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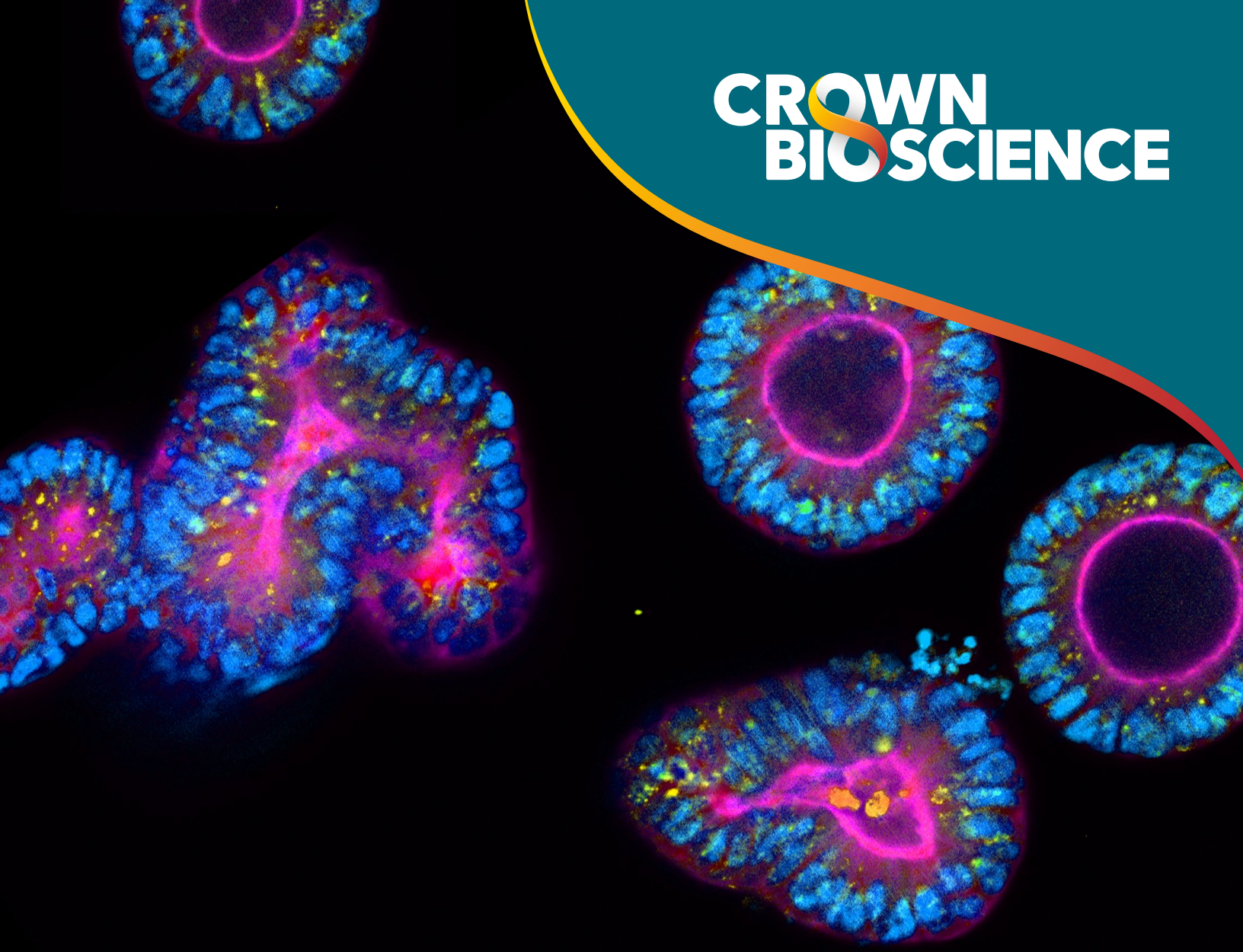

When and How to Use Tumor Organoids in Drug Discovery

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WHITEPAPER

V1.0

The Challenge: How and When to Best Implement Tumor Organoids into *In Vitro* Drug Discovery Programs

Drug attrition rates for anticancer agents are extremely high with only 5% of agents in preclinical development eventually becoming approved for clinical use. The generation and adoption of more clinically relevant preclinical models from the earliest stages of drug development could help reduce this high attrition rate by driving improved decision making throughout the drug discovery process. Using more clinically relevant models enables the identification and prioritization of agents that will have the best chances of succeeding in clinical trials.

In vitro 3D tumor organoids, developed using Hubrecht Organoid Technology (HUB) protocols, are playing an increasingly important role in drug discovery, particularly in identifying anticancer agents with improved translational potential. These tumor models faithfully recapitulate the phenotypic and genetic features of the original tumor, providing enhanced predictivity from the beginning of drug development.

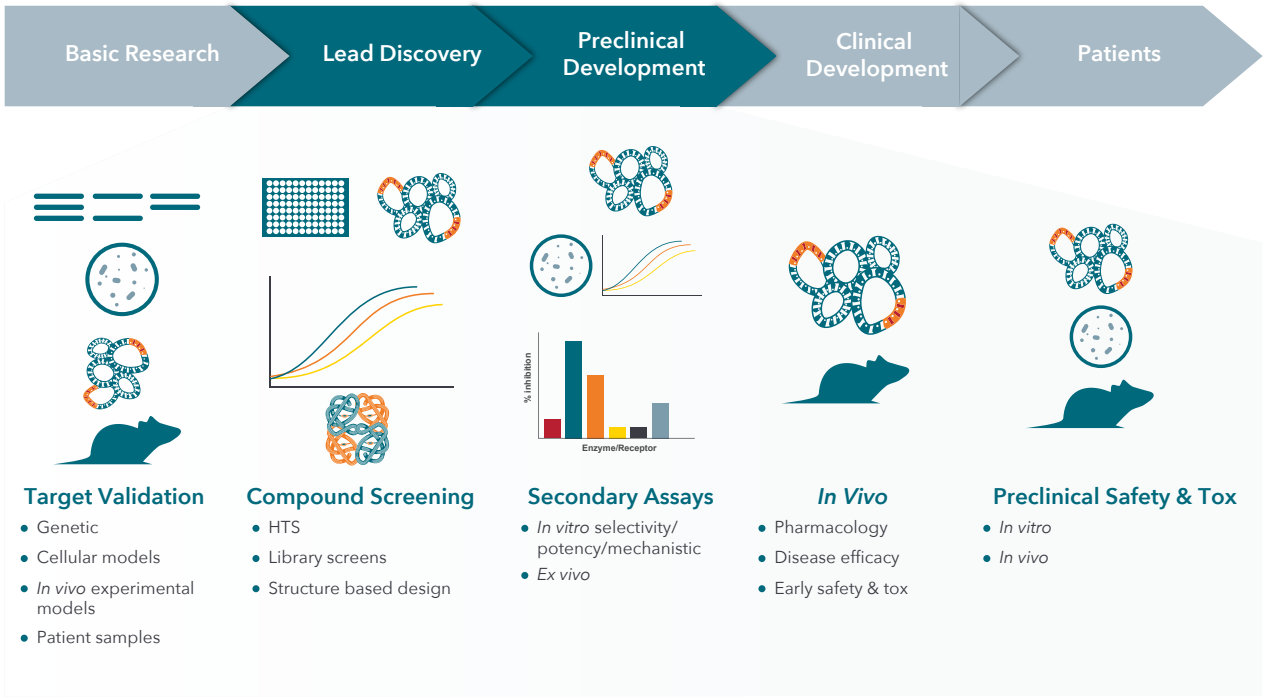
This White Paper highlights the benefits of tumor organoids, and how they compare to standard 2D and 3D *in vitro* culture systems. Optimal applications of tumor organoids are also introduced, to allow researchers to explore when and how to best implement these revolutionary tumor models alongside conventional platforms within drug development programs.

The Need to Develop Advanced Clinically Relevant *In Vitro* Models

Drug development is a lengthy, expensive, and typically linear process. For anticancer agents this process is currently highly inefficient, with 95% of agents that begin preclinical development failing to reach patients. This high attrition rate is shining a light on some failings within the current drug development process. One element is a lack of patient-relevant, clinically predictive models for early oncology drug discovery.

In vitro lead discovery and preclinical development is still heavily dominated by high-throughput screening (HTS) using 2D cell line cultures (Figure 1). These models are easily scalable, have extensive historical molecular and pharmacological characterization, and are readily commercially available. While they still have an important role in basic biology research, to understand disease pathways, investigate drug mechanisms of action, and test target engagement, 2D cell line cultures have inherent limitations.

Figure 1: Screening Tools for Drug Discovery



2D cell cultures which have been adapted to grow outside the natural tumor microenvironment (TME) display genetic changes inconsistent with the clinical *in vivo* setting, as well as having activated stress-response pathways. Immortalized cell lines grown *in vitro* and engrafted *in vivo* also show minimal resemblance to the tissue or tumor of origin and instead have a higher resemblance to each other[1].

More patient-relevant models are therefore needed to predict drug response and generate reliable efficacy data to enable improved decision making earlier in drug discovery.

Advantages of 3D Cultures Compared to 2D Cultures

3D cell cultures are increasingly being recognized as important tools bridging the gap between traditional *in vitro* and *in vivo* models, since they provide enhanced physiological relevance compared to 2D cell lines (Figure 2). A 3D environment creates more physiological cell-cell and cell-matrix interactions. Cells are also exposed to differential gradients of nutrient supply, oxygen, and environmental stressors. These complex interactions influence cell signaling, proliferation, differentiation, survival, as well as drug response.

Technological advances in cell culture are facilitating 3D model expansion for largescale drug screening applications, and to optimize the costs associated with their development and adoption, relative to *in vivo* models. 3D cell culture systems are often preferred to 2D platforms when a relatively smaller number of compounds are being screened and drug developers are seeking a more physiologically relevant model.

3D cell culture can be optimized for a variety of cell-based models including immortalized cell lines and more advanced, patient-derived models. This White Paper focuses on patient-derived primary cells and organoids, which offer more clinically relevant drug development systems.

Types of Patient-Derived *In Vitro* 3D Models

Primary Cells

Primary cells are obtained directly from patient tissue or tumors (or from mouse tissue) and can be grown in both a 2D or 3D format. Primary cells are not adapted to in vitro growth and more faithfully recapitulate the original tissue/tumor morphology and genetic background than immortalized cell lines.

3D cultures of primary cells from patient tumors offer a compelling model for investigating clinical response to anticancer agents thanks to their physiological relevance. However, these cell based models present multiple challenges including:

- The limited availability of patient tumor tissue for preclinical studies which restricts the possibility to perform repeat experiments. Biobanking of patient tumors represents an opportunity for further model development; however, these primary cell models lack the robustness required for *in vitro* growth and reproducibility can become an issue
- The inherent instability of primary cells *in vitro*, which undergo senescence when cultured on plastic making them suboptimal for expansion for large scale screening applications

Figure 2: Clinical Relevance of *In Vitro* Model Systems

Condition	Human Patient	3D Cultures	2D Cultures
Oxygen (hypoxia)	0-3%	<5%	21%
Stiffness	200-4,000 Pa	200-4,000 Pa	~$3x10^9$ Pa
pH	Acidic (<7)	Acidic (<7)	Buffered (pH 7.2)
Dimension	3D (site)	3D (site)	2D
Glucose	Limiting	Limiting	Not limiting
Immune cells	Full	Admix	None
Growth factors	Human	Human	Bovine
Tumor growth	Months/years	Weeks/months	Fast
TME complexity	Complex	Moderate (human)	Limited
Heterogeneity	Complex	Model specific	Limited

3D *ex vivo* assays have been established using primary tumor cells and successfully used for drug testing. For example, ex vivo patient tumor tissue slices have been shown to exhibit matched clinical response[2].

In order to overcome the lack of available patient tissue, patient tumors expanded in mice (i.e. patient-derived xenograft or PDX tumors) have been used in 3D *ex vivo* assays, and shown to reflect patient response[3,4]. Using PDX tumors for 3D cultures has the benefit of matched *in vivo* models, either in live passage or biobanked, that are used to replenish the culture and validate findings. Although a high number of models can be screened simultaneously using already established PDXs, the need for obtaining cells directly from PDX tumors (either fresh or frozen) limits the throughput of this model system.

Figure 3 illustrates a 3D PDX assay workflow and how it can be integrated with *in vivo* PDX findings. The utility of this approach was validated with non-small-cell lung cancer (NSCLC) models and the 3D matrix Cultrex®, a laminin-based basement membrane extract[3]. Tumor cells were cultured alone or co-cultured with stromal cells (CAF or bone-marrow-derived mesenchymal stem cells) to test whether stromal cells influence response to targeted agents, chemotherapeutics, or their combination. In some cases, such as treatment with the EGFR small molecule inhibitor erlotinib, an increased IC_{50} was observed in the presence of stromal cells which is consistent with the role of CAFs in promoting drug resistance (Figure 3).

In a slightly different approach, a second study used cryopreserved (banked) PDX material to overcome the need for *in vivo* systems in live passage. This strategy improved the throughput of the system, however, a correlation from *in vitro* data to the matched in vivo response was only observed for a subset of models, showing a less robust predictive power (Figure 4).

Figure 3: 3D PDX *In Vitro* Screen and *In Vivo* Corroboration

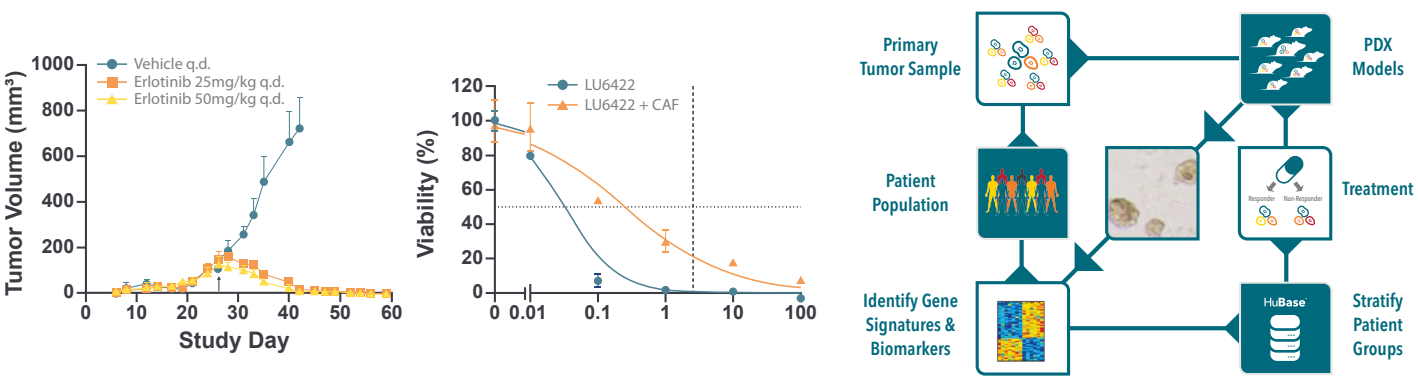
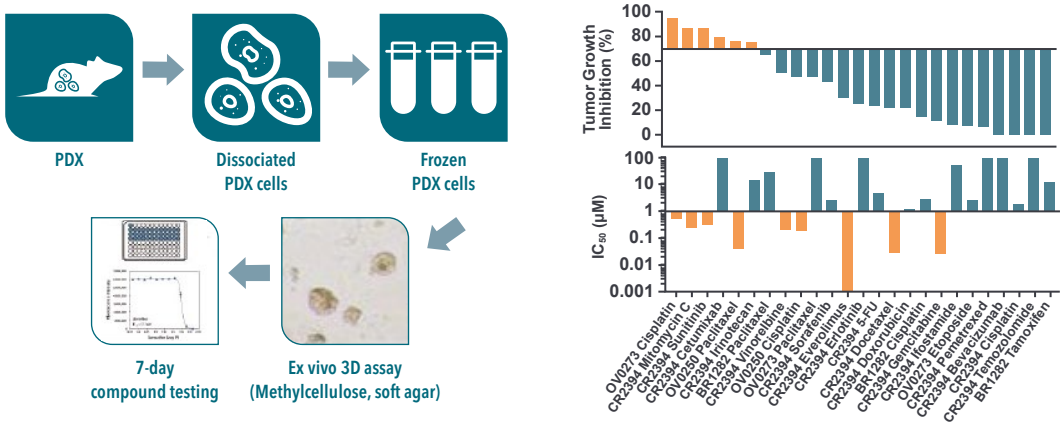


Figure 4: 3D Screening with Banked Frozen PDX Models



Organoids

Organoids represent the most promising recent development in 3D modeling, with improved predictivity of patient response and the most faithful recapitulation of original patient tissue and tumor morphology and pathophysiology.

Organoids are microscopic self-organizing 3D structures that are grown from embryonic or adult stem cells[5]. These "mini-organs" recapitulate the key structural and functional properties of the original organ *in vivo*. The technology for deriving organoids from patient tissue was based on seminal discoveries identifying adult stem cells on all epithelial organs, which was then further refined by Hubrecht Organoid Technology (HUB) for the development of tumor organoids. Organoids and tumor organoids provide an advanced 3D *in vitro* oncology system offering a wealth of benefits, which are summarized in Figure 5.

3D tumor organoid models are generated directly from patient tumors and matched healthy tissue (patient-derived organoids or PDOs) or from patient tumor tissue expanded in mice as PDXs (PDX-derived organoids or PDXOs). Both model types recapitulate original tissue morphology and molecular features and offer an efficient and clinically relevant alternative to primary cells.

PDOs and PDXOs both preserve the key features of their matched in vivo tumor tissue and can serve as patient avatars[6-8]. In addition, organoids can be leveraged to generate new PDX models for *in vivo* studies, offering unprecedented opportunities for preclinical drug development through paired *in vitro* and *in vivo* models. Organoid value has been further increased with the development of biobanks capturing patient population heterogeneity, supporting large-scale studies, and ensuring availability for repeat studies[9].

Key Elements for an Organoid-Based Drug Discovery Platform

Organoids offer the predictive power observed for other patient-derived models such as PDX combined with the superior scalability and robustness of well-established *in vitro* assays. To fully leverage these advanced preclinical models and provide significant benefits, organoid-based platforms for drug development need to meet a series of requirements which are shown in Figure 6.

Figure 5: The Benefits of Organoid Models for Drug Development

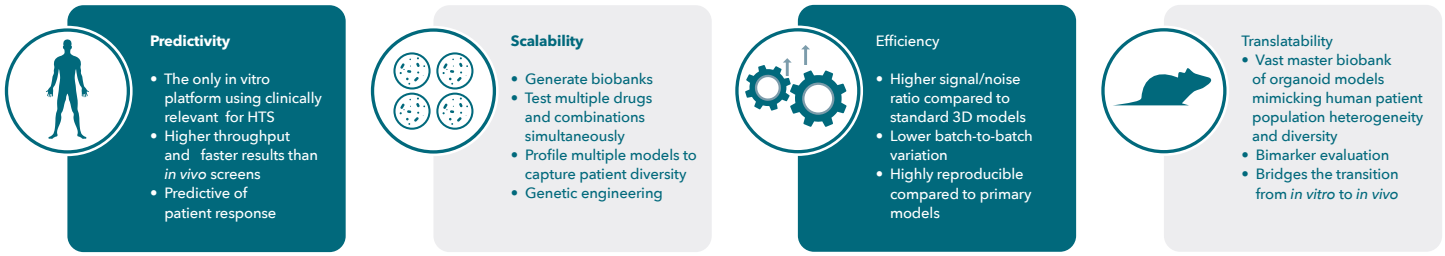
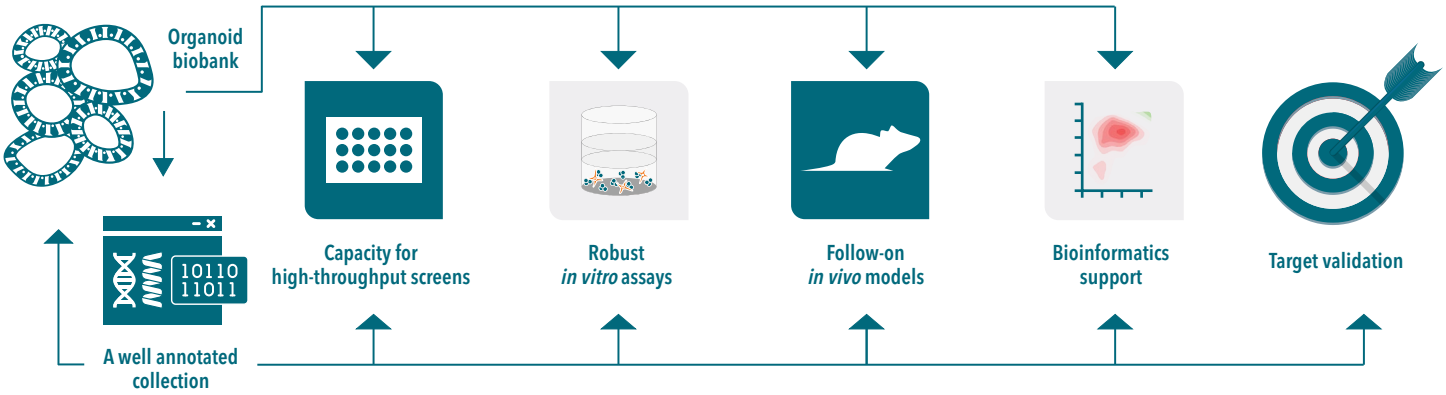


Figure 6: Elements of an Organoid-based Drug Discovery Platform



A Large Biobank of Models

To truly reflect the diversity of the patient population, a tumor organoid model collection should be extensive. The collection should include multiple models per tissue type as well as models from several cancer indications to cover a variety of mutational and pharmacological profiles. This provides the opportunity to assess response across numerous models simultaneously and to identify predictive biomarkers.

A Well-Annotated Collection

To be informative, organoid models need to be comprehensively annotated with genomic profiling data, which can be leveraged for gene signature and biomarker discovery for both new agents and repurposed drugs.

Robust Assay Platforms

An efficiently designed organoid-based screening platform must offer robust assays with well-established read outs. This includes cytotoxicity assays to detect organoid cell killing or validated assays to assess immuno-oncology agents. This provides confidence in the data generated, and allows identification of responder and non-responder models for the drug target.

Capacity to Perform High-Throughput Screens with Bioinformatics/*In Silico* Support

HTS capacity is needed for the rapid screening of large numbers of organoid models and/ or single agents and combination regimens. This capacity provides numerous repeats ("n") to enable powerful statistical analysis to be performed on the generated data. Bioinformatics capabilities are needed to process the information acquired via HTS.

Follow-On *In Vivo* Models

The availability of follow-on *in vivo* models is needed to either test a hypothesis or move to more complex systems. In an ideal scenario the *in vivo* models would be derived from the same patient as the organoid models; however, in principle this is a very rare occurrence. Alternatively, matched organoids and PDX models offer a unique opportunity to validate *in vitro* data in an *in vivo* system predictive of patient response. These matched systems allow biomarkers to be further validated, as some aspects of tumor response cannot be recapitulated *in vitro*.

Each of these applications is described in more detail in the following sections.

High-Throughput Screening using Tumor Organoids

In vitro organoids developed using HUB protocols are genetically and phenotypically stable and readily scaled up for HTS platforms, resulting in highly reproducible results compared with data obtained from primary models. In addition, when already established PDX tumors are used as the source of patient tumor tissue, matched *in vitro/ in vivo* models become available that provide a more informed transition from early *in vitro* studies to late-phase *in vivo* validation. The most common tumor organoid screening strategies are shown in Figure 7.

Alternative strategies are adopted or developed as needed, thanks to the flexibility of the organoid in vitro system. For example, panels of organoid models can be selected for a screen depending on the presence of mutations of interest in key human oncogenes, such as KRAS and/or EGFR. This strategy is useful to identify compounds that show efficacy in subgroups of patients harboring specific genetic alterations.

Organoid Engineering

An interesting feature of organoids that offers unique opportunities for model development is their ability to be engineered. Employing CRISPR, transduction, andother technologies greatly expands the utility of these models in drug discovery. For example, engineering technologies are being used to achieve the following:

- Generation of new in vitro organoid models e.g. isogeneic pairs, drug-resistant models, models with specific antigen expression, fluorescent labeling for co-cultures
- Generation of new xenografts from PDOs to create matched patient-relevant in vitro/in vivo systems
- Development of new in vivo models e.g. bioluminescent labeled orthotopic PDX for in life disease tracking

Tumor Organoids for Immuno-Oncology Applications

The co-culture of tumor organoids with human lymphocytes to test immunotherapies is an emerging area of interest in drug discovery. In vivo models, such as PDXs, are susceptible to graft-versus-host disease, which complicates their use and data interpretation.

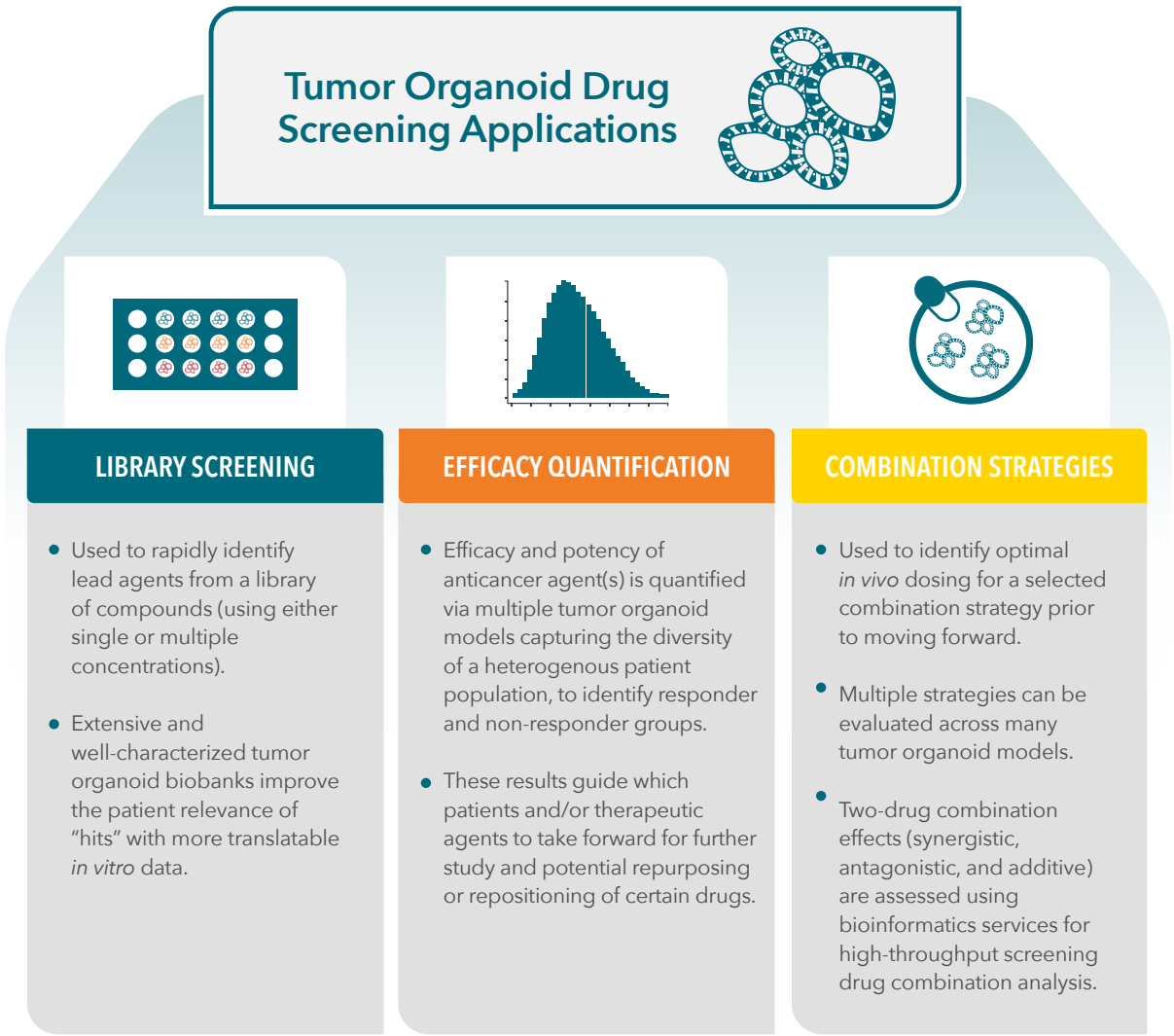
The feasibility of combining tumor organoids with immune cells in co-culture was demonstrated by the generation of a personalized medicine immuno-oncology platform where tumor organoids are co-cultured with autologous patient PBMC[10]. While it is an encouraging step forward, this approach has the following limitations for large-scale drug development:

- Limited volume of blood from cancer patient donors
- Lack of tumor reactive T cells among PBMC from most patients
- Co-culture techniques such as these are still at the research stage

To overcome some of these drawbacks, co-cultures of tumor organoids with nonautologous immune cells from healthy donors have been developed for a number of immuno-oncology applications such as:

- Evaluating the potency of immunotherapies using non-autologous allogenic T cell assays with optimized conditions
- Assessing tumor organoid killing by allogenic T cells and CAR-T cells, or to test ADCC and ADCP effects
- Evaluating tumor reactivity of CAR-T and TCR cells
- Profiling immunotherapy target gene expression on tumor organoids or identifying antigens of interest (e.g. tumor-associated antigens, immune checkpoint molecules)

Figure 7: Tumor Organoid Screening Strategies



Tumor organoids can be co-cultured with various immune cell types, including TILs, CAR-T, or specific TME components. T cell activation and tumor organoid killing is monitored via ELISA, FACS analysis, and morphology evaluation (Figure 8).

Organoids can also be engineered to express specific immune cell targets conjugated with a reporter such as luciferase to quantitatively evaluate the antitumor effects of immunotherapies. A proof of concept study co-culturing liver cancer organoids engineered to express luciferase labeled CD19 (solid tumors normally lack CD19) demonstrated the feasibility to detect specific killing of tumor organoids by CD19 CAR-T cells over 48 hours (Figure 9).

Finally, tumor organoid and immune cell co-cultures are also being utilized to assess ADCC effects on tumor organoid survival. For example, HER2+ ovarian cancer organoids co-cultured with an anti-HER2 antibody (Herceptin®) and PBMCs from a healthy donor resulted in significant tumor organoid cell killing by ADCC (Figure 10).

Figure 8: Applications of Tumor Organoids in Immunotherapy Evaluation

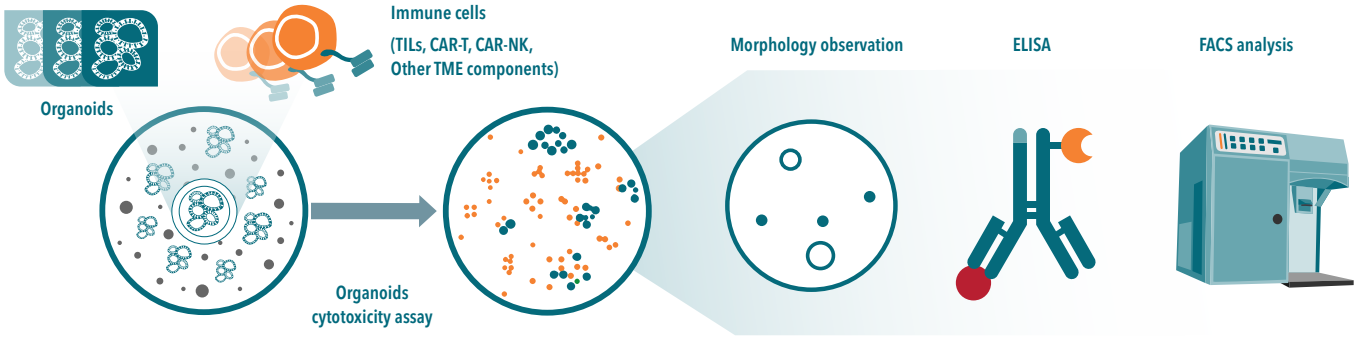


Figure 9: Co-Culture of Engineered Tumor Organoids with CD19 CAR-T Cells

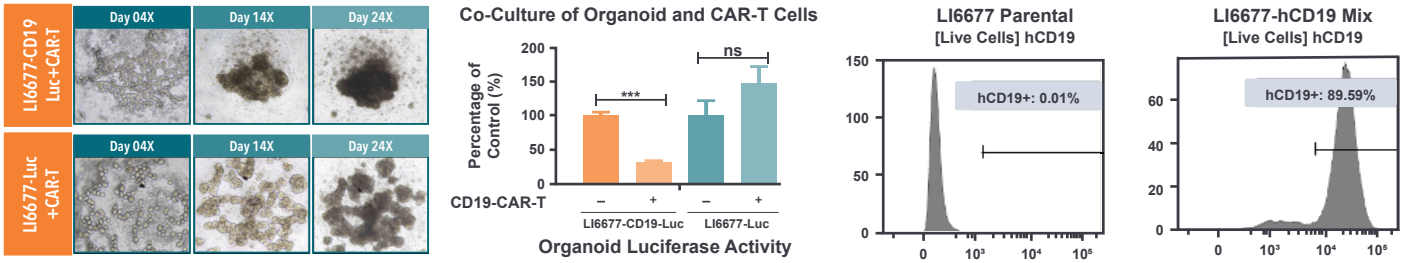
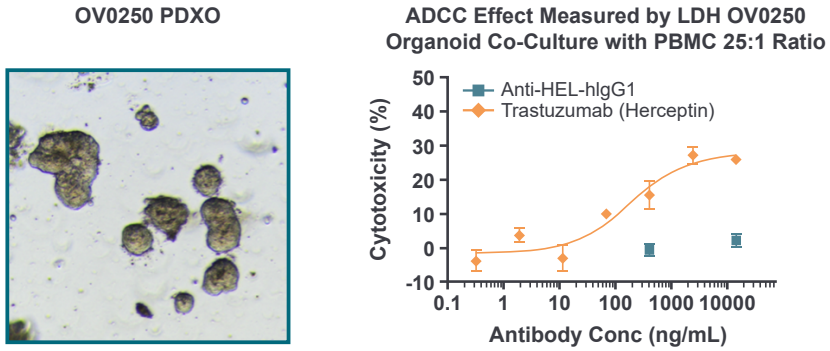


Figure 10: ADCC-Mediated Killing of Tumor Organoids



Organoids for In Vivo Model Selection and New Model Development

As described earlier, organoids preserve many of the features of the parental tumor including architecture, gene expression, mutation profiles, and drug response. Therefore, established and well-characterized collections of PDXs can be leveraged to generate new organoid models to cover more cancer indications, mutational landscapes, and pharmacological profiles than what is currently available (Figure 11).

Using organoids developed from libraries of already established PDX provides an opportunity for a unique matched *in vitro/in vivo* platform that ensures a more informed transition from early *in vitro* target and efficacy studies to late-stage validation trials *in vivo*. This approach results in superior data quality and faster results.

Conversely PDO or engineered organoids can be engrafted to generate new PDX models. Organoids maintain their tumorigenic potential when engrafted *in vivo*, allowing the development of new models from innovative engineered organoids, for example allowing the generation of bioluminescent orthotopic PDX models which have been previously hard to develop.

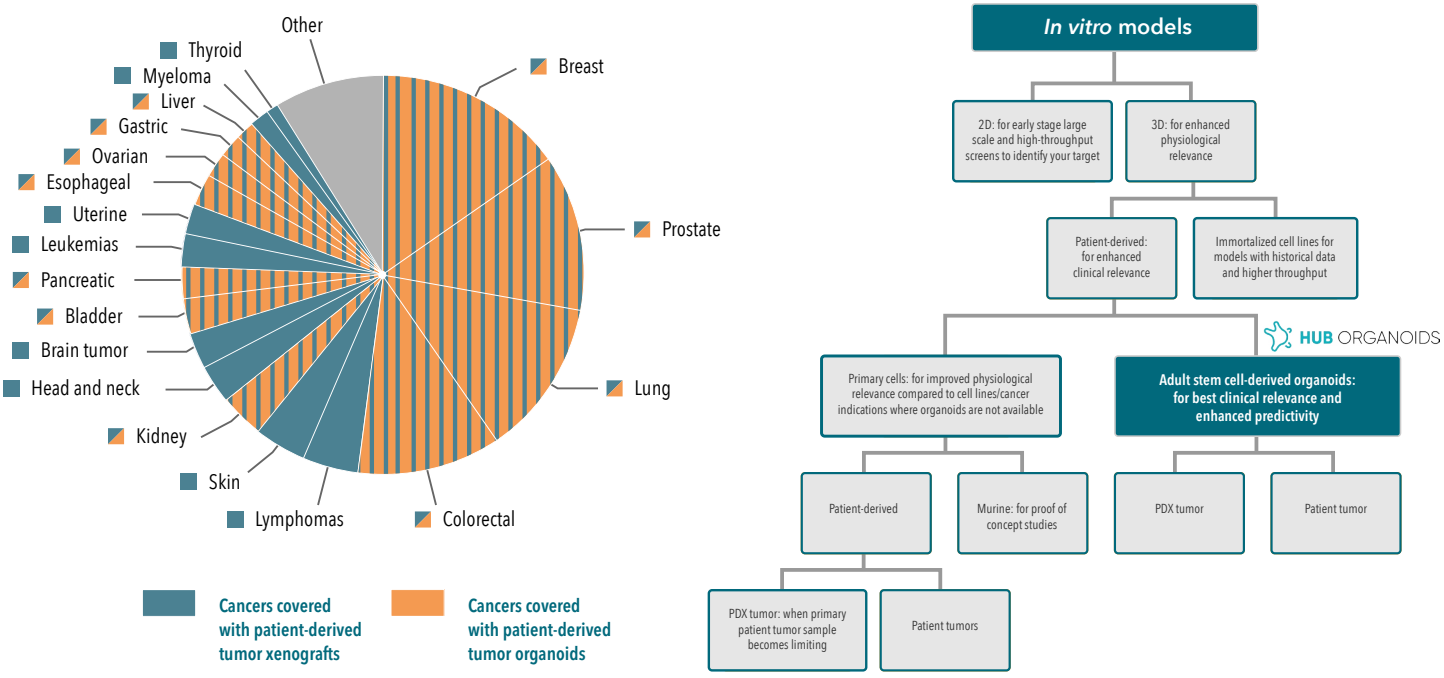
Summary

Many of the early decisions in drug discovery are made in the *in vitro* setting. Deciding when to use specific models in your *in vitro* drug discovery workflow depends on a variety of factors, and choosing the most appropriate model enables better decision making and improves downstream success rates.

Simple 2D cell monolayer and 3D primary cell cultures have their place in *in vitro* oncology drug discovery, based on the features and benefits discussed in this White Paper. Fully understanding when and how to implement tumor organoids alongside conventional platforms in your drug discovery programs allows these innovative and revolutionary models to be leveraged to their full potential.

Organoids provide superior patient-derived 3D *in vitro* models offering scalability and enhanced predictivity of *in vivo* response compared to other 3D in vitro systems. Optimal organoid oncology drug discovery applications include exploring multiple agents simultaneously in large scale screens or testing combination strategies more efficiently, leading to lower attrition rates, and facilitating the development of companion diagnostics.

Figure 11: Different Cancer Types that can be Grown as PDX and PDOs



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