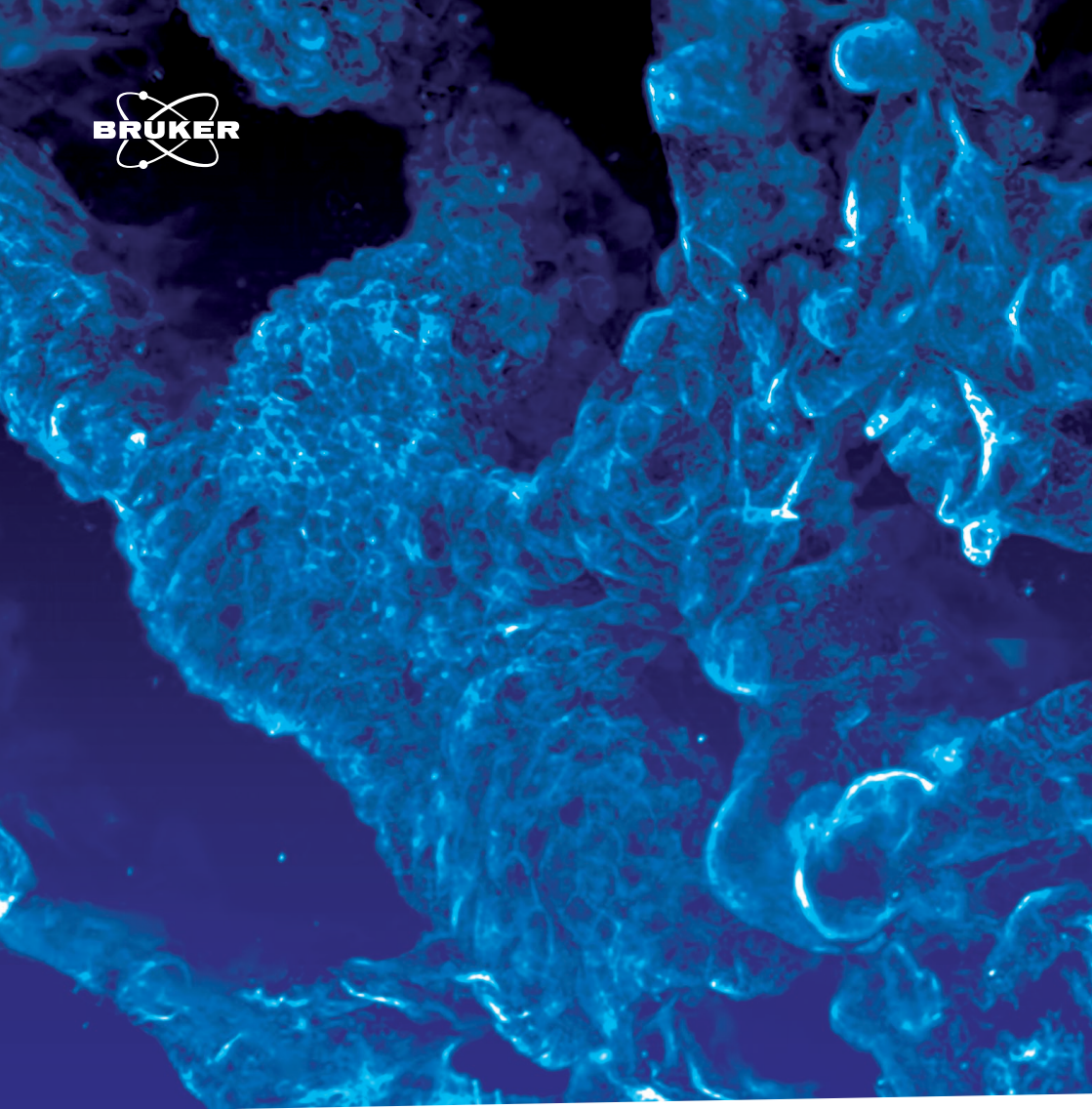




FLUORESCENCE MICROSCOPY

Imaging 3D Cell Cultures

Modeling biology in 3D cell cultures across scales with automated widefield and light-sheet microscopy.

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Introduction to 3D Cell Cultures

Cell cultures are an essential tool in life sciences for studying biological processes in a controlled environment and as an alternative to animal models. In recent years, studying cells in 3D rather than 2D has become increasingly important. This typically happens with a gel-like matrix or scaffold that mimics the extracellular matrix and microenvironment found in tissues.¹ In addition to the matrix, additional cues such as cell-type specific ones (e.g., cell-specific needs, cell-to-cell interaction, cell-to-matrix interaction), physical factors (e.g., stiffness), and soluble factors (e.g., growth factors) impact 3D cell cultures.

In 2023, “methods for modeling development” was named the Nature Method of the Year.² This award came following advances in our understanding of human blastocytes and the “black box” of development, alongside other studies that successfully recreated key embryonic states in vitro.^{3,4}

3D cell culture is a general term for growing cells in a three-dimensional environment. Spheroids are simpler 3D cell aggregates, while organoids and assemblies are more complex. The choice of approach depends on the research objectives and the complexity level needed for experiments. All of the above are used in laboratory settings, and their origin ranges from primary cell cultures from different organisms to tumors or stem cells and depends on the 3D cell culture technique. The establishment of stem cell and organ models is ongoing, establishing an “organoid zoo” that allows comparative studies.⁵ Several different forms of cell cultures will be discussed in this eBook, including:

3D cell culture

This is a generic term referring to cultivating cells in a 3D environment that closely resembles natural tissues or organ architecture.

Spheroids

These are 3D cell aggregates that form when cells self-assemble into spherical structures. Different cell types can be used, but need culturing in a non-adhesive environment. Spheroids can be generated through various techniques, lack a defined tissue architecture, and often have a random cell distribution.

Organoids

Organoids are 3D cell cultures that closely mimic the characteristics of specific organs or tissues and exhibit a high level of cellular organization. Organoids are more complex than spheroids and exhibit tissue-specific organization, including the differentiation of different cell types and physiological functions.

Organoids can also be fused to generate a larger complex, such as by merging gut organoids that fuse to an elongated tubular lumen, which resembles the gut organization more closely.⁶ Organoids are generally derived in either of two ways, which are from embryonic stem cells (ES cells) and induced pluripotent stem cells (iPSC).¹

Assembloids

More complex 3D models created by fusing two or more cell types or organoids, e.g., kidney and blood vessels, cortical and striatal organoids, or adding endothelial and smooth muscle cells are referred to as assembloids. The aim is to study interactions between various tissues and specific cell types, as well as to overcome challenges such as lacking organoid vascularization.

A Closer Look at Organoids

Organoids can be established from pluripotent stem cells (PSCs), including embryonic and induced pluripotent stem cells, or from adult stem cells (ASCs). Both types require an extracellular matrix to act as a basal lamina for cell growth and are compatible with standard lab techniques like CRISPR/Cas-9.⁷ However, PSC- and ASC-derived organoids have distinct differences, making them complementary models.

PSC-derived organoids replicate embryonic development, requiring specific growth factor cocktails over two to three months, and are ideal for studying organ development and genetic diseases. These organoids represent all three germ layers: endoderm, mesoderm, and ectoderm.

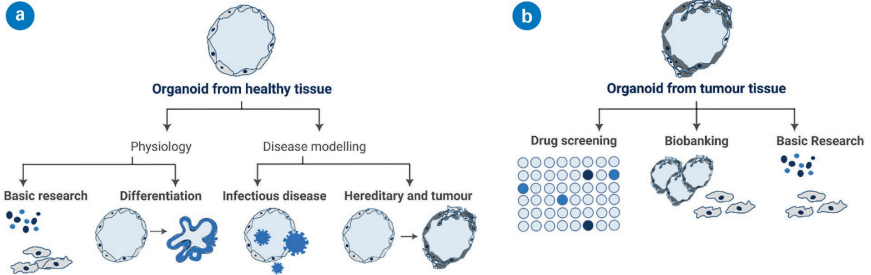
In contrast, ASC-derived organoids model adult tissue repair, only forming from tissues with regenerative capacity. They focus on epithelial structures, lacking stroma, nerves, and vasculature, which simplifies them, but allows the parallel expansion of diseased and normal cells from the same patient. This makes ASC-derived organoids particularly useful for personalized therapy applications.⁸

3D Bioprinting

3D bioprinting organoids involves layer-by-layer deposition of bio-inks containing living cells, growth factors, and extracellular matrix (ECM) components. The bio-inks are printed using precise, computer-guided techniques to create complex tissue architectures. Bio-printed scaffolds can include vascular networks to enhance nutrient delivery, improving the viability and functionality of the organoids.

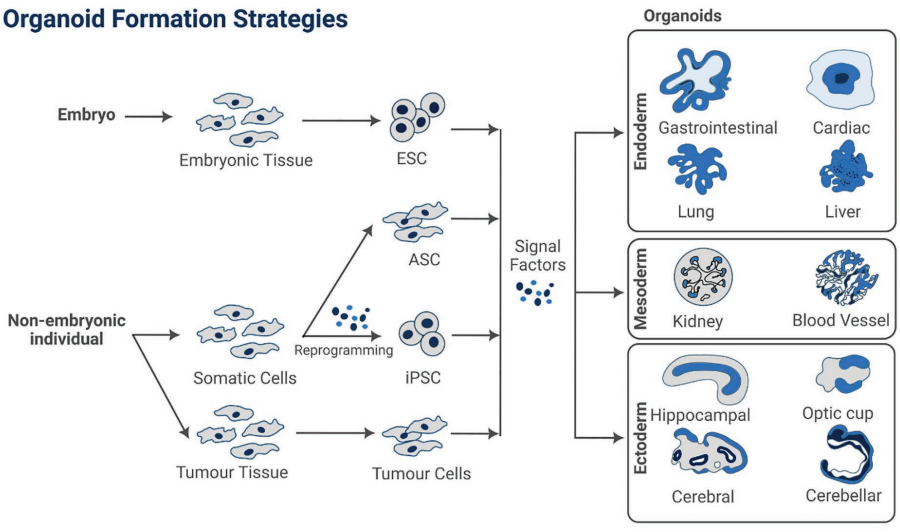
The method allows for precise spatial control over cell placement, enabling the recreation of organ-specific microenvironments. This makes bioprinting especially valuable for studying cell-cell interactions, tissue development, and disease progression. It also allows for high-throughput production of patient-specific organoids using cells derived from biopsies or induced pluripotent stem cells, making it a powerful tool in personalized medicine. By enabling the creation of functional tissue models, 3D bioprinting offers significant advancements in drug testing, disease modeling, and regenerative medicine.¹¹

Organoid Applications



Applications of adult stem cell-derived organoids.⁸

Organoid Formation Strategies



Example workflow to create tissue-specific organoids from embryonic and non-embryonic tissue, including embryonic stem cells (ESCs), adult stem cells (ASCs), induced pluripotent stem cells (iPSCs), and tumor cells.^{9,10}

3D Cell Culture Imaging

The most common cell culture imaging experiments are live timelapse imaging to investigate 3D cell culture development, maturation, and lineage tracing. Additionally, tissue perturbation experiments have become a widely used tool in biophysics, such as using laser ablation for biophysical studies (e.g., tissue tension studies) and uncaging photo manipulation (e.g., release of chemicals). In addition to live imaging, 3D cell cultures can also be fixed, stained, and prepared with tissue clearing, expansion, or sectioning for subsequent imaging.

3D cell cultures enable:

- **Basic Research:** Organoid assays help scientists understand the role of external stimuli and cell-cell interactions.
- **Biobanking:** Tumor biobanks enable drug screening to find the most suitable treatment for patients.
- **Disease Modeling:** Establishing databases of information and cross-correlation information allows novel in silico disease modeling, while in vitro organogenesis allows wet lab modeling of development and disease.
- **Precision Medicine:** Patient-derived organoids allow the establishment of personalized treatments tailored to the patient, reducing costs and side effects.

Imaging Techniques

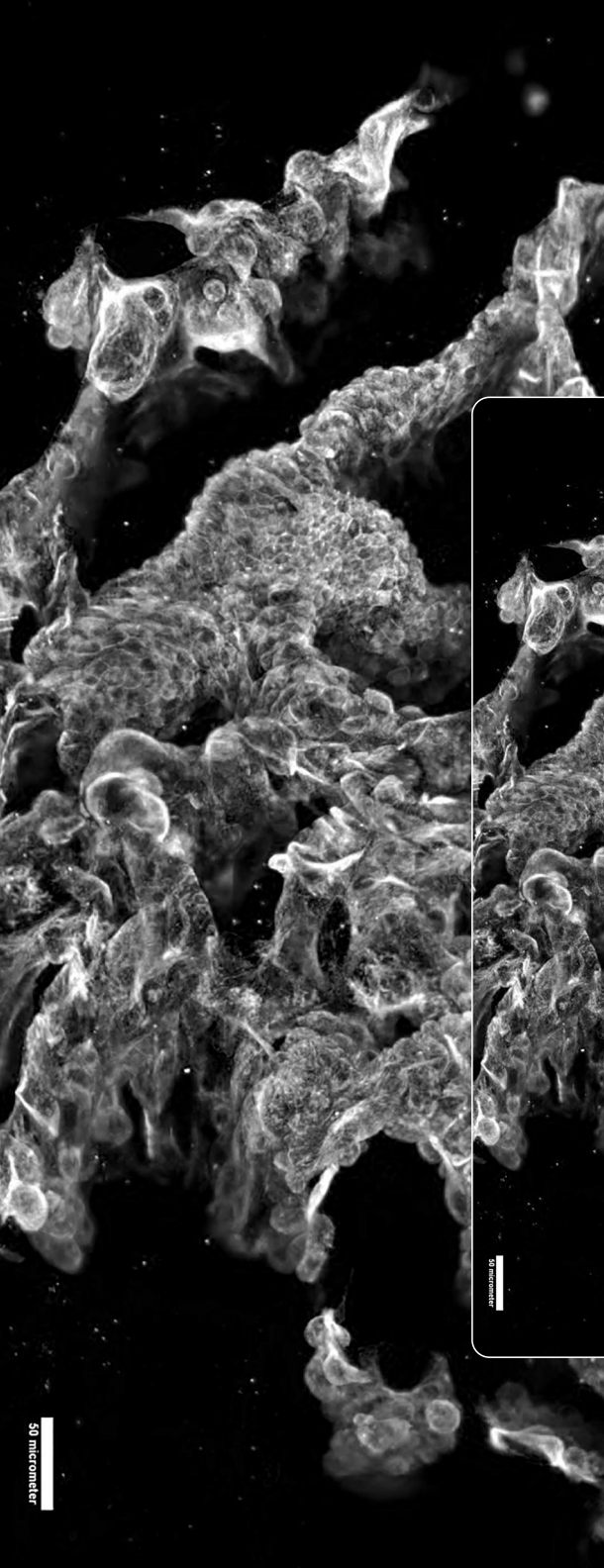
Imaging organoids requires advanced microscopy techniques to capture their 3D structure, cellular interactions, and dynamic processes. Commonly used microscopes and imaging techniques for organoid studies include:

1. **Electron Microscopy (EM):** For ultra-structural analysis, EM is used to investigate fine cellular and extracellular matrix details in organoids, especially when studying tissue organization or viral entry mechanisms.
2. **Bright-field Microscopy:** This is a widely used method for assessing general organoid growth, morphology, and integrity. It provides lower resolution compared to other advanced imaging methods, but does not require extensive sample preparation. It is ideal for routine monitoring of organoid development and for initial checks of overall structure and viability.
3. **Confocal Microscopy:** Confocal microscopy is ideal for high-resolution imaging of fluorescently labeled organoids. It allows optical sectioning through samples, providing detailed 3D reconstructions. It's commonly used to visualize cell morphology, protein localization, and cellular architecture within the organoid.
4. **Light-Sheet Microscopy:** This method is designed for imaging large, intact organoids with minimal photodamage. Light-sheet microscopy enables rapid acquisition of 3D datasets, making it ideal for imaging dynamic processes and whole-organoid structures over time.
5. **Two-Photon Microscopy:** Suitable for imaging deep within organoids, this technique offers greater tissue penetration and reduced phototoxicity compared to confocal microscopy. It's especially useful for live imaging and long-term studies of organoid development or drug response.

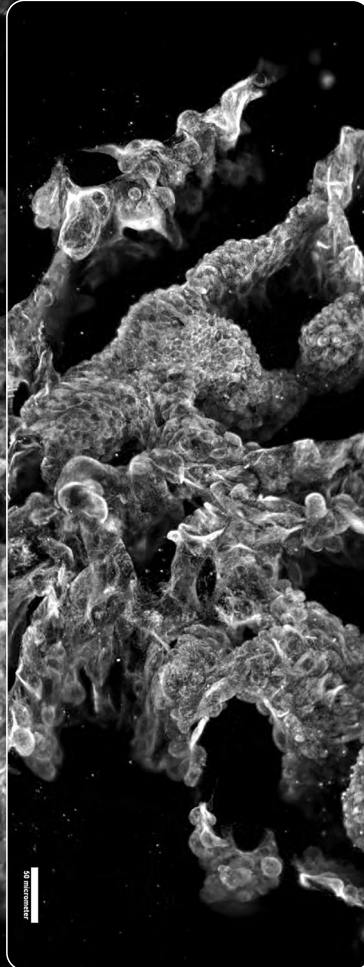
Each of these techniques provides unique insights, and combining them can yield comprehensive data on organoid structure, function, and dynamics.¹¹

Imaging Technique Specifications

Imaging technique	Resolution	Sample preparation	Application
Electron microscopy	~0.1 nm	Fixation, dehydration, sectioning, staining/ sputtering	High-resolution structural information
Brightfield microscopy	~200 nm (xy), 500 nm (z)	Potentially none	General growth, morphology, and integrity assessment
Automated widefield microscopy	~200 nm (xy), 500 nm (z)	Potentially none	High throughput and content screening
Laser-scanning confocal microscopy	~200 nm (xy), 500 nm (z)	Requires fluorescent labeling	Tissue architecture studies; in vivo and fixed sample imaging
Spinning disk confocal microscopy	~200 nm (xy), 500 nm (z)	Requires fluorescent labeling	Ideal for high-speed, live-cell imaging; imaging of subcellular structures; reduced photobleaching compared to point-scanning confocal
Light-sheet microscopy	~200 nm (xy), 500 nm (z)	Requires fluorescent labeling	Long-term imaging of living samples; in vivo and fixed sample imaging; imaging of large, cleared organoids
Multiphoton	~300 nm (xy), 800-1000 nm (z)	Requires fluorescent labeling, deeper tissue penetration	Deep tissue imaging of thick organoids; long-term in vivo imaging with low phototoxicity



hESC-derived
pancreatic
spheres.
Courtesy of Yassen
Abbas.



50 micrometer

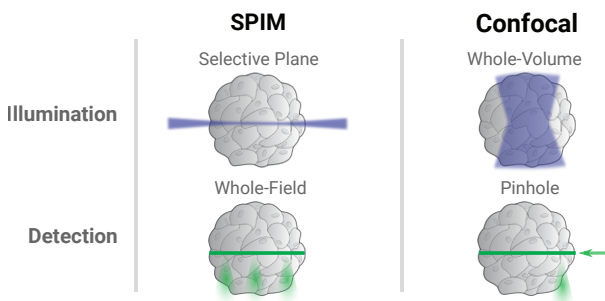
Light-Sheet Fluorescence Microscopy for 3D Cell Cultures

3D cell cultures are fragile systems that require optimized experimental protocols and low phototoxicity during imaging to preserve the samples. Light-sheet fluorescence microscopy, or single-plane illumination microscopy (SPIM), is an imaging technique used in biomedical research.¹² Due to its optical properties, SPIM causes minimal photodamage, allows the acquisition of large 3D samples, and is suitable for live and fixed sample imaging. It is often considered the best overall 3D cell culture imaging method due to its ability to section large, intact 3D structures with minimal damage.

In addition, multi-color fluorescence and multi-modal imaging are possible by combining fluorescence with brightfield microscopy and SPIM with photomanipulation. For researchers, it allows high-throughput imaging with high speed and a large sample number. Therefore, light-sheet fluorescence microscopy is ideal for imaging 3D cell cultures, particularly over time.

Applications of SPIM organoid imaging include:

- Disease modeling,
- Drug screening,
- Personalized medicine,
- Regenerative medicine, and
- High-throughput screening.



Bruker's light-sheet SPIM systems were designed with scientists to ensure sample health, ease of use, and high imaging quality.

Disease Modeling

Light-sheet microscopy allows 3D imaging of organoids that closely mimic human tissues and organs over hours and days. Therefore, it is possible to observe cellular interactions, tissue formation, and disease progression in real time. This can lead to a better understanding of diseases such as cancer, neurological disorders, and infectious diseases at a cellular and molecular level. One such example is invasion assays, which can shed light on how tumor cells invade surrounding tissues in metastasis. Being able to show this in real-time and in 3D provides unique insights into our understanding of cancer progression.¹³

Drug Screening

Comparing 2D and 3D cell cultures can significantly impact drug diffusion, penetration, and ultimately efficacy. In 2D cell cultures, drugs easily diffuse and uniformly reach all cells, which can lead to an overestimation of drug efficacy. In 3D cell cultures and organoids, drug penetration is more complex, with gradients forming as drugs diffuse through the 3D structure. The 3D architecture better replicates the in vivo situation of uneven drug distribution, impacting efficacy and resistance.¹³

SPIM allows for high-resolution, live imaging of organoids exposed to various drug compounds. This enables the observation of drug effects on cellular structures and functions over time, leading to more accurate assessments of drug efficacy and toxicity. Upscaling experiments to high-throughput drug screens significantly increases the speed of the drug discovery process by identifying promising drug candidates early.

Personalized Medicine

Creating patient-derived organoids from patient biopsies allows scientists to test how individual patients might respond to different treatments. For example, organoids can be co-cultured with immune cells from the same patient. This setup monitors how the immune cells react to a drug within the organoid environment, providing insights into potential allergic reactions.⁸ Similarly, patient-derived tumor organoids are beneficial to recapitulate tumor heterogeneity and drug responses.¹⁴ These approaches can help tailor personalized therapeutic strategies, leading to more effective and targeted treatments with fewer side effects.

Regenerative Medicine

Organoids created from patient adult stem cells are established using the natural tissue regeneration processes of the body to establish an organoid directly from the patient's organs, which can be from healthy or diseased epithelium. Studying regeneration is still challenging, but various researchers have made strides to better understand this process. Notable insights have been uncovered about signaling pathways, including growth factors and cytokines, and their specific impact on tissue types.⁸

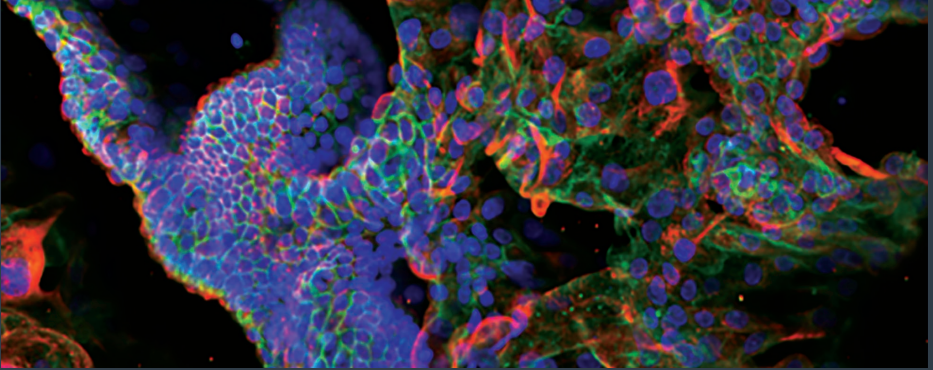
High-Throughput Screening

The main advantage of high-throughput screening is its ability to allow the comparison of various conditions while providing sufficient numbers to account for sample variance.¹⁵ The ability of SPIM to rapidly image large numbers of organoids in a 3D environment makes it ideal for screening applications.

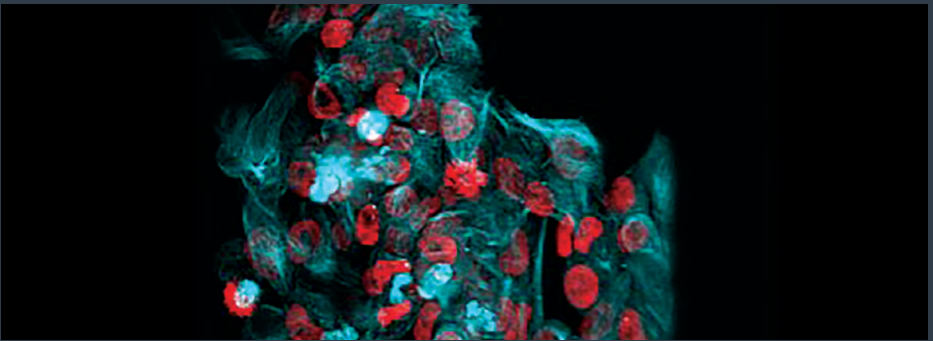
Automated Widefield Screening with the Acquirer IM

Bruker's Acquirer IM is a fully automated widefield microscope with brightfield and fluorescence imaging for a variety of samples. The combination of a static sample holder and a mobile optical unit ensures sample stability during imaging, making it ideal for imaging motion-sensitive samples, such as non-adherent 3D cell cultures, organoids or early embryos.

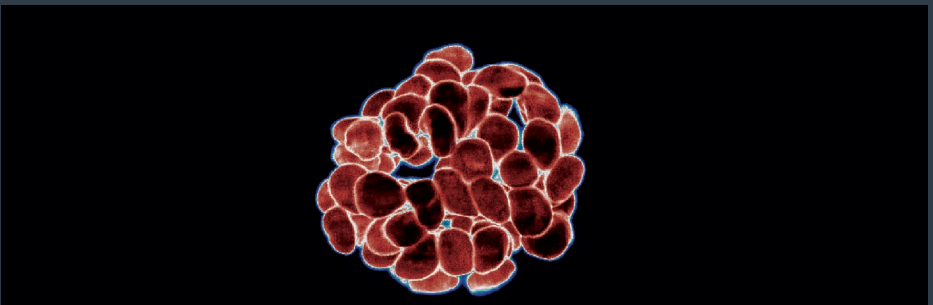
The IM system includes a host of unique features, including built-in incubation, a robotic lid, and an open interface. Seamless integration into automated workflows makes it easy for researchers to perform advanced, long time-lapse experiments, screening, and high-throughput imaging assays.



3D cell culture system of human primary cells ($330\mu\text{m} \times 220\mu\text{m} \times 1200\mu\text{m}$). Courtesy of Yassen Abbas, Turco Lab, University of Cambridge, Cambridge, UK.



HeLa cells expressing GFP and mCherry. Imaged on the InVi SPIM. Courtesy of: Tobias A. Knoch, Erasmus MC, Rotterdam, The Netherlands.

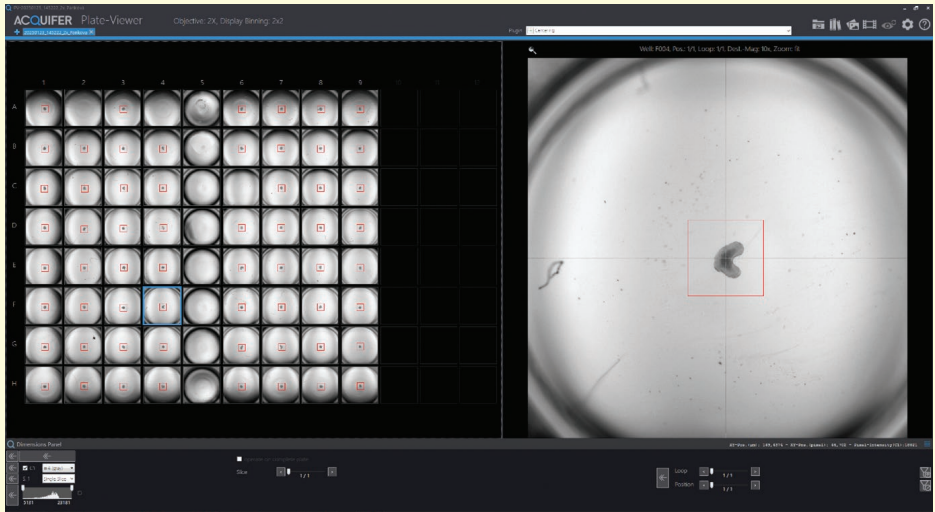


hESC-derived pancreatic spheres imaged on the TruLive3D Imager which enables collecting information from several samples in one experiment. Visualization: Imaris (Bitplane). Courtesy of Yung Hae Kim, Graphin-Botton Group, MPI-CBG, Dresden, Germany.

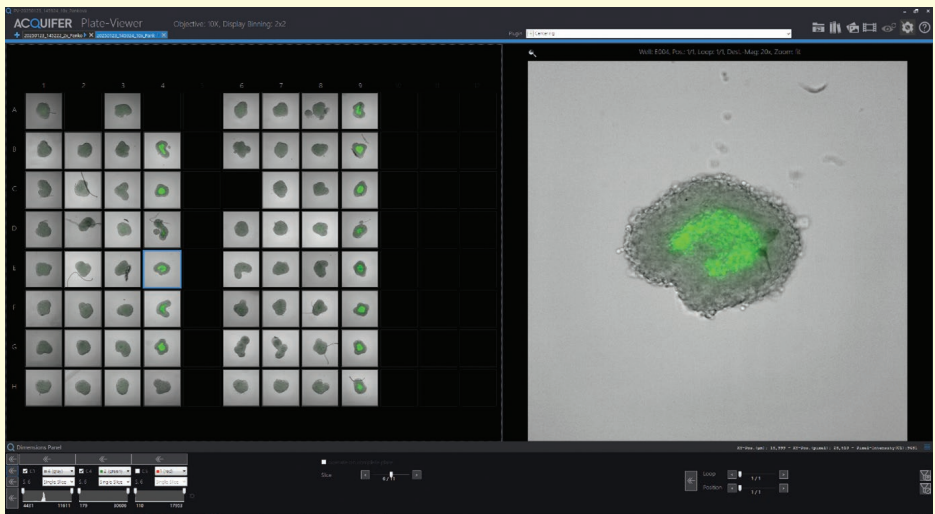
Spheroids

The Bruker Acqifer IM was used to study pancreas spheroids by Dr. Katrin Roth in a drug screening assay. Samples were placed in repellent, round-bottom dishes, that allowed the samples to fall towards the center of the dish. Thanks to the movement of the optics, rather than the stage, the samples remained centered even when moving from one well to another.

In the study, samples were first inspected in the overview, which was followed by zooming in using a region of interest (ROI) detection. Using a Caspase staining, cell death was observed and later quantified relative to a left-to-right and top-down increasing drug concentration. After identifying drug candidates, samples can be further studied in timelapses on the Bruker TruLive3D Imager.



Repellent, round-bottom dish, allowed samples to fall towards the dish center.



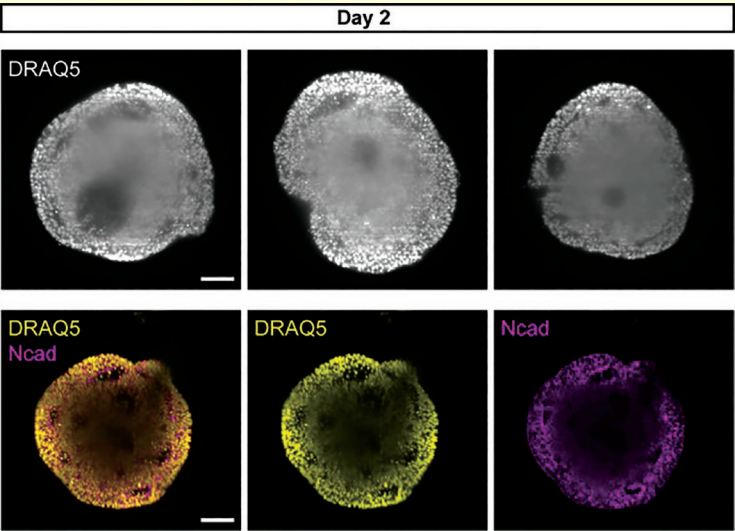
Caspase staining was used to quantify death relative to drug concentration.

CASE STUDIES

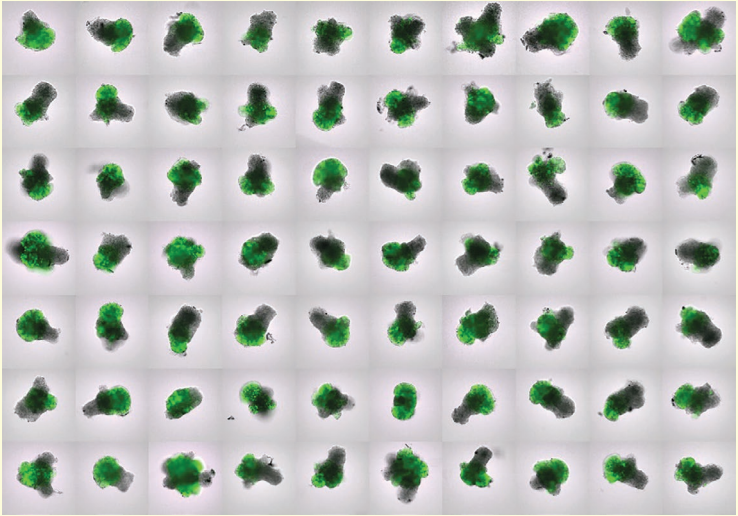
Retinoids

An impactful study from Zilova, L., Weinhardt, V., et al (2021) examines retinal tissue differentiation and development in fish primary embryonic pluripotent cells.¹⁶ Bruker’s Acquirer Imaging Machine (IM) was used by the group to study the processes of aggregation and compaction in cellular aggregates.

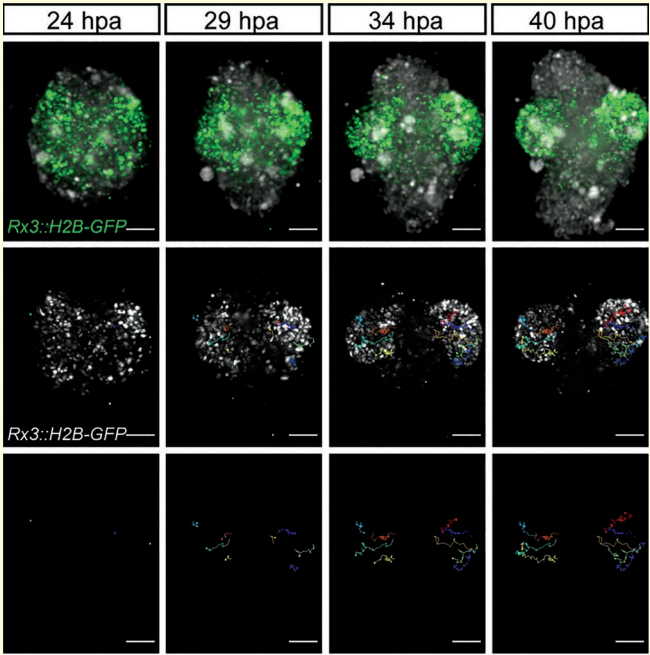
In this same study, Bruker’s MuVi SPIM light-sheet microscope was used to investigate the 3D structure and interior composition of organoids.



3D imaging of organoids using SPIM to study organoid interior composition. Reproduced figure 2 from [16] under a CC BY 4.0 DEED and author permission.

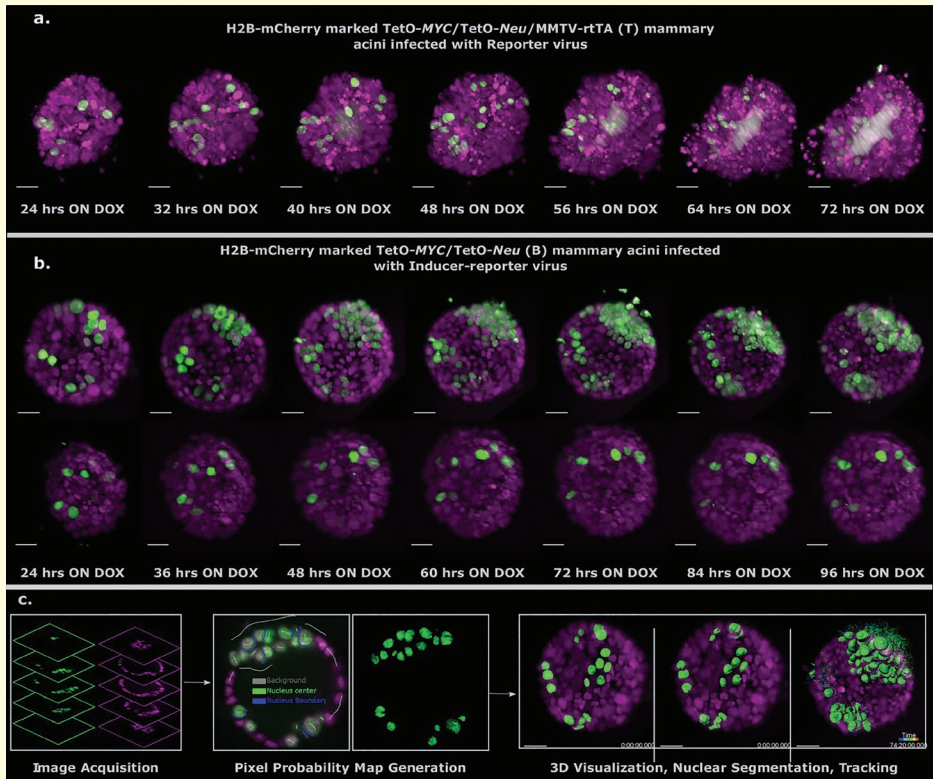


Time-lapse imaging of medaka organoid formation. Acquisition of retinal fate within medaka blastula-derived aggregates. Green: retinal lineage marker Rx3. Data used from [16] under a CC BY 4.0 DEED and author permission.

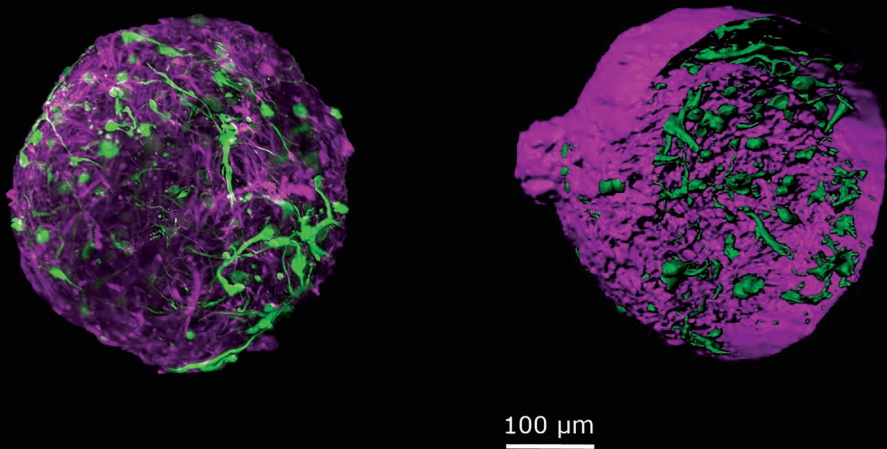


Timelapse of 3D imaging of organoids to study organoid dynamics. Reproduced figure 5 from [16] under a CC BY 4.0 DEED and author permission.

More 3D Cell Culture Light-Sheet Imaging Samples



Characterization and imaging of stochastic tumorigenesis in mammary organoids imaged on the InVi SPIM. Image used from [17] under a CC BY 4.0 license.



Fixed astrocyte spheroid stained with anti-GFAP (Alexa 488) to label astrocytes and anti-Neurofilament200 (Alexa555) to label neurons. Imaged on the InVi SPIM. Courtesy of Markus Bruell, AG Leist, University of Konstanz, Konstanz, Germany.

Challenges for 3D Cell Cultures, Spheroids, Organoids, and Assembloids

The applications of 3D cell cultures, spheroids, and organoids are manifold, but researchers must use different approaches to overcome technological, experimental, and physiological constraints.

Uncovering neuronal endogenous tissue organization is possible, but it is still difficult to visualize tissue specification and functionality due to a lack of symmetry breaking and body axis formation. These are important processes to establish brain regionalization, specification, and functionality. To overcome the lack of polarization, several approaches exist such as using ECM stiffness, microfluidics, 3D printing, or fusion of patterned spheroids.¹⁸ Despite these developments, challenges exist in establishing various axes (anterior-posterior, dorso-ventral, sinister-dexter) and subregions (wrinkles, brain regions).

Further studies that address these challenges and other related topics include cell type specification, cell stress, hypoxia and necrosis, vasculature, glial cells, cytoarchitecture, regional interactions, circuit formation and activity dynamics, and maturation.¹⁹

Vascularization is vital for allowing researchers to understand physiological processes such as drug delivery.²⁰ However, it is difficult to grow 3D cell cultures beyond a certain size with a lack of vascularization, due to local hypoxia beyond 200 μm and subsequent cell death.^{20,21} Growing cell cultures past these sizes is also often needed to study matured structures as core tissues die off.²² To overcome the challenge of lacking vascularization, several biological and technical approaches exist, such as creating assembloids, or integrating organoids with organ-on-a-chip technologies²³ and microphysiological systems (MPS).²⁴

Broadly speaking, 3D cell culture studies are changing their focus from creating 3D tissues to understanding and enhancing their physiology and circuit dynamics.

Ethical Considerations and Debate

3D cell cultures are contributing to reducing the need for animal models, but as a rather new system, the ethical considerations, debates, and legal regulations are not yet as clearly defined as those for longer-standing models or technologies. Ethical considerations are particularly important in view of brain organoids, embryoids, or human-animal chimeras.²⁵

The four main ethical considerations are (i) informed consent, (ii) commercialization, (iii) personalized medicine, and (iv) transplantation into humans. Organoid applications are also complex and ethical considerations need to be refined to include certain technologies, applications, organoid subtypes, and secondary uses.²⁶

The field of bioethics also discussed whether organoids could reach consciousness or sentients at a point, and how this would impact the use of 3D cell cultures. However, 3D cell cultures not only open new avenues for medical explorations, but could help overcome technical limitations to study neurodiseases²⁷ or computational limitations such as the “von Neumann bottleneck,” which suggests that hardware can be a limiting factor for data processing. This opens the discussion of “organoid intelligence” (OI) and how this could impact the future of science.²⁴ A proof of principle for OI was shown in a study that used organoids for artificial computing, called “Brainoware.”²⁸

Advances and Future Directions

Using 3D cell cultures in the lab and clinic is an evolving process with efforts being made to increase what applications are possible for human biology and drug development. The creation of initiatives, protocols, and analysis tools is creating more avenues of exploration. Some examples include:

Human Cell Atlas Organoid Project

The Human Cell Atlas Organoid Project is an initiative aimed at validating organ-specific gene expression profiles within organoids, ensuring that they accurately reflect human organ function. Additionally, the project quantifies biological variation within and between organoid models, which is essential for standardizing research and improving reproducibility. These efforts collectively advance our understanding of human biology and enhance the potential applications of organoids in medicine.²⁹

Organoid Protocols

To ensure scientific reproducibility, standardized and potentially automated protocols for different tissues and conditions must be developed. This is particularly important to decrease variability.

Clearing Protocols

Developing protocols for organoid clearing is needed to gain undisrupted 3D insights and increase experimental efficiency.

Multi-Modal Imaging and Data Analysis

Integration data from multiple advanced imaging techniques and integration with quantitative computational analysis to extract quantitative data from organoids will provide in-depth and novel insights.

Conclusion

Together, the era of 3D cell cultures, spheroids, and organoids opens new opportunities for personalized medicine (in vitro) and organoid transplantation (in vivo). However, technical and ethical challenges remain and impact the bench-to-bedside translation. Regardless, automated widefield and light-sheet fluorescence microscopy are uniquely well suited for live and fixed imaging of 3D samples.

The optical properties of light-sheet microscopes create minimal photodamage and can image samples over time, which has allowed researchers to explore novel applications in cellular biology, developmental biology, oncology, and even artificial intelligence. Furthermore, the Acquirer IM widefield microscope seamlessly integrates into automated workflows for advanced, time-lapse experiments, screenings, and high-throughput imaging assays. The unique combination of light-sheet microscopy and widefield imaging provides unprecedented insights into 3D cell cultures, organoids, and embryos.

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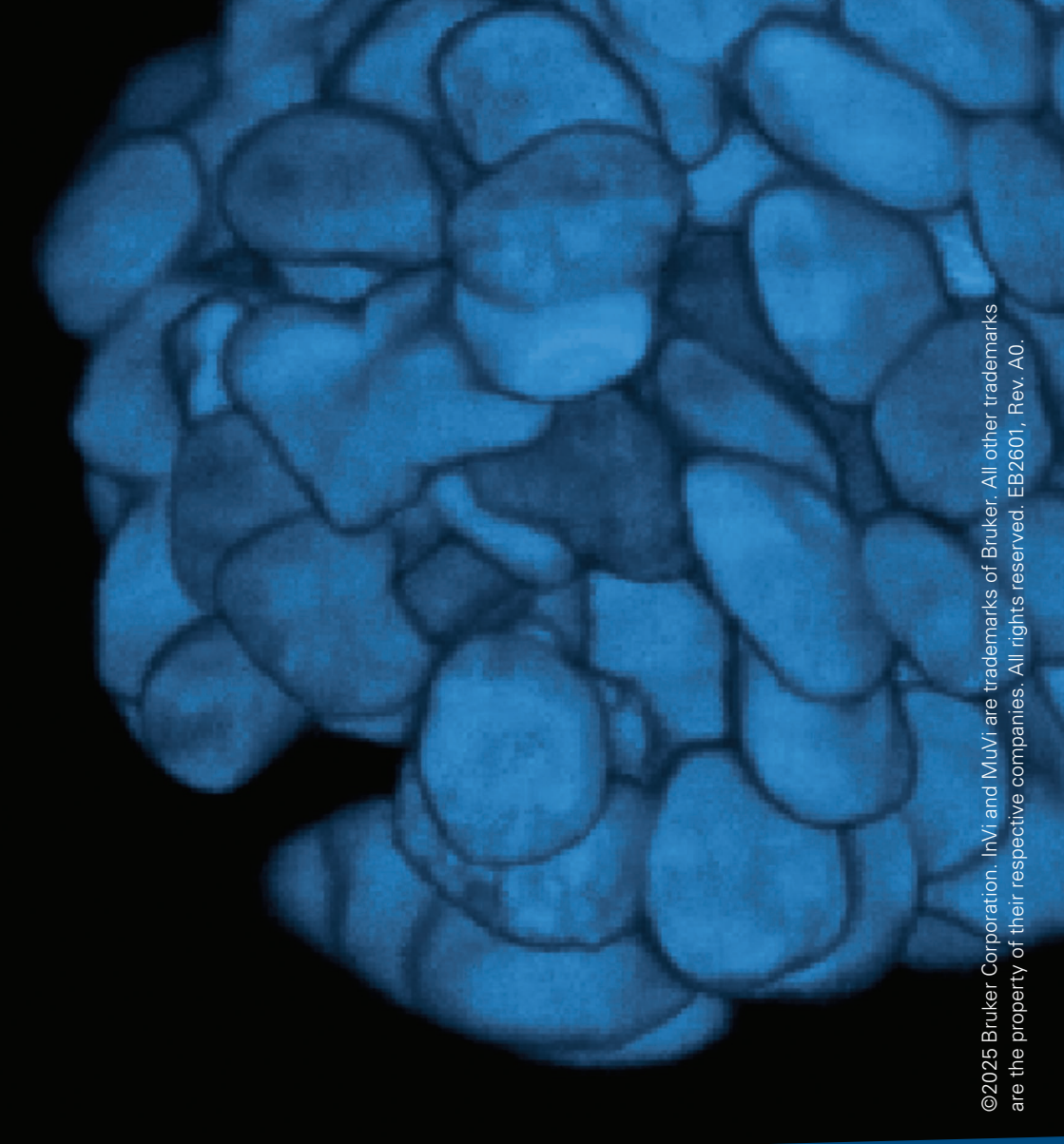
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