational Oncology

High-Throughput Drug Screening in in Situ 3D Systems

In situ 3D co-culture platforms are increasingly applied to high-throughput screening (HTS) to improve early-stage triage of candidate compounds, including natural products, phytochemicals, and synthetic agents. These systems better recapitulate tumor heterogeneity, drug penetration barriers, and microenvironment-mediated resistance than conventional spheroid or monolayer assays.[29] This fidelity is particularly

important for pharmacognostic evaluation, where complex extracts and multi-component mixtures often exhibit context-dependent activity.

Recent advances in lineage-tracing technologies highlight the importance of clonal expansion dynamics in shaping drug sensitivity and resistance trajectories, underscoring the need for HTS platforms that maintain hierarchical cellular organization and evolutionary structure.[30] Miniaturized spheroid cultures integrated into automated microfluidic arrays allow parallel perfusion and real-time imaging, generating dose-response profiles that diverge markedly from 2D assays and revealing compounds that retain potency under hypoxic or ECM-dense conditions.[31] Machine learning-driven image analytics further classify drug response phenotypes and predict synergistic interactions across combination matrices, enabling identification of clinically actionable drug pairs, including natural product combinations often overlooked in monolayer screens.[32]

For successful HTS deployment, critical parameters include assay miniaturization without compromising physiologic gradients, mitigation of edge effects in microplate or chip-based formats, rigorous validation of inter-plate reproducibility, and implementation of automated quality-control thresholds to ensure acceptable coefficients of variation across large-scale campaigns.[33]

Immunotherapy Testing and Immune-Tumor Co-Cultures

In situ 3D co-culture systems provide physiologically relevant platforms for interrogating immune infiltration, checkpoint modulation, and engineered cell therapies under spatially constrained conditions. Patient-derived organoids co-cultured with autologous peripheral blood mononuclear cells (PBMCs) or tumor-infiltrating lymphocytes demonstrate that extracellular matrix stiffness and architectural complexity markedly influence T-cell infiltration and sensitivity to checkpoint blockade.[34]

Microfluidic tumor-immune chips incorporating dynamic perfusion and chemokine gradients recapitulate macrophage polarization and immune-exclusion phenotypes observed *in vivo*, enabling evaluation of TAM-targeting agents, including natural product-derived immunomodulators in combination with checkpoint inhibitors.[35] For CAR-T and other engineered cell therapies, 3D spheroid-based potency assays incorporating stromal and vascular components reveal distinct cytotoxic kinetics and infiltration profiles that suspension-based killing assays fail to capture.[36]

Standardized immune readouts, including cytotoxicity kinetics, multiplexed cytokine panels, and spatial infiltration metrics are essential for reproducibility and cross-study harmonization.[37]

Radiotherapy and Hypoxia-Modulated Responses

Radiation efficacy is strongly modulated by oxygen tension and extracellular matrix composition, both of which are faithfully

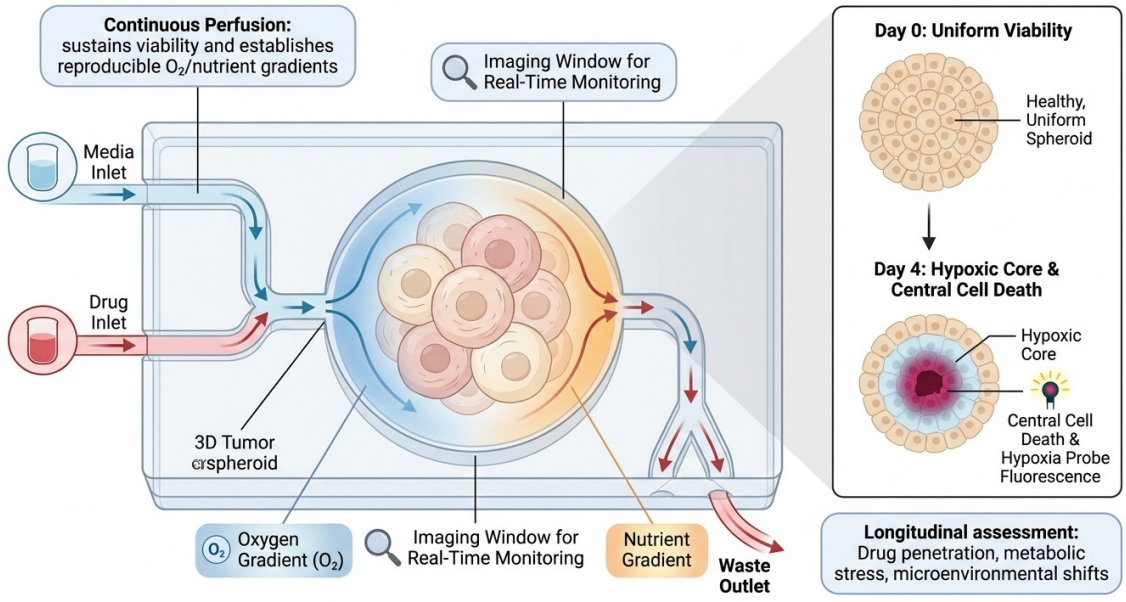


Figure 1: Perfusion-enabled microfluidic architecture supporting dynamic nutrient and drug delivery. Microfluidic tumor-on-chip platform showing dedicated inlets for media and drug perfusion, an imaging window for real-time monitoring, and outlets for waste removal. Continuous perfusion sustains spheroid viability and establishes reproducible oxygen and nutrient gradients. Over four days, constructs transition from uniformly viable spheroids (Day 0) to structures exhibiting hypoxic cores and central cell death (Day 4), visualized using oxygen-sensitive probes. This configuration enables longitudinal assessment of drug penetration, metabolic stress, and microenvironmental shifts relevant to pharmacognostic evaluation.

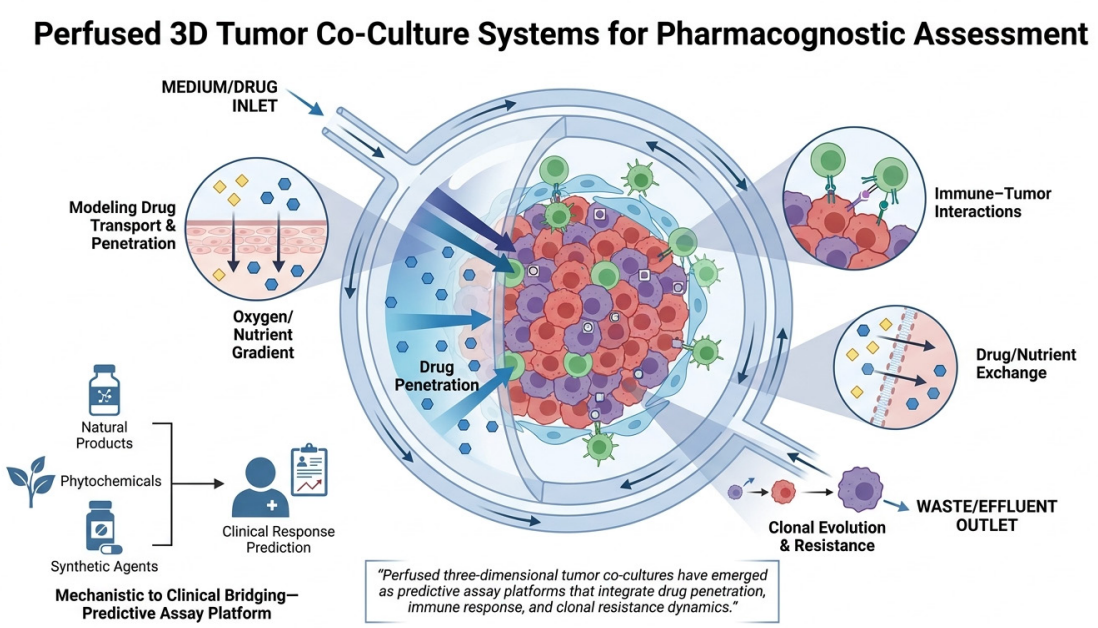


Figure 2: Conceptual overview of perfused 3D tumor co-culture systems for pharmacognostic assessment. Schematic representation illustrating how perfused 3D co-cultures integrate multicellular architecture, vascular-mimetic perfusion, and spatial gradients to model drug transport, immune-tumor interactions, and clonal evolution. These systems bridge mechanistic biology with clinically relevant therapeutic response modeling, supporting evaluation of natural products, phytochemicals, and synthetic agents under physiologically relevant conditions.

reproduced in perfused 3D systems. These platforms reliably develop hypoxic cores that mirror radioresistant phenotypes observed clinically, enabling evaluation of radiosensitizers, including natural product-derived agents targeting hypoxia-inducible factor (HIF) signalling and metabolic vulnerabilities.[38]

Emerging strategies integrating 3D constructs with calibrated dosimetric irradiation and longitudinal imaging allow spatially resolved tracking of DNA damage and repair kinetics, uncovering subpopulations with enhanced repair capacity and informing rational combinations of radiotherapy with DNA repair inhibitors.[39] For translational rigor, radiotherapy studies require precise dosimetry calibration, standardized quantification of oxygen partial pressures, and benchmarking against established *in vivo* models.[40]

Metastasis and Invasion Modeling

Metastatic progression is driven by dynamic cell-matrix interactions, stromal modulation, and intravasation events that are poorly represented in monolayer cultures. *In situ* 3D co-culture systems incorporating spatially organized stromal compartments and perfusable endothelial channels model epithelial-mesenchymal transition (EMT), matrix remodelling, and transendothelial migration, yielding invasion phenotypes and transcriptomic profiles that closely mirror patient-derived metastatic signatures.[41]

These systems support mechanistic interrogation of anti-metastatic agents, including natural product-derived inhibitors of adhesion molecules, proteolytic enzymes, and chemokine signalling axes.[42] Whilst replicating systemic organotropism remains challenging, *in situ* models are particularly effective for dissecting discrete steps such as invasion, intravasation, and extravasation, and should be complemented by *in vivo* validation to capture the full metastatic cascade.[43]

Targeted Therapy Development and Pathway-Specific Testing

3D co-culture systems provide enhanced fidelity for evaluating targeted therapies by incorporating stromal cues that modulate oncogenic pathway dependencies. Patient-derived tumoroids embedded in defined extracellular matrices demonstrate that kinase inhibitor sensitivity is highly context-dependent, with stromal secretomes and matrix stiffness, reshaping downstream signalling and altering drug efficacy relative to 2D monolayers.[44]

These platforms support rational combination therapy design, including pairing natural product-derived modulators with pathway-specific inhibitors. Translational application requires robust biomarkers of pathway engagement and standardized multiplexed readouts, such as phosphoproteomic signatures and functional response assays to inform actionable therapeutic decisions.[45,46]

Toxicity and Safety Profiling

In situ 3D co-culture systems provide physiologically relevant platforms for preclinical safety assessment by preserving multicellular interactions, extracellular matrix architecture, and

long-term functional outputs. These features enable detection of delayed or mechanism-specific toxicities frequently overlooked in short-duration 2D assays. Engineered hepatic 3D co-cultures incorporating hepatocytes and non-parenchymal cells recapitulate fibrotic remodeling and chronic drug-induced hepatotoxicity observed clinically, including stellate cell activation and collagen deposition following low-dose, repeated exposures.[47]

Similarly, 3D engineered myocardium integrating cardiomyocytes, fibroblasts, and electromechanical stimulation reveals late-onset arrhythmias and contractile dysfunction induced by kinase inhibitors, effects not captured in acute 2D cardiomyocyte assays. Neural 3D models embedding neurons and astrocytes within brain-mimetic hydrogels uncover neurotoxic cascades triggered by chemotherapeutics such as cisplatin, driven by glial-neuronal interactions absent in monolayer cultures.[48]

Best practices include chronic exposure protocols, functional endpoints (e.g., contractility, insulin secretion, electrophysiology), multiplexed biomarker panels, and orthogonal validation against *ex vivo* human tissue or *in vivo* comparators.[49]

Tumor Microenvironment Studies

In situ 3D co-culture systems offer a flexible framework for dissecting the cellular and matrix constituents of the tumor microenvironment (TME), preserving spatial architecture whilst enabling controlled perturbation of individual components. Cancer-associated fibroblasts (CAFs) embedded in tunable hydrogels actively remodel the extracellular matrix, modulating stiffness and biochemical composition in ways that redirect invasion trajectories and impede drug diffusion.[50]

Tumor-associated macrophages (TAMs) introduced into perfused microfluidic constructs exhibit polarization plasticity along the M1-M2 axis in response to local cytokine gradients and matrix cues, shaping T-cell recruitment and fostering immunosuppressive niches that diminish checkpoint blockade efficacy.[51] Hypoxia recreated through engineered oxygen gradients reprograms tumor metabolism via HIF-dependent signaling, promoting glycolytic, pro-invasive phenotypes and upregulating drug resistance genes and immune-evasive ligands.[52] Integrated spatial transcriptomics and single-cell profiling reveal how proximity to stromal or vascular compartments correlates with resistant transcriptional states, establishing mechanistic links between microenvironmental context and therapeutic failure.[53]

Heterogeneity Analysis

In situ 3D co-culture systems provide robust platforms for interrogating intratumoral heterogeneity by preserving spatially defined niches and enabling longitudinal tracking of sub-clonal dynamics under therapeutic selection pressure. When coupled with single-cell RNA sequencing or lineage-tracing reporters,

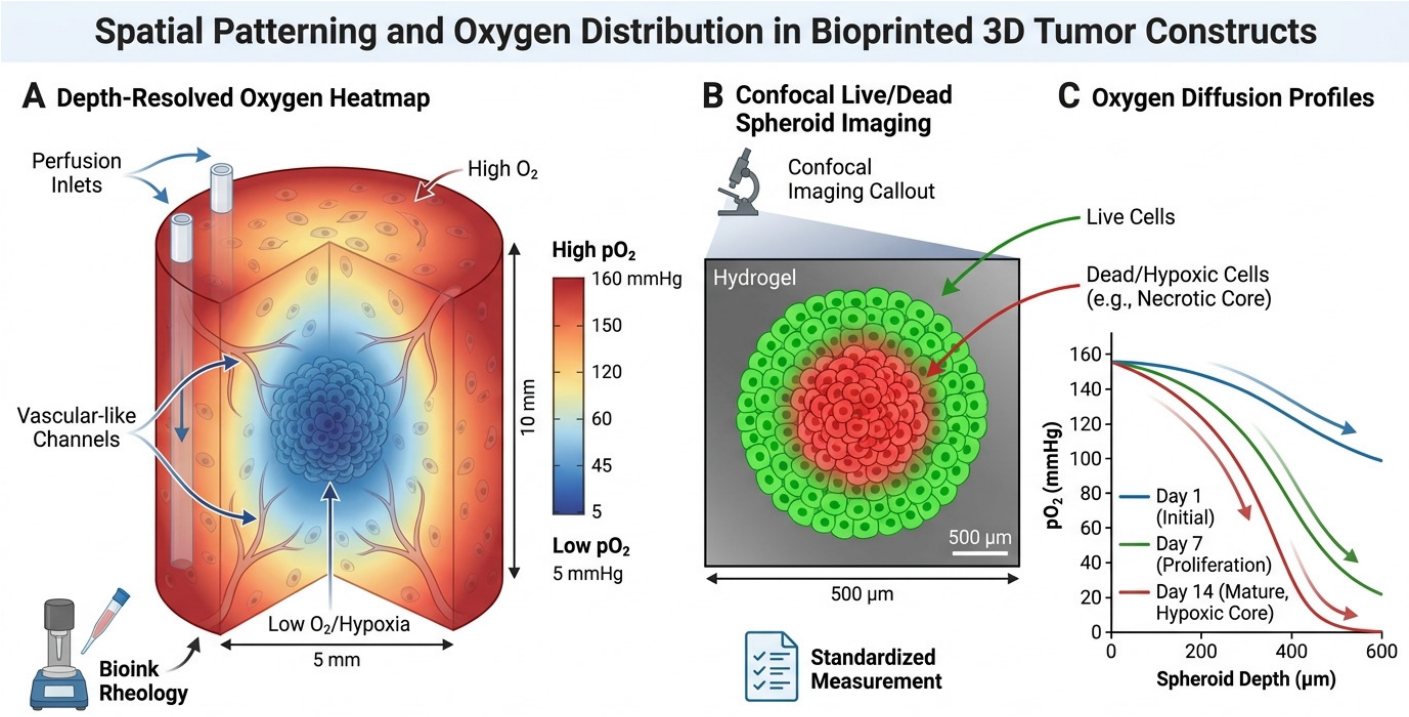


Figure 3: Spatial patterning and oxygen distribution in bioprinted 3D tumor constructs. (A) Depth-resolved oxygen heatmap illustrating intraconstruct gradients within perfused bioprinted tumor models. (B) Confocal live/dead imaging of spheroids (400-600 µm) showing increased hypoxia-associated cell death toward the core. (C) Oxygen diffusion profiles demonstrating progressive depletion of pO_2 with increasing spheroid depth. These data highlight the importance of vascular integration, bioink rheology, and standardized reporting for reproducible assessment of drug penetration and natural product bioactivity.

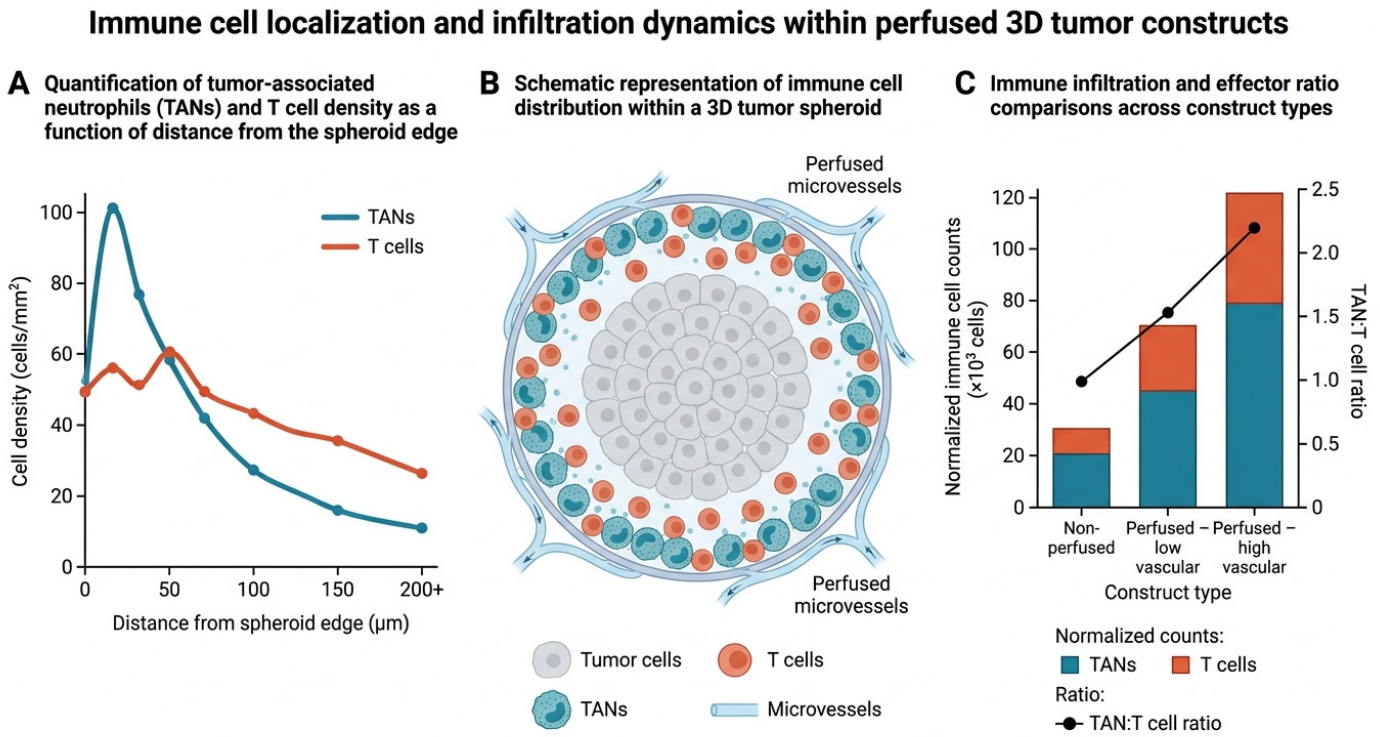


Figure 4: Immune cell localization and infiltration dynamics within perfused 3D tumor constructs. (A) Quantification of tumor-associated neutrophils (TANs) and T-cell density as a function of distance from the spheroid edge, revealing distinct spatial localization gradients. (B) Schematic representation of immune cell distribution, showing preferential accumulation near the spheroid periphery. (C) Comparison of normalized immune cell counts and TAN:T-cell ratios across construct types, demonstrating how vascular architecture shapes immune infiltration patterns and effector balance. These features are critical for evaluating immunomodulatory natural products and checkpoint-directed therapies.

these models uncover differential drug sensitivities among spatially segregated subpopulations and identify persistent cellular states that evade treatment and contribute to relapse.[54,55]

Organoid and tumoroid co-cultures derived from anatomically distinct tumor regions capture inter-regional heterogeneity and facilitate parallel therapeutic interrogation, generating response landscapes that more accurately reflect patient-specific outcomes.[56] High-content imaging integrated with machine learning-driven phenotypic clustering enables quantitative mapping of intratumoral diversity, linking spatial features such as proliferative rims, hypoxic cores, and invasive margins to discrete molecular programs.[57]

Drug Resistance Mechanisms

In situ 3D platforms reveal diverse microenvironment-mediated resistance mechanisms frequently attenuated or absent in conventional 2D systems. Extracellular matrix stiffness and composition dynamically modulate integrin signalling and downstream survival pathways, attenuating apoptotic responses to cytotoxic agents and targeted inhibitors.[58] Hypoxic conditions stabilize HIFs, driving transcriptional programs that promote angiogenesis, metabolic reprogramming, and epithelial-to-mesenchymal transition (EMT), collectively contributing to chemoresistance and immune escape.[59]

Stromal constituents, including CAFs and TAMs further complicate therapeutic response by secreting cytokines and extracellular vesicles that activate compensatory survival circuits and upregulate drug efflux transporters such as P-glycoprotein, limiting intracellular drug accumulation.[60] Functional assays measuring drug penetration, intracellular concentrations, and target engagement within 3D constructs are essential for distinguishing pharmacokinetic barriers from intrinsic cellular resistance.[61]

Tumor Evolution and Clonal Selection

Long-term culture within stable 3D microenvironments provides a powerful framework for interrogating clonal selection under therapeutic pressure, offering mechanistic insight into how treatment regimens, including natural products, phytochemicals, and synthetic agents shape tumor evolutionary trajectories. Perfused 3D co-culture systems maintain spatial gradients, stromal interactions, and nutrient dynamics that influence clonal competition and lineage dominance, features that are poorly represented in conventional 2D assays.[62] Figure 5 illustrates clonal evolution and lineage dynamics within perfused 3D tumor co-cultures, showing declining Simpson's Index, spatial expansion of dominant clones, and proliferative advantages of resistant lineages that demonstrate how these systems enable longitudinal tracking of clonal selection under therapeutic pressure.

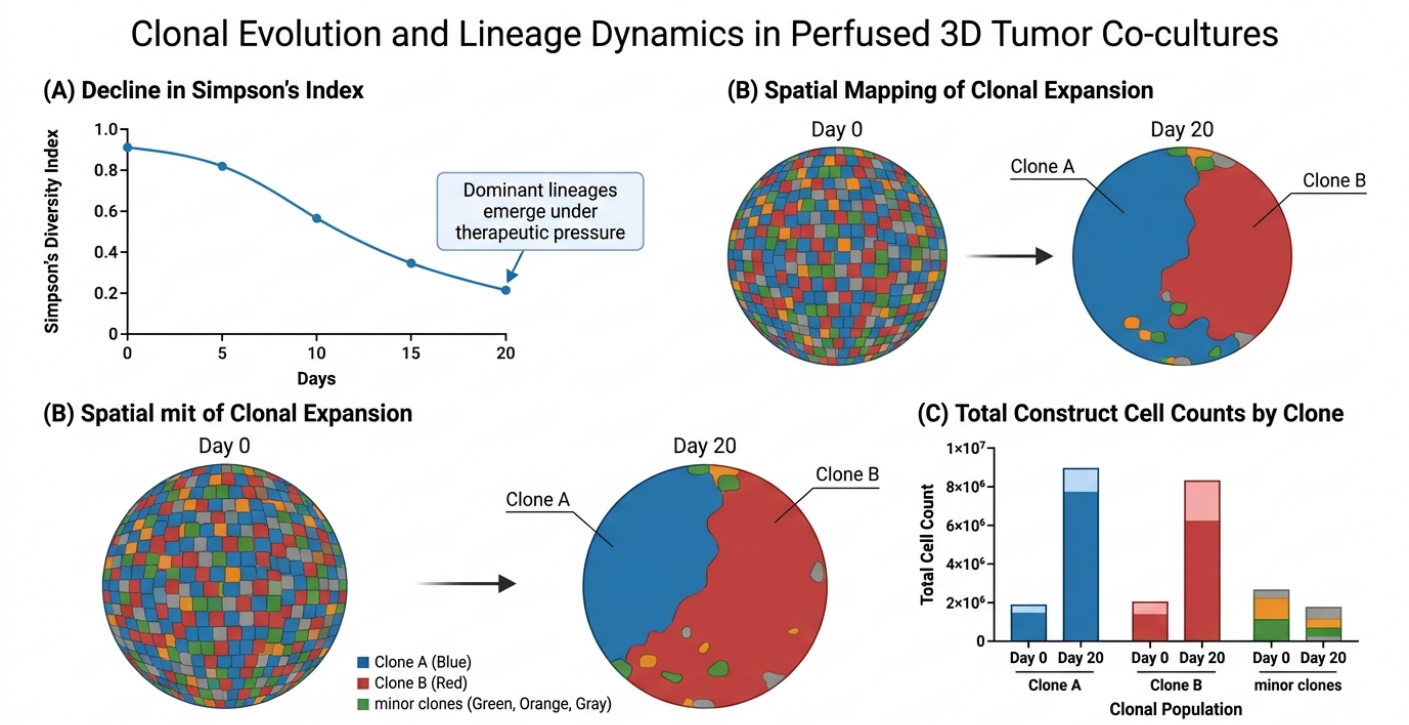


Figure 5: Clonal evolution and lineage dynamics in perfused 3D tumor co-cultures. (A) Decline in Simpson's Index over 20 days, indicating reduced clonal diversity and emergence of dominant lineages under therapeutic pressure. (B) Spatial mapping of individual clones showing preferential expansion of specific lineages over time. (C) Total construct cell counts demonstrating significant proliferation of major clones (A and B) relative to minor populations. These data illustrate how perfused 3D systems enable longitudinal tracking of clonal selection, supporting pharmacognostic evaluation of natural products and combination therapies targeting resistant subpopulations.

Time-resolved single-cell sequencing of serially sampled constructs reveals preferential amplification of resistant phenotypes, enabling identification of transcriptional programs associated with persistence, immune evasion, and metabolic adaptation.[63] Such analyses are particularly relevant for pharmacognostic research, where natural products often exert multi-target effects that may differentially impact subclonal populations.[64] By preserving spatial niches and enabling longitudinal sampling, perfused 3D systems allow researchers to distinguish between direct cytotoxic effects, microenvironment-mediated protection, and adaptive lineage expansion.[65] These platforms also facilitate evaluation of combination strategies aimed at preventing or delaying clonal dominance.[66] For example, integrating stromal-targeting agents, immune modulators, or natural product-derived metabolic inhibitors with conventional therapies can suppress expansion of resistant clones and maintain higher clonal diversity.[67] Perfused 3D constructs thus serve as tractable models for studying evolutionary bottlenecks, adaptive resistance, and the emergence of persistent cellular states under complex therapeutic pressures.[68]

Therefore, clonal evolution studies in perfused 3D systems highlight the need for pharmacognostic approaches that account for spatial heterogeneity, microenvironmental modulation, and evolutionary dynamics.[69] These insights support rational design of therapeutic regimens, including natural product combinations that target both dominant and emerging resistant lineages within clinically relevant microenvironments.[70]

CONCLUSION

Perfused 3D tumor co-culture systems represent a transformative advance for both pharmacognostic research and translational oncology, offering microenvironmental fidelity that cannot be achieved in conventional 2D or static 3D formats. By integrating vascular-mimetic perfusion, multicellular co-cultures, and depth-resolved imaging, these platforms enable rigorous evaluation of drug transport, immune modulation, and resistance mechanisms under physiologically relevant conditions.

The ability to preserve spatial gradients, stromal remodelling, and immune-tumor interactions provides a powerful foundation for assessing the bioactivity of natural products, phytochemicals, and complex extracts whose pharmacological behaviour is strongly shaped by microenvironmental context. Perfused systems also support longitudinal interrogation of clonal evolution, revealing how therapeutic pressure, including exposure to natural product-derived agents drives lineage selection, metabolic adaptation, and immune evasion. These insights are essential for designing rational combination strategies that target both dominant and emerging resistant populations.

Despite their promise, widespread adoption of perfused 3D platforms requires harmonized reporting standards, validated quality-control metrics, and cross-site benchmarking through reference constructs and ring trials. Standardization of hydrogel formulations, perfusion parameters, sensor calibration, and spatial readouts will be critical for ensuring reproducibility across laboratories and for enabling regulatory engagement. Integrating these systems into preclinical pipelines will also require comparative validation against *in vivo* and clinical outcomes, including responses to natural products and multi-component mixtures.

Collectively, perfused 3D tumor co-cultures provide a robust and versatile framework for advancing pharmacognostic assessment, mechanistic oncology research, and precision therapeutic development. The capacity to model drug penetration barriers, immune dynamics and evolutionary adaptation positions perfused 3D co-cultures as essential tools for bridging fundamental biology with clinically relevant response prediction. As the field moves toward standardized methodologies and translational validation, these platforms are poised to play a central role in next-generation natural product discovery, mechanistic pharmacognosy, and personalized oncology.

ABBREVIATIONS

2D: Two-Dimensional; **3D:** Three-Dimensional; **CAFs:** Cancer-Associated Fibroblasts; **CAR-T:** Chimeric Antigen Receptor T-cell; **ECM:** Extracellular Matrix; **EMT:** Epithelial-Mesenchymal Transition; **HIF:** Hypoxia-Inducible Factor; **HTS:** High-Throughput Screening; **PBMCs:** Peripheral Blood Mononuclear Cells; **P-gp:** P-Glycoprotein; **PEG:** Polyethylene Glycol; **pO_2**: Partial Pressure of Oxygen; **TAMs:** Tumor-Associated Macrophages; **TANs:** Tumor-Associated Neutrophils; **TEER:** Transendothelial Electrical Resistance; **TILs:** Tumor-Infiltrating Lymphocytes; **TME:** Tumor Microenvironment.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS CONTRIBUTION

Conceptualization was carried out by **V.A, I.E.C. T.A.N.:** Provided additional references for curating the different sections. Methodology was developed by **V.A.:** With image software support provided by **V.A.:** Validation was performed by **V.A, T.A.N., I.E.C.:** Formal analysis and investigation were conducted by **V.A.** Resources and data curation were provided by **T.A.N, V.A.:** The original manuscript draft was written by **V.A.:** With subsequent review and editing undertaken by **T.A.N., V.A., I.E.C:** Visualization was completed by **V.A., I.E.C:** While supervision was provided by V.A. and I.E.C. Project administration was managed by **V.A.** All authors have read and approved the final version of the manuscript.

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