Comparison of Label Free Imaging Approaches and Destructive *In Vitro* Cell-based Assays for the Assessment of Growth and Viability of Primary Patient-derived Organoid Cancer Models

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Abstract

One major challenge in cancer biology is the lack of physiologically relevant models that have long term expansion potential *in vitro*, but still maintain the heterogeneity of the patient tumor. Recent advancements in *in vitro* 3D culture systems such as primary patient-derived organoids can meet this need by providing novel cancer models that better mimic the microenvironment of the originating tumor and exhibit a stable phenotype. A key feature of organoid culture involves embedding cells within a non-defined extracellular matrix that permits the cells to grow in three dimensions into large, complex structures with varying morphologies. However, these features can also make routine quantification of culture health and proliferation challenging. Unlike simpler 2D monolayer cell cultures, organoids do not proliferate as single cells, which can make cell counting and viability quantification approaches difficult. The extracellular matrix in which organoids are embedded may also require removal, which necessitates additional hands-on processing. Additionally, growth in 3D can interfere with simple visual assessment via brightfield or phase contrast imaging. Here we applied several common approaches for quantification of cell culture health and proliferation to primary patient-derived cancer organoids from multiple tissues, donors, and cancer types. Approaches including commercially available kits to quantify metabolism or ATP and the common trypan blue dye exclusion assay were utilized. The results were compared with label-free imaging approaches from multiple instrument platforms, which assess growth based on morphological features in brightfield or phase contrast images over time. Additionally, a small-scale toxicity assay was performed with various chemotherapy drugs to assess the discrimination ability of the assays. Results varied between models, donors, tissues and cancer types. All methods were able to capture long term changes in organoid proliferation, though all faced unique challenges, typically around sample preparation. Traditional *in vitro* assays designed for 2D monolayer cultures could be impeded by the presence of extracellular matrix and had difficulty in penetrating large, multicellular organoids. This resulted in lower signals or higher backgrounds, unless the samples were pre-processed. Imaging-based approaches required significant customization and optimization on a per-model basis. Overall, we found that to accurately assess the growth properties of such complex three-dimensional organoid cultures, significant optimization and validation may be required. Depending on the specific application, either imaging based or cell-based assay approaches may be suitable.

3D embedded organoid Culture

I. Culture involves embedding dissociated cells in extracellular matrix

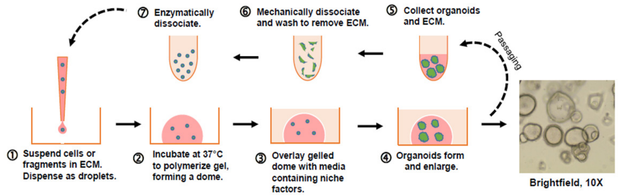


Figure 1. Organoid culture overview. Culture involves multiple dissociation steps and seeding cells in domes created from extracellular matrix.[1]

II. Morphological differences in organoid models

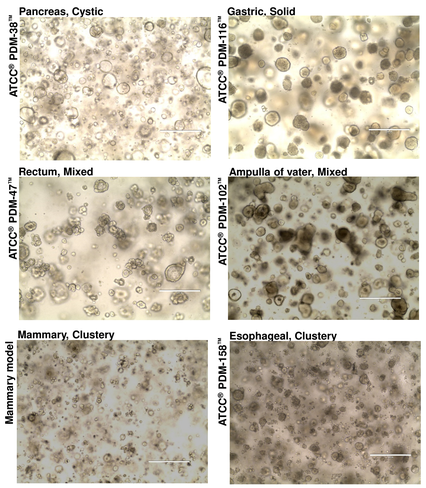


Figure 2. Morphological diversity between models derived from different tissue types

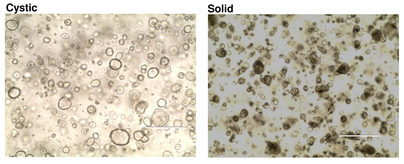


Figure 3. Morphological diversity between two different Gastric models

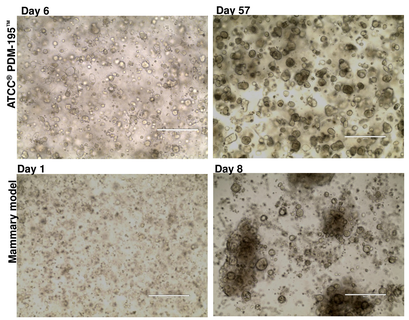


Figure 4. Morphological changes and rearrangement in two different Mammary models.

Assessing growth using live-cell brightfield imaging approach

Culture parameters: Models were generated according to standard protocols.[1] Organoids were seeded in 10 µL domes within Cell Basement Membrane Gel (ATCC® ACS-3035™). One dome was plated per well of a 12-well plate for ease of imaging the entire dome. Models were chosen based on differences in morphology and tissue types to assess whether a standardized analysis protocol could be utilized. Such a protocol could then be applied to generate similar growth profiles across models. Different cell densities were also used to see if density could have an impact on analysis.

Imaging parameters: A 4X phase contrast objective was used to capture brightfield images of the entire dome over time in culture. The center of the dome was defined in software and a 3 x 3 montage was collected from the center point. Z projection images were obtained from 5 optical slices above and below the focal point of the image. Optimal step size was determined automatically by the instrument software. Images were captured automatically by the instrument every eight hours over the course of 7 days. Cellular analysis was performed on montaged and projected images. Masked images show the perimeter of objects/organoids that were counted for analysis (left). Image quantification is shown in the graphs on the right including cell counts, organoid size, organoid area and organoid circularity.

ATCC® PDM-24™- Pancreatic organoid

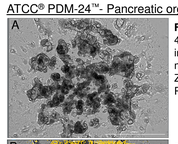


Figure 5A. 4X brightfield image (stitched montage and Z-projection) of PDM-24.

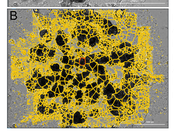


Figure 5B. 4X brightfield image (stitched montage and Z-projection) of PDM-24 with mask.

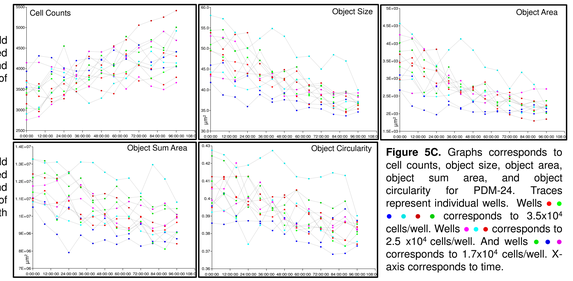


Figure 5C. Graphs corresponds to cell counts, object size, object area, object sum area, and object circularity for PDM-24. Traces represent individual wells. Wells ● ● ● ● ● ● corresponds to $3.5x10^4$ cells/well. Wells ● ● ● corresponds to $2.5\ x10^4$ cells/well. And wells ● ● ● corresponds to $1.7x10^4$ cells/well. X-axis corresponds to time.

ATCC® PDM-158™ - Esophageal organoid

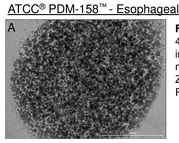


Figure 6A. 4X brightfield image (stitched montage and Z-projection) of PDM-158.

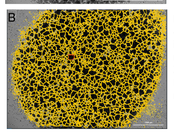


Figure 6B. 4X brightfield image (stitched montage and Z-projection) of PDM-158 with mask.

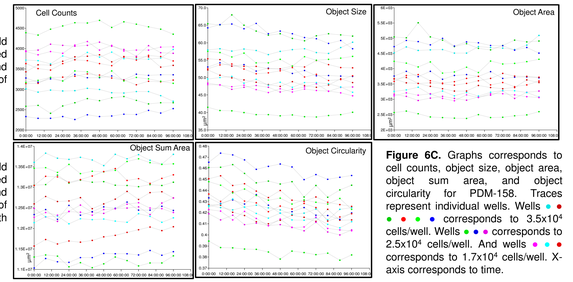


Figure 6C. Graphs corresponds to cell counts, object size, object area, object sum area, and object circularity for PDM-158. Traces represent individual wells. Wells ● ● ● ● ● ● corresponds to $3.5x10^4$ cells/well. Wells ● ● ● corresponds to $2.5x10^4$ cells/well. And wells ● ● ● corresponds to $1.7x10^4$ cells/well. X-axis corresponds to time.

ATCC® PDM 161™ Gastric organoid model

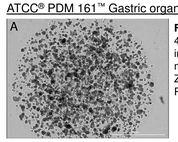


Figure 7A. 4X brightfield image (stitched montage and Z-projection) of PDM-161.

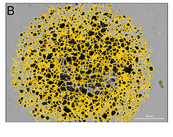


Figure 7B. 4X brightfield image (stitched montage and Z-projection) of PDM-161 with mask.

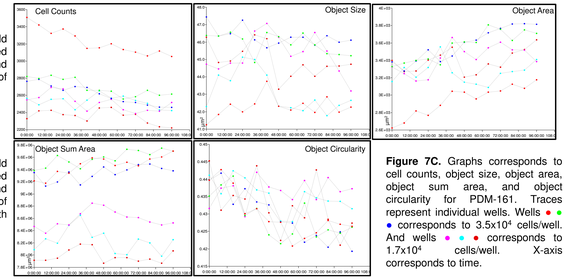


Figure 7C. Graphs corresponds to cell counts, object size, object area, object sum area, and object circularity for PDM-161. Traces represent individual wells. Wells ● ● ● corresponds to $3.5x10^4$ cells/well. And wells ● ● ● corresponds to $1.7x10^4$ cells/well. X-axis corresponds to time.

ATCC® PDM-185™ - Colon organoid

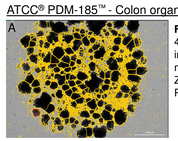


Figure 8A. 4X brightfield image (stitched montage and Z-projection) of PDM-185.

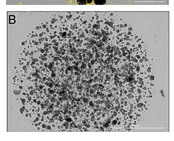


Figure 8B. 4X brightfield image (stitched montage and Z-projection) of PDM-185 with mask.

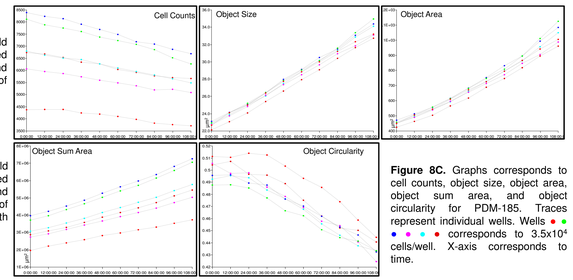


Figure 8C. Graphs corresponds to cell counts, object size, object area, object sum area, and object circularity for PDM-185. Traces represent individual wells. Wells ● ● ● ● ● ● corresponds to $3.5x10^4$ cells/well. X-axis corresponds to time.

Results, summary, and challenges

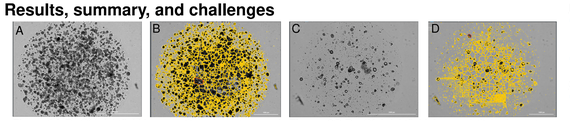


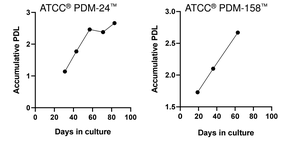
Figure 9A-B. 4X brightfield image (stitched montage and Z-projection) of PDM-185 at $3.5x10^4$ cells/well without (A) and with (B) mask applied.

Figure 9C-D. 4X brightfield image (stitched montage and Z-projection) of PDM-185 at < $1.7x10^4$ cells/well without (C) and with (D) mask applied.

Results:
- Cell counts, object size, object area, object sum area, and object circularity all varied across different models (Figures 5-8).
- Wells plated at different densities showed similar trends within the same model (Figures 5-8).

Challenges encountered:
- Growth protocol generated using the instrument software had trouble delustering and counting fused organoids (Figure 9A-B).
- The protocol had difficulty counting sparse cultures where empty spaces were counted (Figure 9C-D).
- Model to model variability was observed.

Assessing growth using trypan blue exclusion approach



Sample processing and counting: Approximately 1mL aliquot was taken from the original pooled cell suspension. Cell suspension was manually dissociated by pipetting in order to remove any residual Cell Basement Membrane Gel. After this the cells were enzymatically dissociated for 7-12 minutes to single cells. Samples were counted using a Beckman Coulter Vi-Cell XR Cell Counter at default settings.

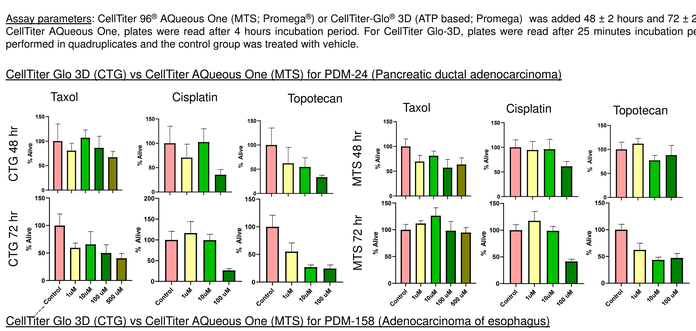
Results:
- Population doublings (PDL) showed an upward trend. However, preprocessing of cells is required in order to obtain cell counts.
- Additionally, this approach did not allow growth monitoring in real time.

Effects of chemotherapy drugs on 3D organoids

Culture parameters: The bottom of each well of 96 well plates were coated with ATCC Cell Basement Membrane Gel (ATCC® ACS-3035™). Organoids were manually dissociated to remove residual extracellular matrix. No enzymatic dissociation was performed. Whole intact organoids were then seeded at $6x10^4$-$7x10^4$ cells/well. Approximately 24 hours post seeding, drug treatments were added to the culture at the indicated final concentration. Cell viability/proliferation was measured at 48 hours and 72 hours post drug treatment. These models were chosen based on differences in morphology and tissue type.

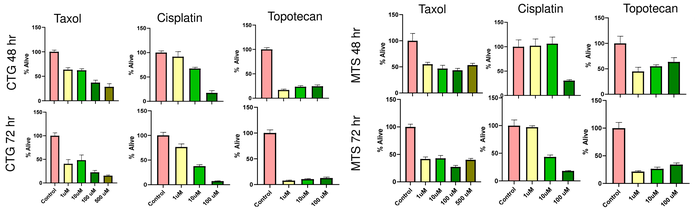
Assay parameters: CellTiter 96® AQueous One (MTS; Promega®) or CellTiter-Glo® 3D (ATP based; Promega) was added 48 ± 2 hours and 72 ± 2 hours post drug treatment. For CellTiter AQueous One, plates were read after 4 hours incubation period. For CellTiter Glo-3D, plates were read after 25 minutes incubation period. Each drug treatment was performed in quadruplicates and the control group was treated with vehicle.

CellTiter Glo 3D (CTG) vs CellTiter AQueous One (MTS) for PDM-24 (Pancreatic ductal adenocarcinoma)



Results:
- Values are plotted as percent values normalized to the control group.
- PDM-24 was less sensitivity to taxol but showed typical response based on dosage for cisplatin and topotecan.
- PDM-158 showed typical dosage sensitive response to taxol and cisplatin. The response for topotecan followed a reverse trend.
- Both CTG and MTS showed similar results for each of the models, however CTG showed a slightly improved signal.

CellTiter Glo 3D (CTG) vs CellTiter AQueous One (MTS) for PDM-158 (Adenocarcinoma of esophagus)



Summary

- Assessment of proliferation was challenging using live-cell brightfield imaging as the analysis parameters showed inconsistencies across model types. The need for model-specific optimization is critical.
- Assessment of proliferation using trypan blue exclusion was able to consistently generate similar population doubling profiles across different model types; however, this approach does not allow for monitoring growth in real time.
- PDM-24 and PDM-158 showed differences in sensitivities to the various chemotherapeutic drugs.
- Differences in response could be multifactorial, including but not limited to cellular, morphological, and epidemiological differences between the models.
- ATP based CTG assay gave cleaner signal compared to formazan-based MTS assay.

Acknowledgements & References

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Clinton J, McWilliams-Koeppen P. (2019) Initiation, expansion, and cryopreservation of human primary tissue-derived normal and diseased organoids in embedded three-dimensional culture. *Current Protocols in Cell Biology* 82, e66. doi: 10.1002/cpcb.66

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