

# Organoid Analysis Guide

Simplifying Progress

**SARTORIUS**

# Introduction

Organoids are rapidly becoming a powerful tool for both basic research and drug discovery, spanning a wide range of biomedical applications including oncology, regenerative medicine, disease modeling, and drug screening. These three-dimensional (3D) organotypical structures can be grown *in vitro* to produce miniaturized versions of the organs from which they were derived. The starting point for development of organoid cultures can be tissue-specific, resident adult stem cells, cancer stem cells derived from patient biopsies, or pluripotent stem cells, either embryonic or induced. When established with 3D extracellular matrices, the cultures can recapitulate the *in vivo* architecture, spatial organization, and genetic diversity of the cell populations found in the original organ with remarkable fidelity.<sup>1</sup> As self-organizing and self-renewing 3D structures, organoids offer a distinct advantage over traditional monolayer culture techniques and offer a more physiologically relevant milieu in which to understand complex biology with greater clarity.

In this eBook, we explore some of the translational applications of organoids, highlight key considerations for successful 3D culture and expansion *in vitro* and describe leading-edge technology for the imaging and objective analysis of these complex, self-organizing tissues.

# Translational Applications

The versatility of organoids, including their ability to be cultured from both healthy and diseased cells and tissues has unlocked a remarkable breadth of research and medical applications. While many of these applications are still in the early stages of development and use, the potential to reveal mechanisms of disease with greater precision and detail and enable more personalized approaches to disease treatment continues to accelerate progress in the field. Undoubtedly, human organoids will provide new opportunities for the study of disease and offer an important complement to existing cell line and animal models. Several recent review articles provide an in-depth look at the development and use of organoid-based disease model systems.<sup>1, 3, 4, 5, 6</sup>

## Hereditary Disease Modeling

Because organoids retain the genetic signatures of the tissue from which they are derived, they are well-suited for modeling genetic diseases such as cystic fibrosis (CF), the first human disease to be modeled using organoids.<sup>7</sup> CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene which encodes a chloride channel protein involved in the regulation of adsorption and secretion of salt and water. The mutations affect epithelial cells in a variety of organs including the lungs and intestines, leading to a build-up of viscous, sticky mucous and infections in the lungs and chronic digestive system problems. Organoids derived from CF patients have continued to evolve and are now used to predict which drugs are most effective against different mutations<sup>8</sup> and to monitor drug levels in the blood of patients as an alternative to conventional pharmacokinetic testing.<sup>9</sup>

## What's in a Name?

An organoid should satisfy several criteria<sup>2</sup>

- Possess a 3D structure containing cells that establish or retain the identity of the organ from which they were derived
- Include the presence of multiple cell types, as in the organ itself
- Exhibit some aspect(s) of the specialized function of the organ
- Display self-organization according to the same intrinsic organizing principles as in the organ itself

Organoids have also been developed as models for other genetic diseases including alpha 1-antitrypsin (A1AT) deficiency<sup>10</sup>, primary microcephaly<sup>11</sup> and Leber congenital amaurosis<sup>12</sup> which affects the retina and leads to blindness. These examples demonstrate the valuable contribution that organoids offer to the discovery and development of new therapeutics by enabling creation of patient-derived, physiologically relevant models of disease.

## Host-Pathogen Interactions

Organoids are also being used to model infectious diseases affecting humans. A major advantage offered by organoids for the exploration of host-pathogen interactions is they can contain virtually all of the cell types present in the organ from which they were derived. As a result, they can closely recapitulate the *in vivo* environment with which a pathogen interacts and causes damage. Organoids have been used to model Salmonella infections in gallbladder<sup>13</sup>, to study cholera toxin inhibitors in the intestine<sup>14</sup> and Helicobacter pylori infection in the stomach.<sup>15</sup>

Forebrain-specific organoids derived from human iPSCs have been used to model the microcephaly associated with ZIKA virus infection during pregnancy. The organoids recapitulate key features of cortical development and ZIKA infection of the organoids leads to growth inhibition and increased apoptosis, implying that the virus alters neurogenesis during brain development which is causative to microcephaly.<sup>16</sup>

## Oncology

Cancer-derived organoids are providing researchers and drug developers with a powerful new tool to both better understand the complexities of this disease and develop more effective treatments. Organoids can be efficiently generated from tumor tissue, are relatively easy to propagate and represent the transcriptional and mutational profile of the original tumor.<sup>17</sup> They also overcome the challenges presented by cancer cell lines which accumulate additional mutations over time and animal models which do not fully reflect the genetic characteristics of human cancers. Tumor organoids also address key shortcomings of patient-derived tumor xenografting (PDX). While PDX more faithfully recapitulates the original tumor, the process is labor-intensive, time-consuming, expensive and not amenable to high-throughput screening.

The potential of patient-derived tumor organoids spans several applications.<sup>18</sup> Organoids from a diverse set of cancer types can be used to create a biobank and then combined with extensive genome sequencing, expression profiling, drug sensitivity and patient clinical data. Access to such an unprecedented repository of information will certainly aid and accelerate research and the search for new therapeutic candidates by facilitating large scale screening efforts. These models can also be used to compare *in vitro* and *in vivo* treatment response; because organoids can be cultured indefinitely, *in vitro* dosing regimens can be varied so that the outcome aligns with that observed in the clinic. Ultimately,

this knowledge can help determine whether standard of care treatment responses of organoids are able to predict the response of patients to treatment. Finally, patient-derived tumor organoids can be used to select specific treatment; this ability will be critical as a growing array of molecular targeted agents are becoming available.

## Regenerative Medicine

Given the ability of organoids to include the cellular lineages present in the organ from which they are derived, they have the potential to serve as an unlimited source for replacing damaged tissues. This approach has been demonstrated using mouse colon organoids expanded *in vitro* from a single stem cell. When the organoids are transplanted into mice with damaged colons, functional crypt units were restored, and epithelial barrier function was fully recovered.<sup>19</sup>

Restoration of function may also be possible by combining organoid technology with gene therapy. Transplantation of organoids in which genetic mutations have been repaired using gene editing technologies could potentially be exploited to treat patients with the disease. As a proof of concept for gene correction in patients with a single-gene hereditary defect, CRISPR/Cas9 gene editing was used to correct a CFTR mutation in small intestinal and rectal organoids from two CF patients; the corrected allele was shown to be fully functional.<sup>20</sup>

# Organoid Culture and Analysis

As self-organizing and self-renewing structures, organoids have a distinct advantage over traditional monolayer culture techniques and hold unprecedented potential for many applications as described above. Adding to their value as a basic and translational research tool is the fact that traditional cell culture techniques and technologies can be used to initiate and expand *in vitro* cultures and maintain *in vivo*-like characteristics, tissue-specific functions and disease-state phenotypes. An informational guide to general organoid culture conditions is available from the American Type Culture Collection (ATCC) and provides guidance on initiation, expansion and cryopreservation.<sup>21</sup> An extensive set of resources and methods is also available on the StemCell Technologies website.<sup>22</sup> In addition, several review articles have compiled comprehensive lists of protocols developed for tissue-specific stem cell- and patient tumor-derived organoids.<sup>1, 5, 23</sup>

While protocols that define culture conditions, seeding densities, matrix recommendations and other critical parameters have been defined for organoids from many types of normal and diseased tissue, quantitative analysis of these differentiated micro-tissues has been challenging. Currently, characterization and optimization of organoid cultures are limited in their ability to objectively monitor these 3D structures as they form and grow over time. Specific shortcomings include:

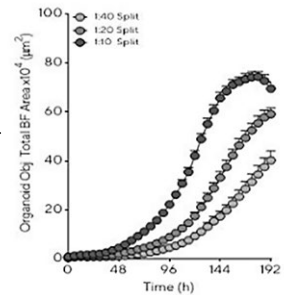
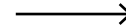
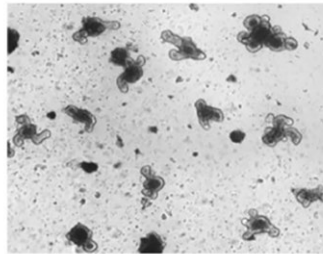
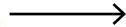
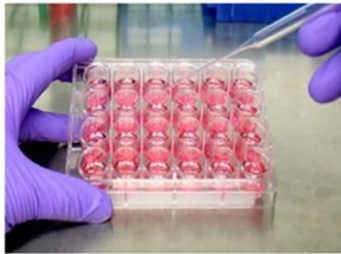
- A lack of validated protocols for reproducible well-to-well organoid formation
- Low data throughput
- Time-consuming and manual processes for acquisition of organoid images
- The need for third party analysis software, with limited quantitative information
- Loss of environmental control during image acquisition which may introduce variability in assay results

While organoids may be established using familiar cell culture tools and technologies, their complexity has driven the need for sophisticated, quantitative analytical methods. The need for an unbiased, quantitative understanding of the dynamic intercellular relationships within organoids has been described as “*essential to fully comprehend the complexity that can be modeled with these systems.*”<sup>24</sup>

# Real-Time Live-Cell Analysis of Organoid Cultures

Quantitative, label-free *in vitro* approach enabled by the Incucyte® Live-Cell Analysis System and organoid analysis software are becoming a requisite for the study of organoids in foundational research, disease modeling and drug screening. This powerful end-

to-end platform offers the ability to kinetically visualize and quantify the formation and growth of organoids cultured in Matrigel® domes (Figure 1).



## 1. Prepare assay plate

Pipette Matrigel® containing organoid cells or fragments into the center of the well of a 24-well or 48-well plate to form a dome.

## 2. Acquire images

Capture images over time in a physiologically relevant environment.

## 3. Analyze organoid

Organoid size, count, and morphology can be assessed using brightfield imaging and segmentation enabling label-free analysis.

Figure 1: Established protocols and Incucyte® analysis tools enable unbiased quantitative characterization of organoids and optimization of culture conditions.

Proprietary brightfield (BF) image acquisition enables real-time kinetic imaging of 3D organoids with size, count and morphology measurements automatically plotted over time, providing quantitative data on differentiation and maturation characteristics.

Figure 2 summarizes the organoid culture quality control workflow. Detailed protocols for the following experiments are available in the Sartorius application note entitled Real-Time Live-Cell Analysis of 3D Organoid Growth in Matrigel® Domes.<sup>25</sup>

1. Harvest and resuspend organoids in Matrigel®



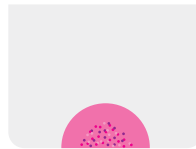
Harvest and resuspend organoid cells or fragments in Matrigel® (50-100%).

2. Pipette Matrigel® in plate



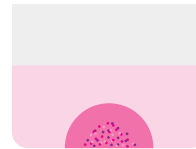
Pipette Matrigel® containing organoid fragments in the center of a 24- or 48-well plate (30-50 µL or 10 µL, respectively).

3. Polymerize Matrigel®



Incubate plate at 37° C for 10-15 mins to polymerize Matrigel®, forming a dome.

4. Add media



Overlay polymerized dome with recommended culture media.

5. Monitor organoid formation and growth



Place plate in Incucyte® to monitor organoid formation and growth.

Figure 2: Incucyte® lab tested protocol for culturing organoids in Matrigel® domes. Protocol allows easy culture, expansion and maintenance of organoid cultures for representation of organ-specific physiology.<sup>26</sup>



## Monitoring and Quantifying Organoid Growth

Figure 3 demonstrates the ability of the Incucyte® Organoid Software Analysis Module to visualize individual organoids embedded throughout the Matrigel® dome. The top row shows a single, in-focus BF 2D image for the three different types of organoids, six days post seeding; the middle row shows enlargements of the boxes in the top row, revealing cell type-specific morphological features. Time-course plots (bottom row) showing individual well total BF area ( $\mu\text{m}^2$ ) over time, demonstrating cell-type specific organoid growth. Mature hepatic and pancreatic organoids showed similar size and rapid growth while the intestinal organoids appeared smaller and exhibited a distinct budding phenotype as they mature.

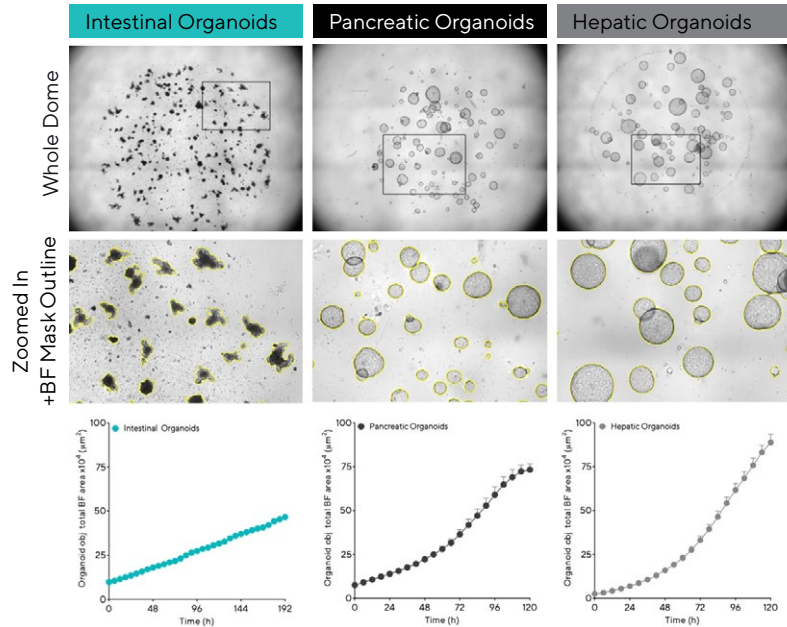


Figure 3: Acquisition and quantification of organoid growth in Matrigel® domes. Mouse intestinal (1:3 split, 50% Matrigel®), pancreatic (1:5 split, 100% Matrigel®) and hepatic organoids (1:40 split, 100% Matrigel®) were embedded in Matrigel® domes in 24-well plates and imaged every 6 h in an Incucyte® system. All images captured at 4X magnification. Each data point represents mean  $\pm$  SEM, n = 4 wells.

## Optimizing Organoid Culture Conditions

Defining optimal culture conditions is essential for establishing healthy organoids for use in downstream studies. Characterization of organoid formation using real-time kinetic measurements on the Incucyte® platform provides the ability to objectively assess the impact of growth conditions and seeding densities and define the conditions necessary to maximize expansion.

Figure 4 summarizes a study used to determine optimal conditions for maximal expansion of mouse hepatic organoids seeded at multiple densities in Matrigel® domes. Organoid size and count were automatically analyzed by the Incucyte® software. BF images taken five days post seeding and time-course plots demonstrated that organoid size and growth rate was directly proportional to cell number. Organoids seeded at the highest density appeared larger ( $> 500 \mu\text{m}$  diameter) and exhibited rapid growth reaching maximal size within 120 hours (BF images and total area time-course, respectively). At lower densities, the organoid maturation phase was extended, and the greatest growth potential (size) was observed (average area time-course).

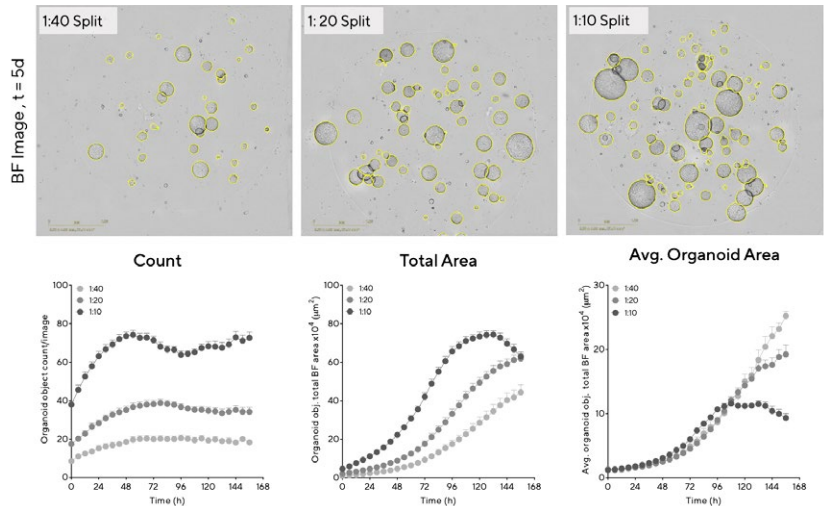


Figure 4: Determine optimal conditions for maximal organoid expansion. Mouse hepatic organoids were embedded in Matrigel® domes (100%) in 48-well plates at multiple seeding densities. BF images were taken five days post seeding. Data were collected over 168 h at 6 h intervals. All images captured at 4X magnification. Each data point represents mean  $\pm$  SEM,  $n = 14$  wells.

## Defining the Optimal Organoid Maturation Phase

Morphology metrics are used to determine the appropriate passaging frequency for organoid cultures. These metrics are cell-type specific and include indicators of maturation such as budding and accumulation of debris within the organoid lumen. Real-time tracking of changes in organoid eccentricity (object roundness) and darkness (object brightness) with the Incucyte® platform enables rapid, unbiased assessment of optimal culture passage periods.

To define the optimal passaging frequency for mouse hepatic organoids embedded in Matrigel® domes, cultures were imaged using the Incucyte® platform for eight days (Figure 5). Hepatic organoids are typically ready for passaging when the majority of

organoids have reached their maximum growth and have not collapsed.<sup>27</sup> Representative BF images show that two days post seeding, cultures were not ready for passaging as the majority of organoids were less than 100 µm in diameter and had clear lumens. A decline in eccentricity was observed within 48 hours as organoids formed and became more rounded (time-course data; far right panel). The optimal period for passaging this culture occurred between days four and five, when most organoids within the dome had reached maximal size, exhibited a rounded morphology and had not collapsed. The image and time-coursed data from day six indicated that the organoids which had reached maximal size had collapsed and darkened.

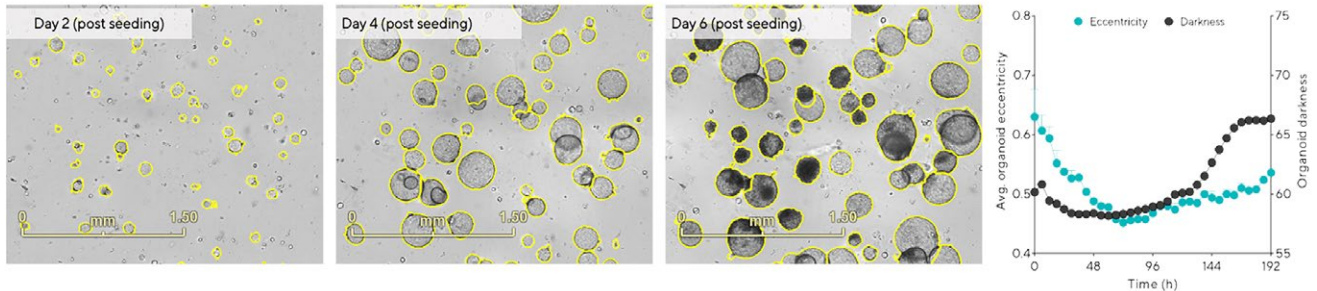


Figure 5: Define cell-type specific passage frequency using integrated morphology metrics. Hepatic organoids were embedded in 100% Matrigel® domes (1:10 split) in 24-well plates. Data were collected over 192 h at 6 h intervals. All images captured at 4X magnification. Each data point represents mean ± SEM, n = 6 wells.

## Tracking Organoid Differentiation and Growth Efficiency

Under routine culture conditions, organoid morphology and growth capabilities are expected to remain consistent across multiple passages. Monitoring of key parameters using qualitative approaches to confirm this, however, can be time- and labor-intensive and introduce bias.

In contrast, the Incucyte® platform can be used to kinetically and objectively quantify multiple parameters to assess organoid expansion and growth efficiency during extended passaging (Figure 6). When maintained at a consistent density, intestinal organoids exhibited comparable count, area, eccentricity, and darkness measurements over time. In addition, representative BF images at Day 7 post seeding confirmed the maintenance of a distinct budding phenotype. These results demonstrate the ability of this imaging and analysis approach to support robust and reproducible assessment of long-term organoid expansion.

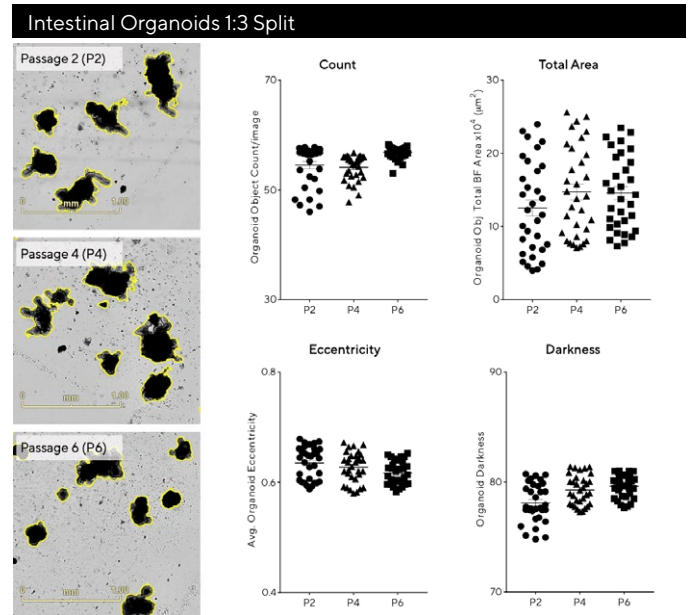


Figure 6: Assess growth and differentiation efficiency of organoids across multiple passages. Intestinal organoids were embedded in 50% Matrigel® domes (1:3 split, 24-well plate) over multiple passages and evaluated for growth and differentiation consistency over time. Data were collected over 192 h at 6 h intervals. All images captured at 4X magnification. Each data point represents mean  $\pm$  SEM, n = 6 wells.

# Unlocking the Potential of Organoids

The ability to establish organoids from human progenitor cells has ushered in a new era in both fundamental and translational research. As self-organizing tissues, organoids can recapitulate organ architecture, offering a more physiologically relevant *in vitro* model of normal and disease processes. While detailed protocols exist for establishing organoid cultures for dozens of tissues and cancers, technological advancements are essential to enable rapid, accurate and unbiased imaging and analyses. Neither qualitative assessments nor techniques used with 2D monolayer cell cultures offer the ability to confidently determine the health and quality of these complex 3D tissue structures—which can impact the accuracy and reproducibility of downstream assays.

The Incucyte® Live-Cell Analysis system, in combination with the Incucyte® Organoid Analysis Software Module, facilitates kinetic, qualitative and unbiased evaluation of organoid formation and growth. This breakthrough technology for organoid imaging and objective analysis provides important advantages including the ability to:

- Automatically analyze 3D organoids embedded within Matrigel® domes
- Use integrated, real-time label-free metrics to optimize and define culture conditions and regimes

- Determine the optimal periods for passaging or extension of organoid cultures based on integrated morphological parameters
- Assess culture quality during extended passaging

Undoubtedly, human organoid systems will continue to improve and provide unprecedented opportunities to better understand complex disease mechanisms. The ability to objectively define parameters such as seeding density, passage frequency and morphology will be critical to establishing healthy cultures for use in downstream studies and unlocking the full potential of organoids.

The ultimate goal of *in vitro* 3D cell culture is to enable more physiologically relevant downstream analyses of cells. Through comprehensive characterization, scientists can develop a deeper understanding of biology, ensure more robust maintenance of stem cells during extended cultures, and monitor cells at every step of the 3D organoid workflow. A key element in controlling adverse variables is to standardize workflows (including organoid culture QC and assay steps) and metrics, thereby eliminating human subjectivity and interpretation.

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## North America


Essen BioScience Inc.  
300 West Morgan Road  
Ann Arbor, Michigan, 48108  
USA  
Phone +1 734 769 1600  
Email [orders.US07@sartorius.com](mailto:orders.US07@sartorius.com)

## Europe

Essen BioScience Ltd.  
Units 2 & 3 The Quadrant  
Newark Close  
Royston Hertfordshire  
SG8 5HL  
United Kingdom  
Phone +44 1763 227400  
Email [euorders.UK03@sartorius.com](mailto:euorders.UK03@sartorius.com)

## Asia Pacific

Sartorius Japan K.K.  
4th Floor Daiwa Shinagawa North Bldg.  
1-8-11 Kita-Shinagawa  
Shinagawa-ku, Tokyo  
140-0001  
Japan  
Phone: +81 3 6478 5202  
Email: [orders.US07@sartorius.com](mailto:orders.US07@sartorius.com)

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visit [www.sartorius.com](http://www.sartorius.com)