Feasibility of manufacturing-scale bioproduction of novel next-generation 3D organoid cancer models in support of the Human Cancer Models Initiative

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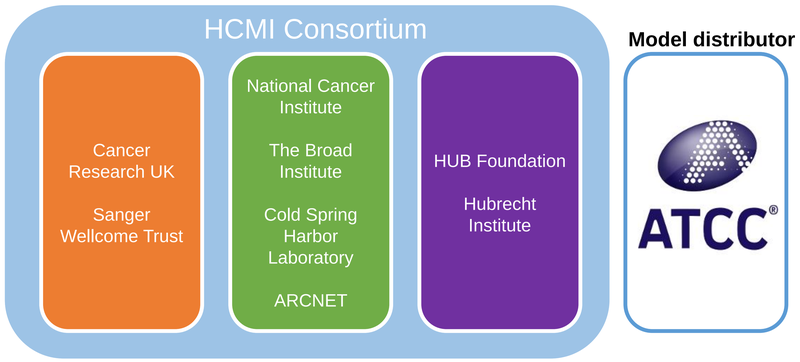
Abstract

To meet the need for improved approaches to study cancer *in vitro*, there has been a surge in the development of novel research models utilizing advanced culture methods. These methods permit *in vitro* growth of cancer types previously not possible, and/or models with enhanced *in vivo* relevancy compared to traditional continuous cell lines. However, availability of these early-stage research models is currently limited and there is a lack of data on the ability to scale up production of these models to support the needs of the global research community. We sought to investigate the protocols, expansion capacity, cryopreservation ability, genetic stability, and feasibility of larger-scale bioproduction of a subset of the models generated by the Human Cancer Models Initiative (HCMI), an international collaborative effort between Cancer Research UK, the foundation Hubrecht Organoid Technology, the National Cancer Institute, and the Wellcome Sanger Institute. The HCMI's initial goal is the development of 1,000 novel human cancer models, paired with bioinformatics and patient clinical data, particularly from rare or underrepresented cancer types. One advanced culture method being utilized, three-dimensional organoid "microtissue" culture, potentially poses challenges for traditional large-scale bioproduction processes. It requires growth embedded within an undefined extracellular matrix and complex media formulations containing multiple small molecules and recombinant proteins with unknown stability and shelf-life. Additionally, organoid growth media typically includes multiple sources of undefined conditioned media containing critical growth factors. We cultured organoid models derived from human colon, pancreas, esophagus, and mammary tissues developed by laboratories contributing to the HCMI. Multiple unique donors were available for all tissues and both cancer and non-cancer models were available for two tissue types. Most models were maintained in culture continuously for at least 60 days (7-27 population doublings, > 10 passages). Tissue and donor variability was evident in model characteristics, including morphology (assessed by microscopy and immunocytochemistry), growth rate, and genetic stability (measured by short tandem repeats analysis). All models were amenable to scale up beyond multiwell plates, and all models could recover from cryopreservation. While organoid culture represents a significant divergence from typical two-dimensional monolayer culture of continuous cell lines, our results show that these next-generation *in vitro* models are suitable for larger-scale bioproduction. This is vital to ensure the widespread availability of these models within the research community to facilitate applications like pre-clinical drug discovery and basic cancer research.

Human Cancer Models Initiative



Human Cancer Model Initiative goals

- Generate 1,000 new cancer models
- Utilize novel culture techniques that better mimic *in vivo* physiology
- Models derived from underrepresented tissues and genetic backgrounds
- Supported by patient clinical and genomic data

Pilot phase goals

- Generate 100 new cancer models
- Transfer to ATCC for expansion and distribution

Feasibility study goals

- Replicate and optimize protocols
- Assess potential for scale-up

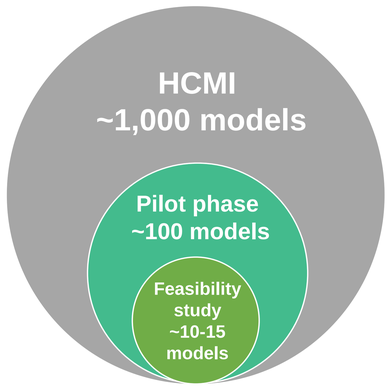


Figure 2. Estimated model counts per project phase

Results

Table 1. Feasibility study model information. Of the target 10-15 models for the feasibility study, 16 were received in time for inclusion in the pilot phase feasibility study, 14 were cultured, and 12 completed all testing. Models provided were intended to be a representative selection of the 100 models to be produced during the pilot phase of the HCMI project.

Total number of models	16 received, 14 cultured, 10 banked
Number of tissues	4 (colon, esophagus, pancreas, mammary)
Disease states	3 (tumor, normal, other)
Model providers	5

Protocols, Reagents and Formulations

7	6	2	14-16	3	+
Protocol variants	Media formulations	ECMs	Media components	Sources of conditioned media	Proprietary components

Figure 2. Diversity in protocols and formulations. All protocols described small scale culture in multiwell plates. Four of five CMDCs routinely cultured models with antibiotics. Media components were sourced from 10 individual suppliers. However, 10 of the components were present in all media formulations. All media formulations utilized at least one component that was a supplier-proprietary reagent.

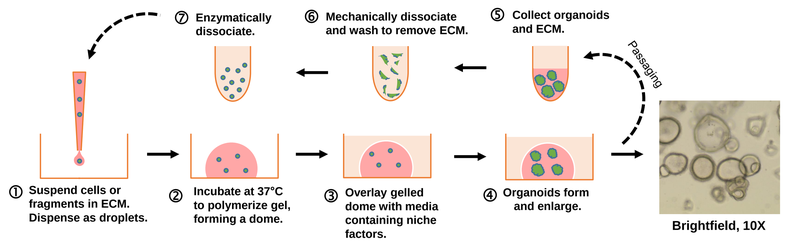


Figure 3. Standard single dome three dimensional organoid culture. Culture methods were broadly similar between protocols, utilizing a typical "embedded" three dimensional culture system overlaid with medium, though specific dissociation procedures, media formulations, and ECM varied.

Expansion Potential and yield

Table 2. Scale-up plan for organoid culture. CMDC protocols specified no seeding densities and suggested split ratios were 1:4 or less, if stated at all. We routinely seeded organoids at $5x10^4$/~10 **µL** ECM/cm^2, which was typically equivalent to split ratios of 1:6 or greater though it varied by model. We utilized a "multi-dome" approach where organoids were seeded in multiple small domes of ~10 µL within each vessel to maximize gas and nutrient diffusion.

Format	Surface area (cm^2)	Total ECM (µL)	No. of domes (~10 µL/each)	Seeding Density	Observed yield
6-well	9.5	100	8-10	$5x10^5$	0.8 – $3.2x10^6$
100 mm dish	55	600	40-60	$3x10^6$	5.5 – $16.8x10^6$
T150 flask	150	1800	120-180	$9x10^6$	30.8 – $51.5x10^6$

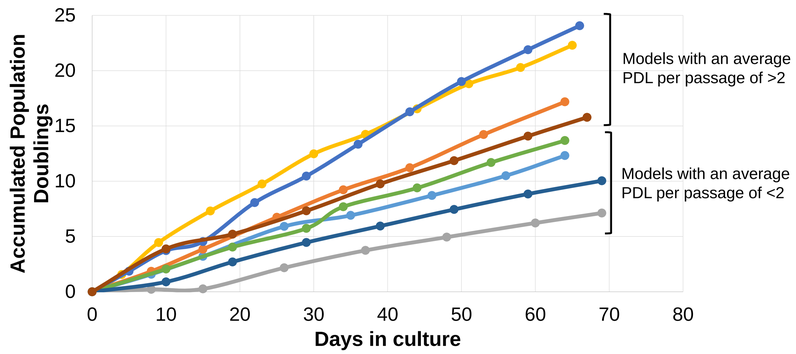


Figure 4. Model growth rates as measured by population doubling level (PDL). A subset of models (eight) were maintained in culture for >60 days (equivalent to 6-10 passages) to assess expansion potential. Average PDL ranged from 1.2 – 2.4 depending on the model. Models were considered "fast growers" if the average PDL per passage was greater than 2, and "slow growers" if less than 2. No models showed indications of senescence.

Model Morphology

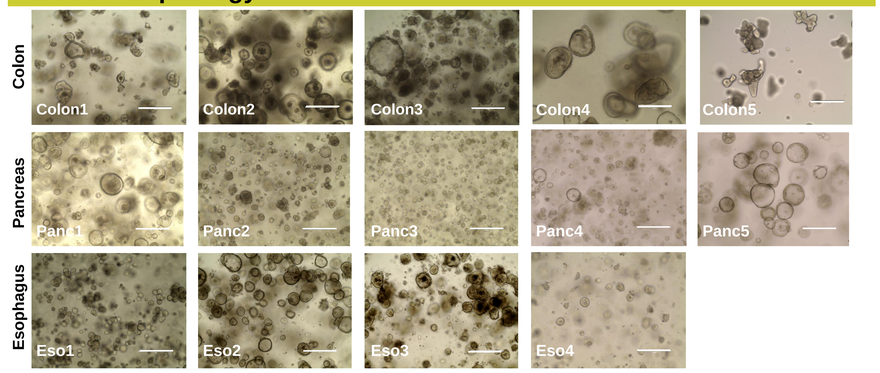


Figure 5. Brightfield photomicrographs displaying culture morphology seven to ten days post-passage. Models exhibited heterogeneous morphology between tissues, donors, and across the course of culture (not shown). Models could be broadly described as "cystic" (large balloon-like spheres), "solid" (small and lacking an obvious lumen), or "mixed" (exhibiting a combination of solid and cystic organoids). Images captured with 10X objective. Scale bars are 400 µM.

Immunocytochemistry

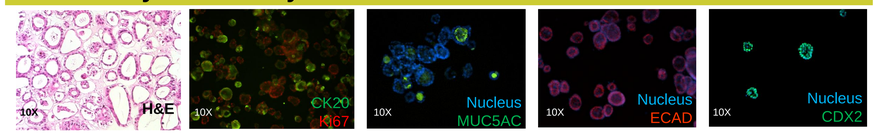


Figure 6. Immunostaining of colon models reveals tissue and organoid relevant protein expression. Organoids were fixed and paraffin embedded, and sectioned. Organoids showed expression of CK20 (cytokeratin-20; cytoskeletal), Ki67 (proliferation marker), MUC5AC (Mucin 5AC; goblet cell marker), ECAD (e-cadherin; epithelial marker), and CDX2 (caudal type homeobox 2; colon marker).

Genetic stability of individual models

Figure 7. Short tandem repeat (STR) analysis. STR profiling was performed after bank generation and compared with source or early passage material, assessing alleles at 18 loci. One of 10 models tested exhibited drift, a gain and loss of separate alleles. Overall this suggests that organoid models are largely stable over time in culture.

	Organoid Models				
Colon	1	2	3	4	5
Esophagus	1	2	3		
Pancreas	1	2			

STR MATCH PERCENTAGE: 100% | <100%

Viability during culture, cryopreservation, and recovery

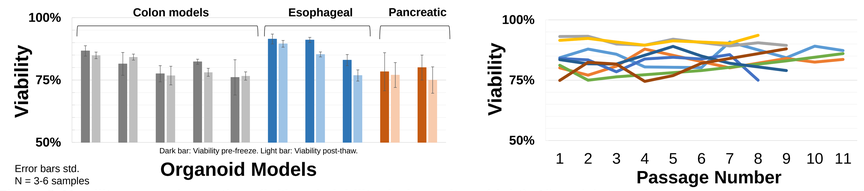


Figure 8. Viability pre- and post-thaw (left) and viability during culture (right). All models tested were successfully cryopreserved and recovered. No differences were seen between two commercially available cryomedia (data not shown). During routine culture viability was largely stable, ranging from 71% to 94% depending on the model. Across all models the average viability was 83%. Viability was assessed by trypan blue exclusion after enzymatic dissociation using an automated cell counting instrument.

Summary

- All models tested in this study were amenable to moderate scale up (generation of at least $100x10^6$ cells in <2 months), and our bank of cryopreserved organoids now totals approximately $1x10^9$ cells.
- We developed and demonstrated the feasibility of utilizing a "unified" organoid culture procedure to replace multiple disparate laboratory/tissue specific protocols. This standardization facilitated larger-scale bioproduction activities and will be published to support the research community in their use of organoid models.
- Remaining challenges for human cancer organoid culture include the need to prepare complex, model-specific culture media, and the requirement for multiple types of undefined material (conditioned media and ECM).

Acknowledgements

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