Tumour Microenvironment 3D-modelling: Simplicity to Complexity and Back Again

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Outstanding Questions

- What are the best approaches to study cross talk between multiple TME cell types within the same model system?

- How best to validate 3D model systems against the host tissue they purport to represent?

- Will marrying 3D model systems with clinical data reduce the need for *in vivo* experiments in pre-clinical validation?

- Practicality is crucial to adoption of a 3D model. Can accessibility to cell sources and methodological pragmatism be improved to allow widespread uptake?

Highlights

- The tumour microenvironment is host to multiple cell types that play supportive and suppressive roles in cancer progression

- Multi-cellular 3D models that incorporate cells of the tumour microenvironment are fundamental to cancer research.

- 3D models have revealed novel insights into the biology of cellular interactions within the tumour microenvironment using highly tractable, physiologically relevant systems.

- Tissue banks, animal material, and induced pluripotent stem cells, provide researchers ready access to a range of cell types for use in model systems.

- The increasing complexity of 3D models presents challenges for informative analysis. Nascent methods allow improved visual, genetic, and proteomic profiling.

Abstract

Tumours are surrounded by a host of non-cancerous cells that fulfil both supportive and suppressive roles within the tumour microenvironment. The drive to understand the biology behind each of these components has led to a rapid expansion in the number and use of 3D *in vitro* models, as researchers find ways to incorporate multiple cell types into physiomimetic configurations. The use and increasing complexity of these models does however demand many considerations. In this review we discuss approaches adopted to recapitulate complex tumour biology in tractable 3D models. We consider how these cell types can be sourced and combined and examine methods for the deconvolution of complex multicellular models into manageable and informative outputs.

3D Modelling of the Tumour Microenvironment

The drive to reduce the use of animals in research has seen 3D *in vitro* models develop into a critical part of the researcher's toolbox over recent years. Offering the ability to model complex cellular behaviours under physiomimetic conditions, 3D models present the ideal bridge between simple 2D culture and complex *in vivo* systems. Modelling multiple cell types in one tractable system also allows cellular interplay to be studied in real-time and dissected with exquisite precision.

An area where use of 3D models offers outstanding insight is in the study of the tumour microenvironment (TME). Tumours interact with and co-opt a diverse range of both tissue-resident, and recruited non-cancerous cells, which exhibit both pro- and anti-tumourigenic roles (Box 1). Different cancers generate distinct microenvironments with their own repertoire of cell types and functions that show distinct host organ-dependent phenotypes. A good example to illustrate this is the distinctive TMEs displayed by breast and pancreatic cancers. Breast cancers harbour ductal myoepithelial cells and have a large adipocyte component [1], while pancreatic tumours are hyopovascular, and have a rich, dense stroma [2] (Figure 1).

Studies into the cellular interactions within the TME have led to new advances in our understanding of cancer progression. Moreover, new therapeutic approaches that specifically target the TME offer the opportunity to enhance current anti-cancer therapies and improve patient outcomes. Numerous 3D model systems currently exist to examine the TME, ranging from the simple co-culture of cells in hydrogels, to complex, multi component microfluidics, each with their own advantages and limitations [3] (Figure 2). Novel models are continually being reported, offering researchers a veritable smorgasbord to choose from to answer particular research questions. Common cancer models, such as spheroids and organoids, are now used routinely to reveal new biological insights and develop therapeutic strategies, or complement complex and far more time-consuming *in vivo* and clinical studies.

In this review we provide an overview of common 3D model systems used to incorporate cells of the TME, highlighting how researchers have used these systems to gain new insights into biology. We also discuss challenges associated with 3D modelling of the TME, including sourcing suitable TME cells, and strategies to interpret increasingly complex, multi-cellular 3D models.

Sourcing Tumour Microenvironment Cells

Modelling the TME requires careful selection of TME cell types to ensure adequate, context-specific recapitulation of the target tissue (Figure 3). Cell lines represent a common source of TME cells, as they are accessible, abundant, and generally easy to cultivate. While often a useful tool for TME studies, researchers should be wary of lines that harbour genetic alterations that may affect function and thus their ability to reflect the intended cell type. As an example, a common method to immortalise cells is the introduction of SV40 large T antigen, which encourages the sustained modification of a number of signalling pathways including p53 and thus may have unintended consequences on the biology of the cell [4].

Cell lines and isolated cells can also be conditioned to mimic cells of the TME. A classic example is macrophages, which can be differentiated to either a pro- or anti- inflammatory state through stimulation with either interferon-γ and lipopolysaccharide, or interleukin-4, respectively [5]. Mature adipocytes can also be differentiated from pre-adipocytes [6], fibroblasts [7], or mesenchymal stem cells [8], providing a more consistent source of these for cancer models than afforded by primary tissue. Differentiation state is critical, as mature adipocytes have more impact on cancer cell growth and invasion than immature counterparts [9], emphasising the need for careful cell selection and preparation.

Primary, patient-derived, cells offer an ideal source of cells for modelling purposes. If tumour-derived, the cells will offer the additional benefit of being TME-educated, and thus functionally distinct from normal counterparts. Access to primary TME cells traditionally required research groups to be located at hospital sites, with a bespoke team in place for consenting, collection, and processing, which was beyond the reach of most research groups. However, with the increase in tissue banking, researchers now have enhanced access to primary TME cells [10]. Tumour samples from these banks also provide the opportunity to validate findings in clinical samples, with potential to access outcome data, thus bolstering the value and pre-clinical relevance of the model system. Reproducibility may be an issue between patient samples, but should be seen as a benefit, as common findings may reflect a critical process, and differences may reveal distinct subtypes with clinical relevance, for example in terms of interpatient, intertumour or intratumour heterogeneity [11].

Induced pluripotent stem cells (iPSCs) present a means to derive cell types of choice for research purposes [12]. iPSCs are also amenable to genetic alteration, allowing manipulation of cell types that are otherwise difficult to modify, such as iPSC-derived macrophages. IPSCs can be used to generate macrophages that are transcriptionally similar to blood-derived counterparts and can be activated to distinct phenotypes. Genetically modified macrophages derived from modified iPSCs

can also be produced to ease visualisation via fluorescent tags [13], or to further produce phenotypically distinct macrophages [14].

TME cells can also be derived from multiple species for subsequent use in model systems. For instance, distinct fibroblast populations have been isolated from murine pancreatic tumours and characterised *ex vivo* [15]. Whole tissues, such as aortae and ganglia [16,17], can also be isolated from rodents and used in 3D models. These have the benefit of retaining their physiological attributes, and contain a number of tissue specific cell types, allowing specific tumour-tissue interactions to be studied in context.

Approaches to Model the Tumour Microenvironment

Having decided on the most appropriate source of cells, one must consider the level of modelling complexity that is best suited to the biology under investigation. Models can range from simple monoculture in strictly defined matrix, to multicellular approaches mimicking physiological conditions such as fluid flow and immune components.

Hydrogels

The basic component of most 3D models used to study the TME is the hydrogel, in which multiple cell types can be embedded into a highly tuneable liquid matrix, which is then polymerised and maintained in culture over days/weeks. Cells can then orientate themselves, remodel the matrix, and interact with each other within a 3D environment.

Hydrogels are most commonly used with extracted matrix proteins such as rat-tail/bovine collagen and Matrigel, which remain liquid at 4°C and subsequently polymerised when placed at 37°C. Individual cells, multi-cellular structures, and additional matrix components can easily be placed within the gel and removed at the end of culture with enzymatic digest of the matrix components. Synthetic hydrogels, produced with polymers such as polyethylene glycol, are also being used increasingly as they allow for much tighter control over matrix properties. Synthetic hydrogel components can be modified to incorporate different integrin binding motifs, mimicking distinct ECM properties, or the binding of multiple growth factors [3,18].

Hydrogels present a highly tuneable system with which to study matrix composition and biomechanical properties of cells. They have provided insights into the capacity of cancer-associated fibroblasts (CAFs) to remodel the extracellular matrix (ECM), showing CAFs to remodel

existing collagen fibres, alongside producing collagen *de novo*, while contracting the gel, generating a stiff tumour-like matrix, dependent on the mechanosensing ability of CAFs [19,20]. Likewise macrophages cultured in high-density collagen matrices, resembling the dense tumour stroma, show enhanced immunosuppressive effects compared to those cultured in low density collagen [21].

Cells can self-assemble into physiomimetic structures when placed in hydrogels and provided with the correct cues. This is most evident in vascular models, where endothelial cells, often with support cells such as pericytes, assemble into tubular structures. While useful to study endothelial biology, these models do not accurately reflect the distinct stages of angiogenesis, which can limit their use and interpretation [18,22].

A more complex example is the generation of breast ducts. Isolated populations of primary luminal and myoepithelial cells can be placed in collagen gels, wherein they recombine into correctly patterned ducts [23]. This allows genetic manipulation of either cell type, or the addition of other tumour factors. For instance, conditioned medium from inflammatory macrophages promotes luminal filling in breast duct cultures through a pathway involving IKKε regulation of serine biosynthesis [5]. Thus hydrogels are an excellent, versatile tool, to study cell-matrix and cell-cell interactions within a defined 3D environment. Physiological multi-cellular structures can also be created within these systems but care needs to be taken that the resulting structure mimics the host tissue.

Organotypic models

Organotypic cultures adapt hydrogels into roughly compartmentalised systems. A hydrogel, often laden with CAFs or other TME cells is placed on a porous membrane that is either submerged in culture medium or raised to the air-liquid interface. Cancer cells are then seeded on top of the TME-mimetic gel and typically cultured for days to weeks, allowing complex cellular interactions and changes to develop. A caveat is that most cultures require end point histopathological analysis, which is time-consuming and only offers a snapshot of cell behaviour. Nevertheless, these models have revealed fundamental biology underpinning interactions between cancer cells and cells of the TME.

Cancer cells that retain their epithelial characteristics struggle to invade through acellular organotypic gels. However, if cultured with CAFs either embedded within the gel or mixed on top alongside cancer cells, cancer cell invasion becomes pronounced. CAFs can lead cancer cells through the gel matrix by creating protease-dependent tracks that allow cancer cells to follow [24].

Alternatively, CAFs can manipulate the matrix constituents of the basement membrane to allow cancer cell invasion independently of proteases [25].

CAF remodelled hydrogels can be converted to organotypic cultures by placing them on membranes and layering cancer cells on top. Interfering with how CAFs remodel the hydrogel, for instance by inhibiting the cytoskeleton regulator ROCK, can perturb subsequent growth and invasion of cancer cells. This translates therapeutically, as blockade of matrix remodelling with ROCK inhibitors potentiates anti-cancer therapy in mouse models of pancreatic cancer [26].

The ability of CAFs to facilitate invasion in organotypic models has become a staple method to assess CAF-cancer cell interactions across multiple cancer types. More recently the method has been used to demonstrate the growth and invasive capacities of distinct subsets of CAFs in pancreatic cancer, highlighting the heterogeneity among this cell type [11,15].

Organotypic models can be modified to incorporate multiple cell types and use different matrices, including decellularised scaffolds. Cutaneous melanoma has been modelled successfully by culturing keratinocytes on a decellularised dermis infused with fibroblasts, to create a skin mimetic. Introduced melanoma cells proliferate and invade into the model, recapitulating disease progression. Blood derived dendritic cells injected into this model acquire CD14 expression, and show a deficiency in T cell activation, closely resembling dendritic cells isolated from melanoma patients [27].

Recently a model of ovarian cancer omental metastasis has been developed as a tetra-culture organotypic system. An adipocyte-laden collagen gel is formed using primary adipocytes, upon which primary omental fibroblasts and mesothelial cells are cultured, generating a physiomimetic omental layer. Ovarian cancer cells can then be seeded on top to mimic omental metastases and the subsequent interplay between the multiple cell types studied. This approach demonstrated that the further addition of platelets to the model could facilitate cancer cell invasion through a transforming growth factor-β (TGFβ) dependent pathway [28], highlighting the translational power of performing such multicellular studies.

Spheroids

Spheroids present a simple yet relevant method to study cancer cell biology, either alone or as multi-cellular units. Spheroids provide an environment more akin to the tumour, with self-imposed nutrient and hypoxic gradients adding dimensions not experienced with traditional 2D cultures. Commonly,

they are grown in low attachment plates in matrix-free conditions, but embedding spheroids in hydrogels allows for cancer cell interactions with the ECM or additional cell types to be investigated. These cultures are more amenable to live imaging than organotypics, allowing cellular interactions to be monitored in real time, especially when combined with fluorescent reporters. As spheroids grow in size, oxygen and nutrient diffusion is reduced, creating a hypoxic core that mimics the environmental conditions of many tumours, although this can limit the culture time of this model due to the build up of toxic by-products [3].

As with organotypic models, spheroids embedded in hydrogels are an excellent tool to study cancer cell-CAF interactions, demonstrating that CAFs can physically pull cancer cells through the ECM via heterotypic cadherin-cadherin interactions. Moreover, CAFs in spheroids can deposit their own matrix and remodel the surrounding gel, influencing cancer cell invasion, for instance through the production of fibronectin [29]. Additionally CAFs can be pre-conditioned before being placed in spheroids, allowing the influence of cancer-derived exosomes to be examined [30].

Additional TME cell types may also be incorporated into spheroid cultures and can exhibit distinct phenotypes depending on whether they are incorporated into the spheroid or placed within the surrounding matrix. For instance, macrophages seeded within a cancer cell spheroid, but not those embedded around the spheroid, upregulate expression of epidermal growth factor (EGF) enhancing spheroid growth and chemo-resistance [31].

Adaptive immune cells can also be added to cultures of cancer cell spheroids, where their tumour killing abilities and the effects of potential immunotherapy can be assessed. For instance, blood derived natural killer and T cells can infiltrate colorectal cancer spheroids and initiate tumour cell killing. This system highlighted the interaction between the NK and T cell receptor NKG2D and its ligand MICA/B as a potential immunotherapy target [32].

Organoids

Rather than forming 3D cultures from differentiated cell types, organoids provide an excellent tool to study tumour growth. Formed from single stem cells, they retain genetic, pathological, and heterogeneous features of the host tumour [33]. With their ability to be passaged and cryopreserved, organoids offer researchers the benefit of a closely native model system with the utility of cell lines. While organoids often have complex culture requirements and are not currently available for all cancer types, the field is constantly expanding and organoid lines are now available through commercial entities.

As with spheroids, additional TME cells can be embedded alongside the organoids. Blood-derived cells can be cultured alongside organoids, promoting the formation of tumour reactive T cells that can be used to study patient-specific tumour-T cell interactions [34]. Positioning can greatly affect TME biology, as vividly demonstrated by co-culture of pancreatic stellate cells with pancreatic cancer organoids, which has enhanced our understanding of CAF heterogeneity. Stellate cells are a key source of CAFs in pancreatic cancer and their culture in direct contact with organoids generated a myofibroblastic CAF, characterised by strong expression of α-smooth muscle actin (α-SMA). Conversely, culture of stellate cells with organoids in the same environment, but spatially distinct, generated CAFs with an inflammatory signature [35].

Direct patient and mouse-derived organoids can also be cultured as cellular aggregates. As these organoids are not cultured from single cells they lack the utility of typical organoids, but they retain numerous stromal components allowing native tumour-stroma interactions to play out in an *in vitro* system. As such, patient-derived organoids retain their host immune components and respond to immune checkpoint inhibitors, which predicts *in vivo* efficacy [36,37].

Mouse mammary organoids implanted in ECM retain their luminal and myoepithelial cell polarity and can be used to study the influence of the myoepithelial cell on cancer progression in real-time. Expression of the pro-metastatic gene *Twist1* within the luminal compartment encourages the dissemination of luminal cells. However, this escape is restricted by the myoepithelial cells, which capture and restrain luminal cells within the organoid. Disruption of myoepithelial cell function, through knockdown of either αSMA or P-Cadherin, markedly increased luminal cell escape, demonstrating a protective effect for myoepithelial cells in progression [38].

Normal human primary breast fragments can also be placed in matrix-laden hydrogels, whereupon they form multi-branched structures with correct luminal and myoepithelial patterning [39]. Tumour cells can then be placed inside the branches, and their invasion out into the matrix used as a model of breast cancer progression. Using this approach, SMARCE1 was observed to regulate multiple matrix and protease genes required for invasion through the ductal barrier [40].

Explant Cultures

The 3D models described so far often require suspensions of single cells to be inserted into hydrogels. Recapitulation of the physiological state is then created either by allowing the cells to form and remodel their surroundings into their desired state, or layering the model in a predesigned

formation. Structures formed this way may therefore not reflect the native physiology or miss undervalued components. An alternative strategy is to remove a functional unit from a patient or animal and use this in a 3D model as an explant. Explants can retain many of the physical attributes of the host and have been used to more accurately model TME / cancer interactions. A caveat of this system however, is that that genetics of the explant are not as easily manipulated without transgenic animals. Equally, maintaining the balance and differentiation status of cell types within a cultured explant is challenged by cell type specific acclimatisation to culture conditions *ex vivo*.

An example of this if the aortic ring assay, which can better model distinct stages angiogenesis than simpler hydrogel models [16,22]. Aortae are dissected from mice, cut into rings and embedded in collagen I, whereupon endothelial proliferation, vessel sprouting and branching can be studied in response to pro-angiogenic factors. Multiple cell types, for example pericytes, are also present within aortic ring cultures, allowing their roles to be examined [41].

The use of explants to study TME and cancer interactions is exemplified by the dorsal root ganglion (DRG) culture used to study the invasion of cancer cells along neurons, termed perineural invasion [17,42]. First described with prostate cancer cells, an excised DRG is implanted in Matrigel along with cancer cells. Over time the DRG sprouts neurites along which cancer cells can invade [43]. This model has been used extensively to demonstrate reciprocal signalling of axon guidance cues to direct neurite outgrowth and cancer invasion [44-46]. Schwann cells, glial cells known classically for their role in axon myelination, are also present in DRG cultures and have been shown to direct the migration and attachment of cancer cells to neurons through NCAM1 mediated cell-cell contacts [47].

Tumour explants can also be excised and maintained in culture, allowing intact microenvironment interactions to be examined in real time. Further, distinct tumour regions such as the core and invasive front can be examined through dissection and culture of relevant areas. This approach was used to visualise the migration of cancer cells within the core of murine gut tumours by combining transgenically labelled cells with two photon microscopy to allow depth of view and live tracking [48]. As the core of tumours is often inaccessible to imaging techniques, explant cultures offer a powerful means to examine this region of tumours.

Microfluidics

None of the model systems considered thus far incorporate fluid flow, which is a key limitation for the study of some aspects of cancer biology. Microfluidics presents an ideal technology to study the

tumour vasculature *in vitro* as they allow a perfused vasculature to be maintained and visualised in real time [49,50]. Multiple cell types can be incorporated in these systems, either perfused or embedded in separate compartments within the device, allowing their interactions to be examined. This had led to advances in our understanding of tumour extravasation, intravasation, and immune recruitment to tumour sites.

This approach has been applied to investigate the metastatic niche preference of cancer cells. Perfused microvasculature was cultivated with matrix compartments containing osteoclasts and mesenchymal stem cells, to form a bone microenvironment, or myoblasts, to create a muscle microenvironment. Breast cancer cells perfused through these systems preferentially extravasate into the bone microenvironment, signifying that microfluidic vasculature modelling faithfully recapitulates *in vivo* metastatic homing [51].

Neutrophils perfused together with tumour cells form clusters in the microvasculature, which destabilise the endothelial barrier, enhancing extravasation [52]. Microfluidics has also been used to establish macrophages as a key player in establishing the pre-metastatic niche. Matrix-embedded macrophages produce MMP9, which destabilises endothelial tight junctions and increases extravasation of perfused cancer cells, whilst also producing tracks within the matrix that facilitate invasion of newly extravasated cancer cells [53].

Tumour cell intravasation can also be examined by placing tumour organoids next to perfused vessels. Organoids integrate with vessels, creating mosaic vessels comprised of both endothelial cells and tumour cells in an *in vitro* recapitulation of vascular mimicry. These mosaic vessels exhibit basement membrane dysfunction and leakiness, characteristic of the tumour vasculature, which facilitate tumour cell intravasation [54].

Deconvolution Strategies for 3D Model Analysis

3D modelling has offered researchers valuable insight into the biology of the TME. With the increasing prevalence and complexity of 3D models, their interpretation will become more challenging, as individual cell type and positional informational can be difficult to resolve. Granularity of resolution is an important consideration; identifying differences between cell types may require less resolution than examining heterogeneity within a specific cell type, where single cell level data demand more complex analysis. Here we discuss methods that have been used to deconvolute 3D model data, as well as omics approaches that could further improve the power of model systems (Figure 4).

Compartmentalisation

One of the simplest approaches to extract and analyse individual components is to use compartmentalised 3D approaches, such as microfluidics. As an example of this approach, in a modified DRG model rat DRGs were cultured in microfluidic devices that compartmentalised the neuronal bodies away from tumour cells. This allowed axonal growths from the DRG to interact with tumour cells, allowing individual capture and examination of the DRGs and cancer cells while maintaining their interactions. Using this system, exogenous serine, secreted by tumour-associated neurons, was shown to be essential for effective mRNA translation in nutrient-deprived pancreatic tumours [55].

Imaging

Imaging is usually the first choice for analysis of 3D models. Multiple markers, either through immunofluorescence or expression of fluorescent reporters in target cell types, can be used to distinguish cell type and interrogate behaviour, while retaining spatial information. Some 3D models, such as organotypics [56], can be fixed and embedded in paraffin, allowing individual sections to be cut and examined for gene/protein expression. This provides an objective means of visualising cell behaviour while maintaining some spatial characteristics, but essentially reduces a 3D model down to 2D snapshots. Optical projection tomography (OPT) can also be used to reveal the cellular architecture of 3D gels and can act as an alternative to histopathological analysis of organotypic cultures, allowing invasion in these models to be visualised in the 3D space [57,58].

Wholemount immunofluorescence imaging of 3D structures provides a clearer picture of cellular detail as well as whole sample architecture, but has been limited by signal dissipation in thicker samples caused by light scattering. Tissue clearance techniques provide a means to overcome this scattering issue, with detailed methods now available to improve imaging in complex 3D model systems [59,60]. 3D models are also amenable to multiplex imaging, allowing multiple cellular states to be visualised in a single sample [61].

Gene Expression

Gene expression analysis is a powerful tool to study how cell behaviour changes under different conditions. With 3D models incorporating numerous cell types, identifying genetic changes within each cellular compartment becomes a challenge. One way to overcome this is to use cell types from

multiple species. Bulk RNA sequencing data from the model system can then be separated by post hoc, species-specific, bioinformatic deconvolution [62], generating cell-type specific transcriptomic data. Alternatively, single cell RNA sequencing offers unparalleled insight into the genetics of each cell that comprises a 3D model [63]. The strength here is that data are not complicated by any issues of crosstalk being compromised by use of cells from different species. However a concern exists regarding whether fidelity of gene expression profile is preserved during the process of single cell isolation [64,65].

Newer single cell RNA sequencing techniques such as SPLiT-Seq and sci-RNA-seq allow multiplexing of cells and samples [66,67]. This removes the need to encapsulate single cells for processing, removing a costly and limiting step for single cell RNA sequencing. Importantly these techniques are compatible with fixed samples, preserving the expression state of cells. For 3D modelling applications the challenge then becomes isolating single cells from fixed systems. Methods have been described for single cell isolation from fixed organoids in Matrigel [68], but more complex 3D models with additional matrix components will prove more of a challenge.

Proteomics

Proteomic approaches allow the signalling state of cells to be interpreted by capturing the protein abundance and post-translational modifications within a cellular system. However, as with gene expression analysis, a challenge for heterocellular systems is separating the signalling state of individual cell types [69].

Strategies now exist to label the individual cell types of multicellular systems to allow their separation post proteomic analysis. These include cell type-specific labelling using amino acid precursors (CTAP), which involves the introduction of distinct lysine synthase enzymes into target cell types. Heterocellular cultures can then be fed with isotopically labelled lysine precursors that label the individual proteomes of target cells [70]. This approach has been used to unravel reciprocal signalling between pancreatic cancer cells and supporting stellate cells in 2D culture [71]. When adapting this technique for 3D multi-cellular systems care needs to be taken to ensure labelled lysine is not shared by cell types, e.g. via gap junctions. Further, as this technique relies on labelled lysine to differentiate cellular proteomes, lysine poor proteins are unable to be analysed. Proteome coverage is also affected by the use of one amino acid label, as opposed to traditional mass spectrometry labelling techniques such as stable isotope labelling by amino acids in cell culture (SILAC), which uses both labelled lysine and arginine.

An alternative method, stochastic orthogonal recoding of translation (SORT), involves introducing atypical aminoacyl/pyrrolysyl -tRNA synthetase/tRNA pairs into target cell types. These tRNAs can then incorporate modifiable amino acid analogues into the proteome of target cells, allowing subsequent deconvolution by mass spectrometry [72]. Using neuronal specific promoters to drive tRNA expression, this technique has been used to selectively label neurons in adult mouse brains, allowing neuronal proteomes to be enriched and analysed from whole brain lysates [73]. The development of additional tRNA synthetase/tRNA pairs to label multiple amino acids will increase the efficiency and coverage of this technique [74].

Mass cytometry offers a means to extract signalling, expression and positional information from individual cells in a multicellular system, using heavy-metal conjugated antibodies to evaluate numerous markers; many more than that afforded by conventional fluorochromes. Recently described with organoids, the combination of markers for both cell type and post-translational modifications of choice peptides revealed the signalling and cellular state of the individual cells that comprise an organoid, allowing cellular interplay to be interrogated in unprecedented detail [68]. Addition of either fibroblasts or macrophages demonstrated how this technique can be applied to interrogate feed forward and feedback mechanisms between cell types.

Concluding Remarks

3D modelling has allowed the roles of TME cells in cancer progression to be unmasked, revealing new biological insights and therapeutic opportunities. As research into the TME has expanded, so too has the repertoire of model systems available to study nuances in cell subtypes.

Many elements of 3D modelling, for example spheroid formation, are now technically undemanding, providing access for a growing number of researchers, and allowing the formation of increasingly complex, multi-cellular models. The range of model systems, cellular sources, and methods to interpret findings may appear daunting, but each has merits and the choice of model should be considered carefully to best fit the research aims. Explants provide researchers with functional units to study TME biology at the expense of tractability, while hydrogels allow control over gel composition but may create biomimetic structures that do not faithfully recapitulate the host physiology.

Ultimately 3D modelling is a balancing act. The ideal cell to use needs to be balanced with availability and compatibility with the model system. Equally the 3D system needs to reflect the biology under investigation, while facilitating the desired granularity of understanding. With improved access to

clinical tissue and better technologies to visualise cells *in situ* and unpick heterocellular signalling, the use of 3D models to understand the TME holds great promise for addressing translational research questions across biomedicine (see Outstanding Questions).

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BOX 1: Cells of the Tumour Microenvironment

Cancer Associated Fibroblasts

One of the most studied cell types of the TME is the cancer-associated fibroblast (CAF). These cells have central roles in directing tumour invasion and facilitating cancer cell survival and are the key cell type in regulating the tumour matrix. Heterogeneity among CAFs is increasingly apparent, adding additional complexity and nuance to their function [75].

Vasculature

Tumour angiogenesis is a key facilitator of tumour progression and metastasis. The abundant pro-angiogenic signals within the TME lead to the formation of immature leaky vessels characterised by excessive branching, poor pericyte coverage and aberrant morphology. In addition to supplying nutrients and oxygen, and removing waste, blood vessels are critical for delivering systemic therapies. They also provide the means for tumour cell dissemination and metastasis [76].

Immune Cells

Tumours contain a host of immune cells that drive progression, as well as suppress other elements of the immune system that would otherwise clear cancerous cells. The importance of immune cells in cancer progression is underscored by the success of immunotherapy, which makes the further dissection of cancer-immune cell crosstalk all the more important [77].

Adipocytes

The link between obesity and cancer is well established and has focused attention on the role of adipose tissue in cancer progression. Adipose cells are a key source of metabolites, lipids and adipokines that can feed cancer cells and promote their growth, invasion and resistance to therapy [78].

Myoepithelium

Myoepithelial cells are contractile epithelial cells that support the luminal cells of secretory tissues such as the prostate, mammary, lacrimal and salivary glands. Their significance in cancer is highlighted by their role in breast cancer, where an intact myoepithelial ring around cancerous luminal cells signifies the cancer is non-invasive, while their loss marks the progression to invasive disease [79]. However, the contribution of this cell type to cancer progression appears to be context dependent, with evidence for both a suppressive and driving role [80].

Neurons

Neuronal signalling is an important facet of the TME. Cancers can use established neurons as a pathway for dissemination, a process termed perineural invasion, and can co-opt neuronal signalling for their growth and survival. Cancers can even encourage the growth of new neurons into tumours, as well as influencing the phenotype of neurons to become pro-tumourigenic [81,82].

Figure Legends

Figure 1. The rich and varied tumour microenvironment. Schematic representation of the tumour microenvironments in breast and pancreatic cancer, highlighting their distinct features and cell types. Breast cancers contain tissue-specific ductal myoepithelial cells and have a large adipocyte component, while pancreatic tumours are hyopovascular, have a dense desmoplastic stroma and a predilection for perineuronal invasion.

Figure 2. Common 3D approaches to model the tumour microenvironment. Visual guide to common 3D modelling approaches. *Hydrogels:* multiple cell types are suspended together in a matrix where they can interact within a 3D environment. *Spheroids:* one or more cell types are formed together into spheroids that are then cultured in low attachment plates or embedded in matrix. *Organotypics:* cancer cells are cultivated on top of a matrix that is often infused with fibroblasts. *Organoids:* self-organising, multicellular cultures that mimic the host organ/tumour.

Explants: Excised tissues explants can be cultured *ex vivo* where they retain physiological behaviours: e.g. mouse aortae used to study angiogenic sprouting. *Microfluidics:* compartmentalised devices that allow cell behaviour to be examined under flow.

Figure 3. Sources of tumour microenvironment cells. Common sources of cells of the tumour microenvironment for use in 3D models. *Cell lines:* A number of TME cell types have immortalised cell line equivalents offering easy access to an endless supply of cells. *Induced pluripotent stem cells (iPSCs):* iPSCs allow researchers to generate hard to acquire TME cell types in a tractable system that permits genetic manipulation. EB - Embryoid Bodies. *Animal derived:* Animal models provide sources of tumour-educated TME cells and complex tissue explants for 3D modelling applications. *Patient/donor derived:* Biobanks and donors offer researchers access to *bona fide* sources of TME cells that harbour tumour associated physiological changes.

Figure 4. Deconvolution strategies for 3D model analysis. *Transcriptomics:* Using heterospecific cell types in the same model system allows separation and analysis post RNA sequencing by matching reads with host species. Single cell technologies can also be applied to 3D models. *Proteomics:* Amino acid labelling of multiple cell types, using techniques such as CTAP, allows specific proteomes to be labelled continually while in co-culture and facilitates subsequent separation by mass spectrometry. CyTOF can also be used to identify post translational modifications (PTM) at the single cell level using a combination of cell specific and PTM markers *Imaging:* 3D cultures can be antibody labelled and processed for whole mount imaging, which retains complete 3D architecture. Alternatively, models can be paraffin embedded and sectioned, allowing expression and spatial analysis by immunohistochemistry (IHC).

Fig 1

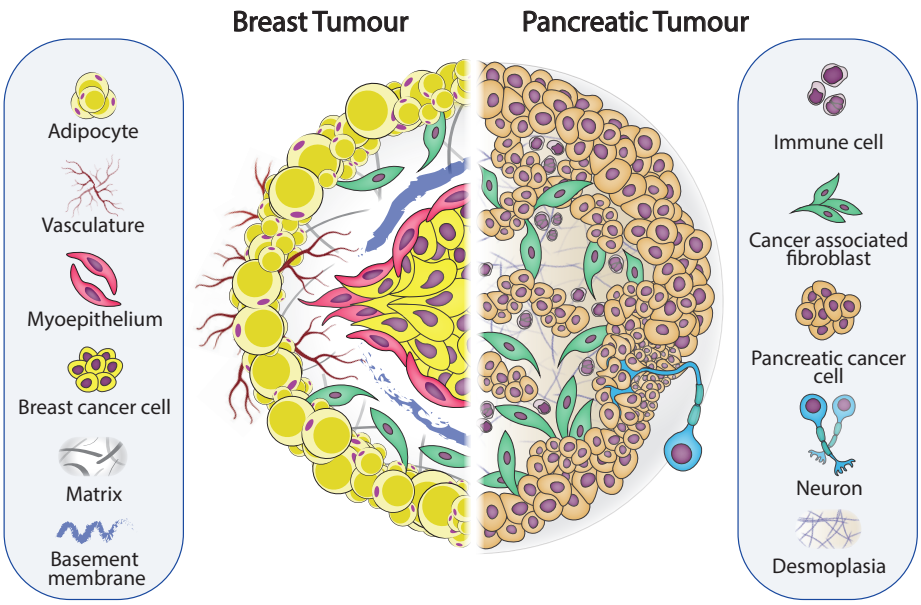

Breast Tumour
Pancreatic Tumour
Adipocyte
Vasculature
Myoepithelium
Breast cancer cell
Matrix
Basement membrane
Immune cell
Cancer associated fibroblast
Pancreatic cancer cell
Neuron
Desmoplasia

Fig 2

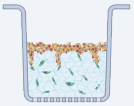
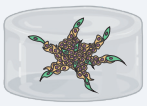
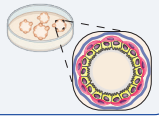
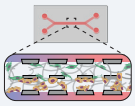
		Advantages	Disadvantages
Hydrogels 	Crosslinked polymer structures sharing similarity to native matrix in which cell types can be embedded	Additional matrix proteins can be incorporated Ability to tailor properties High throughput	Batch to batch variation Basic composition Cell structures may not recapitulate physiology
Organotypics 	Specialised hydrogel approach in which cancer cells are layered on top and submitted to a gradient	Compartmentalisation of different cell types Long term cellular crosstalk can be examined Ability to introduce gradients	Time-consuming Mostly restricted to end-point analysis
Spheroids 	Cellular aggregates consisting of one or more cell types which can be cultured in suspension or embedded within a hydrogel	Establishment of hypoxia/ nutrient gradients Spatially distinct modelling of cancer-stromal crosstalk Real time imaging possible	Dependent on cells' ability to form spheroids Limited to short term culture
Organoids 	Self-assembling multicellular structures which closely resemble organisation of host tissue	Model innate heterogeneity Can be embedded in hydrogels containing additional TME cells Reproducible	High maintenance Limited to specific cancer types
Explants 	Isolated tissue segments, retaining physiological characteristics, capable of being cultured *ex vivo* as a functional unit (e.g. aortic ring assay)	Native cell type interactions retained Close recapitulation of tumour-TME interactions	Challenging to manipulate genetically Access to tissue may be limited Technically demanding
Microfluidics 	Bespoke bioengineered chips facilitating the study of compartmentalised cell types under fluid flow	Recapitulates circulation Real time imaging possible Flexibility in design of cellular compartments	Expensive manufacturing process Highly specialised

Fig 3

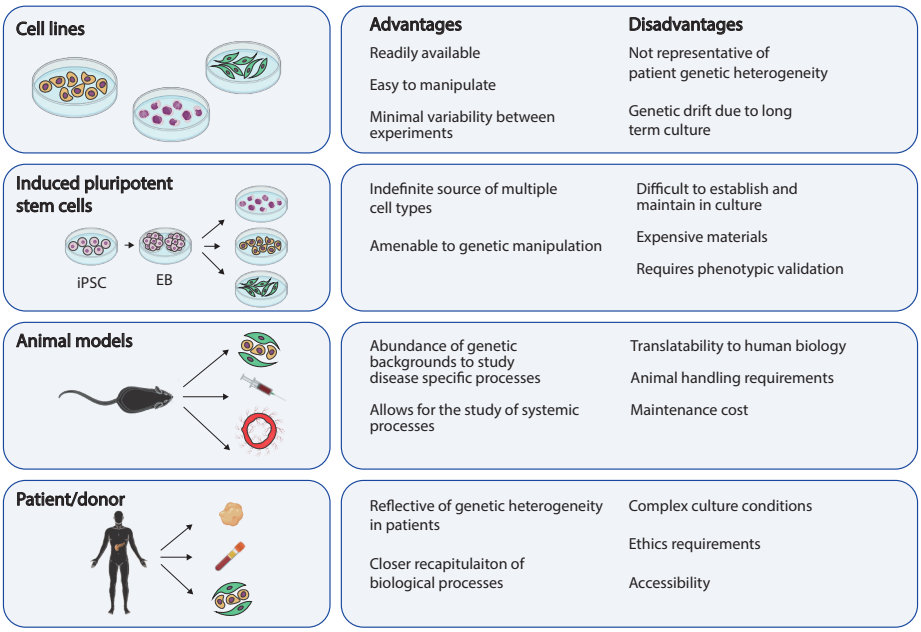
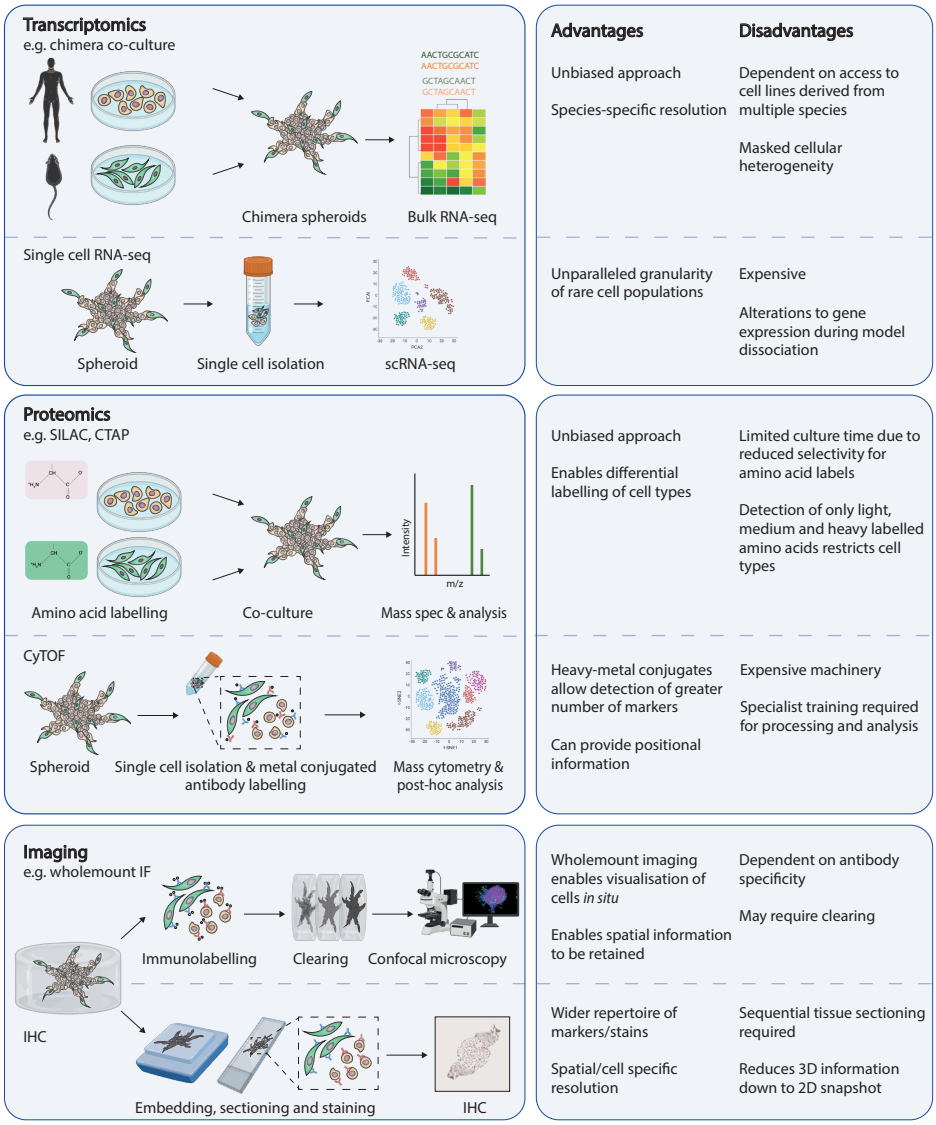

Cell lines
Advantages
Readily available
Easy to manipulate
Minimal variability between experiments
Disadvantages
Not representative of patient genetic heterogeneity
Genetic drift due to long term culture
Induced pluripotent stem cells
iPSC
EB
Indefinite source of multiple cell types
Amenable to genetic manipulation
Difficult to establish and maintain in culture
Expensive materials
Requires phenotypic validation
Animal models
Abundance of genetic backgrounds to study disease specific processes
Allows for the study of systemic processes
Translatability to human biology
Animal handling requirements
Maintenance cost
Patient/donor
Reflective of genetic heterogeneity in patients
Closer recapitulaiton of biological processes
Complex culture conditions
Ethics requirements
Accessibility

Fig 4



Advantages	Disadvantages
Unbiased approach	Dependent on access to cell lines derived from multiple species
Species-specific resolution	Masked cellular heterogeneity
Unparalleled granularity of rare cell populations	Expensive
	Alterations to gene expression during model dissociation
Unbiased approach	Limited culture time due to reduced selectivity for amino acid labels
Enables differential labelling of cell types	Detection of only light, medium and heavy labelled amino acids restricts cell types
Heavy-metal conjugates allow detection of greater number of markers	Expensive machinery
Can provide positional information	Specialist training required for processing and analysis
Wholemount imaging enables visualisation of cells *in situ*	Dependent on antibody specificity
Enables spatial information to be retained	May require clearing
Wider repertoire of markers/stains	Sequential tissue sectioning required
Spatial/cell specific resolution	Reduces 3D information down to 2D snapshot