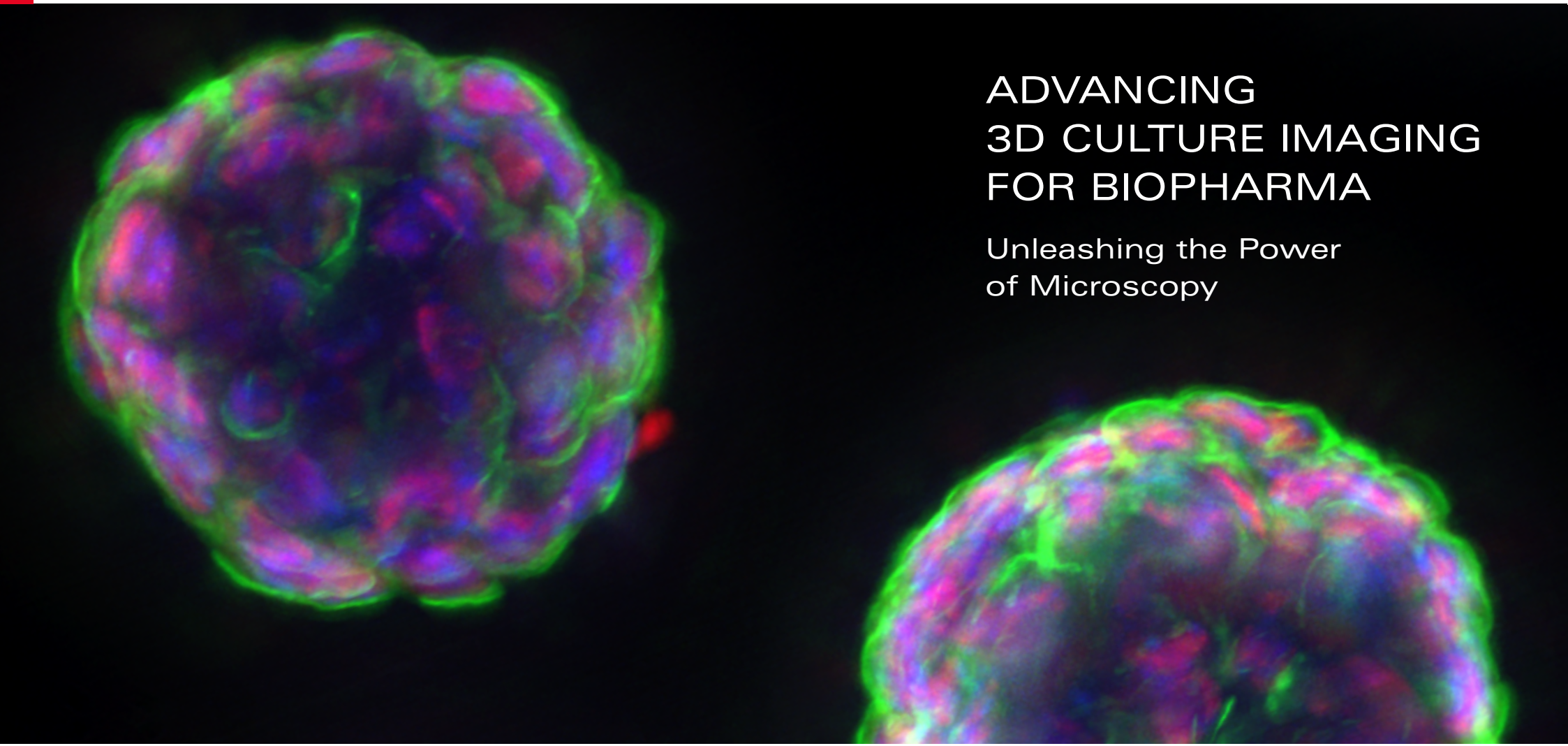
From Eye to Insight

Leica MICROSYSTEMS

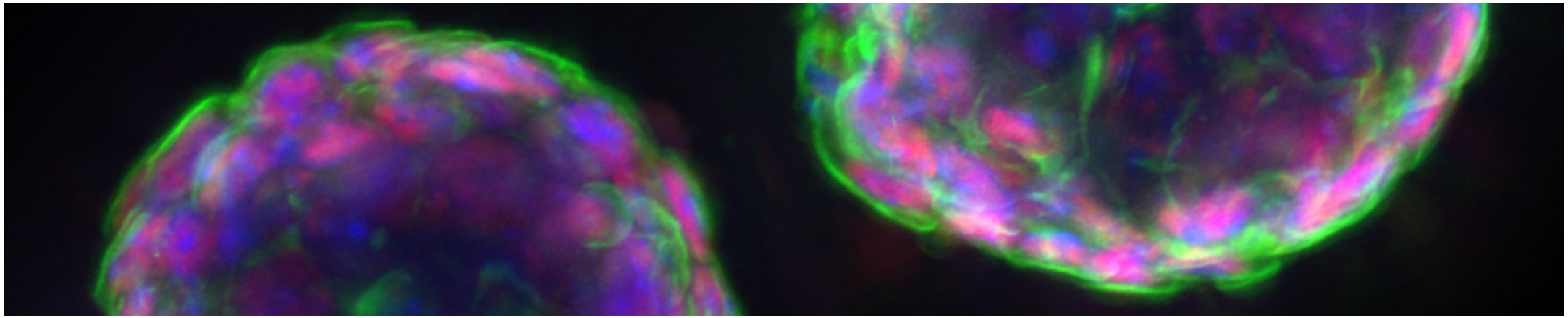


ADVANCING 3D CULTURE IMAGING FOR BIOPHARMA

Unleashing the Power of Microscopy

In association with

Drug TARGET REVIEW



CONTENTS

INTRODUCTION

In recent years, drug discovery and development processes in biopharma have undergone remarkable advancements with the emergence of three-dimensional (3D) culture systems, including organoids and spheroids. These models provide an environment that better mimics the natural 3D architecture and interactions found *in vivo*.

The potential applications of such 3D model systems are extensive. Because of their more physiologically relevant nature, they can better facilitate investigations into the mechanisms underlying drug resistance, enable the screening of potential therapeutics, and can provide a more precise and predictive evaluation of compound toxicity. By better identifying and evaluating drug targets at earlier discovery stages, they can increase the quality of therapeutic candidates in the drug development pipeline. The use of these model systems can also reduce the need for animal models.

As scientists delve deeper into the intricacies of spheroids, they gain insights that help drive advancements in regenerative medicine and our understanding of complex diseases. The translatable insights that these model systems can offer has particularly benefited research into cancer, neurodegenerative disease and immunological disorders.

Why are organoids and spheroids so relevant?
Organoids consist of organ-specific cell types that can replicate the spatial organization and some functions of actual organs. They offer a highly physiologically relevant platform for investigating complex questions related to disease onset and progression, tissue regeneration, and inter-organ interactions.

The landscape of 3D culture systems extends beyond organoids, encompassing other notable models such as spheroids and organ-on-a-chip platforms. Spheroids in particular have garnered considerable attention as more predictive models for drug discovery. These spherical cell aggregates self-assemble within a cultured environment, successfully mimicking the architecture and functionality of natural tissues. In conventional two-dimensional (2D) cultures, cells grow as monolayers on planar surfaces. In contrast, spheroids create a more realistic microenvironment that facilitates intricate cell-cell interactions, the formation of complex cellular junctions, and the establishment of nutrient and oxygen gradients akin to those found within living organisms.

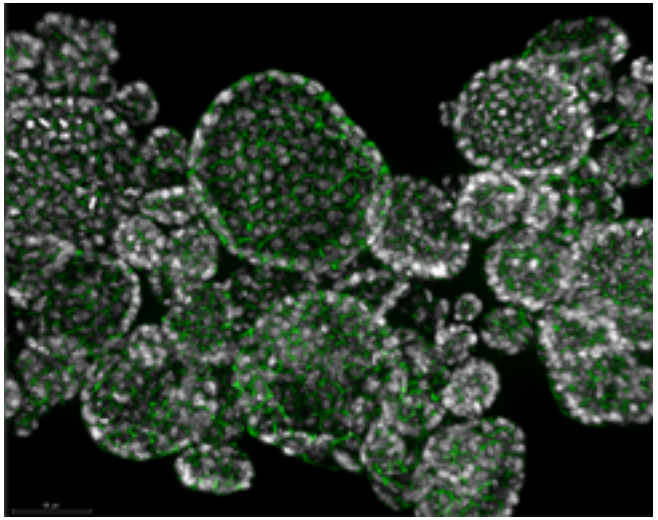


Figure 1: Organoid Cluster – Organoid Cluster acquired using a THUNDER Imager, stained with DAPI (nucleus) and GFP (plasma membrane. Image courtesy of Dana Krauß, Cancer Research Institue, Medical University of Vienna, Vienna, Austria.

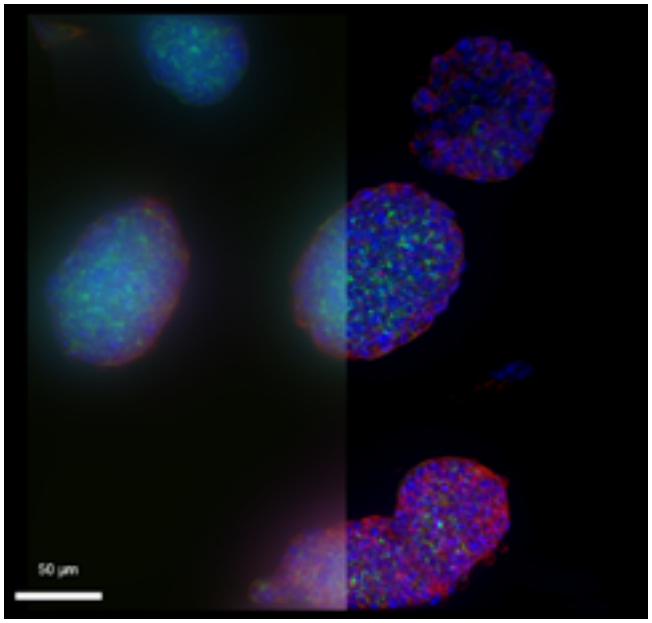


Figure 2: MIN6 cells grown as pseudoislets (pancreatic beta cells). DAPI (blue), Insulin (Alexa488, green), membrane receptor (Alexa594, red), phalloidin (Alexa647, white). Sample courtesy Dr. Rémy Bonnavion, MPI for Heart and Lung Research, Bad Nauheim (Germany).

By reproducing the complexity and physiological relevance of native tissues, spheroids can unlock profound insights into cell signaling pathways, drug responsiveness, and the impact of the microenvironment on cellular phenotypes.

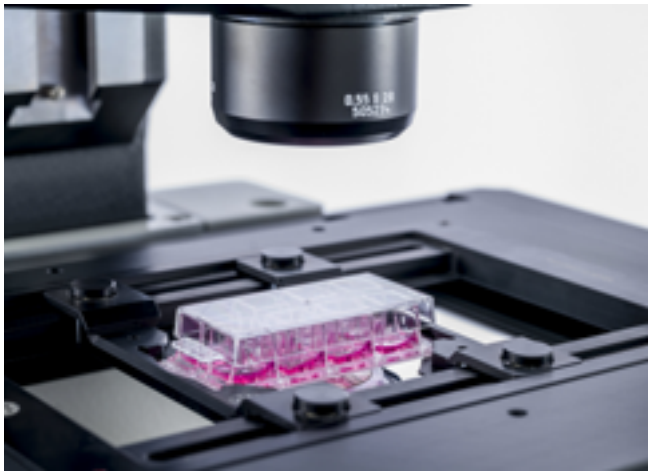


Figure 3: Imaging 3D cultures using a Leica THUNDER Imager.

Standardization and reproducibility of cultures, scalability for high-throughput applications, vascularization to support larger sizes, long-term viability, imaging deep inside 3D structures and heterogeneity in cellular composition are among the key challenges faced when working with organoids and spheroids. Overcoming these challenges will further enhance the potential of these 3D models for understanding complex biological processes and improving drug discovery and development.

Organoids and spheroids under the microscope

Microscopy plays a crucial role in studying both organoids and spheroids, revealing details of cellular structures, interactions within the microenvironment and live cell dynamic processes. Imaging these models, however, does present unique challenges. Their compact and complex nature often defies conventional imaging techniques, impeding efforts to capture the entirety of the spheroid or obtain high-resolution images of its constituent cellular components. The analysis of spatial cell distribution within an organoid or spheroid, and the quantification of cellular interactions, requires specialized imaging and computational tools.

In this eBook, we explore some of the challenges encountered when imaging these types of 3D models, shedding light on innovative microscopy solutions that can empower scientists to make new advances in areas such as regenerative medicine, drug discovery, and disease research. ■

Taylor Mixides
Editor, Drug Target Review

CHALLENGES OF IMAGING ORGANOIDS AND 3D CULTURES

Imaging 3D cultures presents unique challenges compared to traditional 2D cultures, which can impact the quality and interpretation of the acquired images. Several factors contribute to these challenges, including the larger volumes involved, out-of-focus blur, the requirement to maintain cell viability during longer term imaging experiments, the necessity to prevent physical damage to delicate structures, and the need for accurate images for analysis. These challenges are explained further below.

Imaging deep within 3D samples

Traditional imaging techniques optimized for 2D cultures may not adequately capture the intricate structures and spatial organization of cells within organoids and spheroids. Obtaining clear and detailed images throughout the larger volumes seen in organoids and spheroids can be difficult due to light scattering and attenuation.

The specificity of fluorescence microscopy provides a powerful tool to observe and analyze biological processes and structures with accuracy. It allows for the visualization of specific molecules and cellular components within samples, enabling the study of intricate details and dynamic events. Widefield microscopy (sometimes referred to as epi-fluorescence microscopy), illuminates large areas of the sample at a time, which can result in images with out-of-focus blur due to light emitted from regions above and below the focal plane. This contributes to increased background noise, reduces contrast, and makes it challenging to distinguish specific features and boundaries. Thus, it is difficult to accurately segment, analyze and extract relevant information from the images. One strategy to mitigate the issue of out-of-focus blur is to use deconvolution algorithms that computationally restore the in-focus information. Scientists may also rely on specialized imaging methods such as confocal microscopy, multiphoton microscopy, digital light sheet (DLS) microscopy and super-resolution techniques including stimulated emission depletion (STED) microscopy and structured illumination microscopy (SIM) to improve the resolution and accuracy of images obtained from 3D cultures.

Maintaining cell health during long-term imaging

When conducting time-lapse microscopy experiments with organoids and spheroids, the need to keep cells healthy and viable is critical. Prolonged exposure to intense light, elevated temperatures, or toxic imaging agents can cause phototoxicity and compromise cell viability. To address this, it is important to carefully optimize imaging conditions to minimize phototoxicity while still obtaining high-quality images. This may involve reducing the excitation light intensity, minimizing exposure and using specialized live cell imaging techniques.

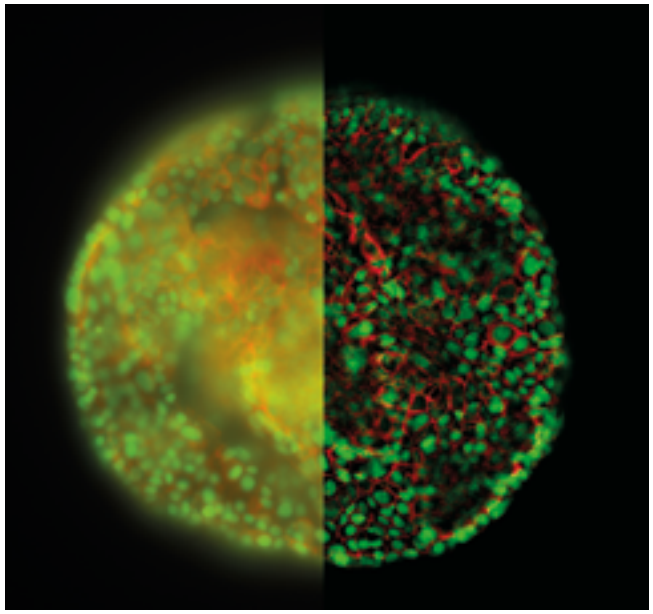


Figure 4: HeLa cell spheroid stained with Alexa Fluor 568 Phalloidin (Actin) and YOYO 1 iodide (Nucleus). Imaged with a THUNDER Imager 3D Cell Culture. Shown are both the raw widefield image and the same image after Computation Clearing is applied.

Deviations from physiological conditions can disrupt cellular behavior and functionality, potentially leading to inaccurate results. The use of imaging platforms that provide environmental controls to maintain an optimum temperature, humidity, and CO_2 levels can help ensure the prolonged survival of samples throughout the imaging process. It is also crucial to provide a stable supply of nutrients to the cells as this is essential for cell homeostasis and proper functioning. Furthermore, preventing impairments caused by evaporation is also critical for preserving the integrity of the experiment and ensuring accurate representation of cellular processes.

Another challenge can arise from changes in the characteristics of organoids or spheroids during the imaging period. As these 3D cultures continue to develop, they may undergo lateral and axial growth, resulting in alterations in their size, shape, and position within the imaging field. These changes can pose difficulties in maintaining the focus of the microscope throughout the time-lapse experiment. Scientists therefore have to adapt to these shifting sample characteristics by adjusting the focal plane and imaging parameters accordingly to ensure consistent and reliable imaging.

Furthermore, maintaining the expression of fluorescent markers at endogenous levels is crucial during long-term imaging. Perturbations in marker expression can impact cellular behavior and functionality, potentially leading to misleading observations and inaccurate interpretations of the results. Experimental conditions must therefore be carefully optimized to minimize any situation that could affect the expression levels of fluorescent markers.

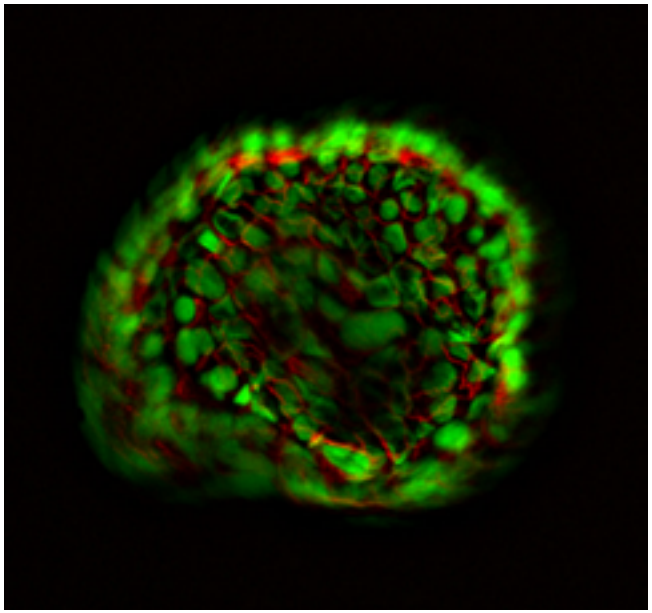


Figure 5: An organoid approximately 150 µm in diameter mounted into a depression slide. Imaged with a THUNDER Imager Model Organism. Shown are both the raw widefield image and the same image after Computation Clearing is applied.

Preventing physical damage to samples

Gentle handling of organoids or spheroids to retain their natural 3D structure and cellular interactions is also an important consideration. If the sample has to be removed from its culture environment to be prepared for imaging, then the mechanical stress caused by physical manipulation can result in a loss of sample material or destruction of delicate 3D structure. The imaging process should minimize interference with the natural growth and behaviour of the spheroid or organoid, allowing the 3D culture to continue developing after imaging.

Clear images for accurate analysis

Once the samples have been successfully imaged, the next crucial step is the analysis and interpretation of the acquired data. It is often necessary to quantify cellular and molecular parameters such as cell proliferation, migration, differentiation, and gene expression. However, due to the complex and heterogeneous nature of organoids, obtaining high-resolution, high-contrast, representative images for analysis can be challenging. This requires careful optimization of imaging parameters such as contrast, signal-to-noise ratio, exposure time, and focus. Many of these imaging settings can be automatically selected and optimized in modern imaging systems. The development of automated image analysis algorithms and machine learning approaches has also proven invaluable in processing and extracting meaningful information from the large datasets generated by imaging 3D cultures.

Addressing all of these challenges is essential to ensuring the successful imaging and analysis of organoids and spheroids, especially if observing these models over time. Only then can scientists start to gain more valuable insights into the complex biology of organoids and spheroids and maximize the potential of these advanced cellular models in both basic and applied research. To do this, specialized techniques and approaches that can overcome the limitations of traditional imaging methods are needed. We will explore this further in the next chapter. ■

SOLUTIONS FOR IMAGING ORGANOIDS AND 3D CULTURES

Leica Microsystems is one of the market leaders in the field of microscopy and has developed a range of innovative imaging solutions that can help overcome many of the challenges of imaging organoids, spheroids and other 3D cultures. These technologies allow deep and fast imaging for both endpoint measurements and for studying dynamic processes in 3D samples over time.

From traditional methods, such as widefield or confocal microscopy, to more advanced imaging methods such as multiphoton microscopy, digital light sheet microscopy and super-resolution techniques including STED and SIM, Leica makes it possible to visualize fine cellular details as well as overall tissue architecture within 3D cell cultures.

In their commitment to supporting the study of organoids, spheroids and other 3D cultures, Leica Microsystems provides resources and expertise to scientists. This eBook focuses on the use of selected microscopy platforms and techniques that can help address challenges related to sample preparation and imaging, the optimization of imaging parameters, the need for accurate analysis, and much more. These technologies can simplify the imaging process, enhance image quality, and enable in-depth exploration of the complexities of these remarkable cellular models for biopharma.

Let's take a look at some of these technologies in more detail.

Mica – the world's first Microhub

Mica is a completely integrated and innovative system that brings together different imaging techniques in a sample protecting, incubating environment. It leverages machine learning software, unique fluorescence unmixing techniques and automation to simplify imaging workflows.

Traditionally, different types of microscopy experiments need to be done on systems which offer different capabilities e.g., confocal systems or widefield systems. Mica brings together both widefield and confocal microscopy capabilities in the same system. This means that users can generate fast overviews in widefield mode at lower magnification, find a point of interest, and then with one click switch the view to confocal mode. Here they can examine the sample in higher resolution, gaining more detailed information.

Figure 6: Mica – the world's first Microhub

SEE MICA IN ACTION ...

Investigating mammary gland and cancer organoid models

To illustrate the impact of Mica in organoid research, this video interview showcases how scientists at TUM (Technical University of Munich) utilize Mica to investigate disease models such as mammary gland organoids and pancreatic cancer organoids. The video demonstrates the practical application and benefits of Mica for studying and understanding the intricacies of the development and behavior of these organoids.

INTERVIEW

Examining Developmental Processes in Cancer Organoids

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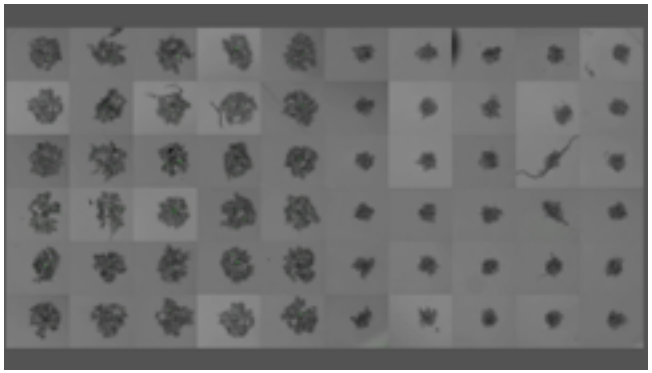

Figure 7: Formation of 3D spheroids from 1000 stably transfected MDCK MX1-GFP cells per well (left half) and 1000 U2OS cells per well (right half). Imaged using Mica in a time-lapse acquisition over 60 hrs with 30 minutes interval. Green, GFP. Gray, integrated modulation contrast.

In addition to widefield and confocal modes, Mica integrates Leica's patented imaging technologies, **THUNDER** and **LIGHTNING**, which both give greater clarity to images. THUNDER technology, as outlined in the next section, detects and removes the out-of-focus blur that occurs with widefield observation, making high speed 3D imaging possible in widefield mode with just the click of a button.

FURTHER INFORMATION

Mica – The world's first Microhub

FIND OUT MORE

With LIGHTNING, scientists can also enhance image quality, extending the image resolution for any channel in real time down to 120 nm.

The fact that Mica is an incubator, offering temperature, CO_2 and humidity regulation, also means that users can maintain samples at close to physiological-like conditions, which is especially important for long term experiments with organoids and spheroids (**Figure 7**). An autoimmersion objective also enables long term live cell imaging.

In another departure from traditional microscopy systems, Mica eliminates the need for special microscopy expertise by replacing manual setup steps (e.g., sample-finding focus strategies and parameter-setting) with a single push of a button. After inserting the sample, it takes just a few clicks to acquire a high-quality image.

THUNDER Imagers

Leica Microsystems THUNDER Imagers are another solution for advanced 3D cell culture assays, offering speed and reliability for tracking the growth and development of spheroids and organoids.

THUNDER is an opto-digital technology that removes out-of-focus blur through a method called Computational Clearing, which is directly embedded in the image acquisition stream. By taking the size of the targeted specimen features into account, Computational Clearing efficiently differentiates between signal and background, generating high resolution, high contrast images, even deep inside thick 3D clusters, in real time.

This ability to acquire sharp images fundamentally changes the way of working imaging 3D cultures. Thicker sections can be used, and larger structures can be imaged, than with a "standard" widefield microscope. By removing the blur and out-of-focus light inherent to widefield imaging, THUNDER Imagers can enhance the visualization of organoid and spheroid structures.

SEE THUNDER IN ACTION ...

Investigating tumor spheroids

An example application of using THUNDER to investigate 3D tumor spheroids can be found in the webinar "The Role of Iron Metabolism in Cancer Progression", featuring Professor Flavia Biamonte from the University of Catanzaro.

Professor Biamonte explores the role of iron metabolism in cancer development and progression, as well as its modulation of the immune response. Understanding how iron influences cancer and the immune system can aid the development of new therapeutic approaches.

WEBINAR

The Role of Iron Metabolism in Cancer Progression

WATCH NOW

Figure 8: THUNDER Imager Live Cell & 3D Assay

For 3D cultures, observing cells in as close-to-natural state is crucial for obtaining meaningful results. The THUNDER Imager Live Cell & 3D Assay addresses these needs by allowing users to optimize experimental conditions such as the lowest light intensity and the shortest exposure times possible. It features a high-end LED source with a small bandwidth optimized for excitation. Even with low illumination and short exposure times, the sensitive high-end sCMOS camera delivers meaningful image data thanks to a quantum efficiency of up to 82%. To minimize photobleaching, the system limits illumination to the actual recording time, synchronizing the camera shutter with the high-speed Lumencor LED light source.

An incubator also ensures optimal physiological conditions for living cell cultures, including stability, humidity, temperature and CO_2 levels (pH). A water immersion microdispenser also allows the use of a water-immersion objective for multiwell workflows, even during long-term experiments. Water immersion objectives enhance light collection, resulting in higher contrast and resolution in cell images.

Imaging live organoid and spheroid samples can be tricky because of drift, morphology change, or cell growth. The THUNDER Imager Live Cell & 3D Assay includes features such as reliable drift correction with Adaptive Focus Control (AFC) and software autofocus that compensates for changes in specimen position, enabling accurate time-lapse multi-position experiments and tracking of cell changes.

By combining the speed and high-throughput capabilities of widefield microscopes with the power of computational clearing, offering high sensitivity for low phototoxicity and photobleaching, and enabling optimal physiological conditions, THUNDER Imagers provide high imaging performance for organoid studies.

FURTHER INFORMATION

THUNDER Imager Live Cell & 3D Assay

FIND OUT MORE

STELLARIS – Confocal Microscopes

Confocal laser scanning microscopy (CLSM) images samples by means of optical sectioning. By limiting the illumination and detection to a single spot on the sample, out-of-focus light is eliminated, resulting in dramatically increased contrast. Complete high-resolution images are created by moving this spot across the entire sample, and 3D images can be reconstructed from the image stacks allowing visualization of processes in 3D models such as organoids and spheroids.

Leica Microsystems STELLARIS systems are based on a completely re-imagined confocal microscope platform and can be combined with modalities including fluorescence lifetime imaging microscopy (FLIM), stimulated emission depletion (STED) microscopy, digital light sheet (DLS) microscopy and Coherent Raman Scattering (CRS) microscopy.

Microscopic images, even confocal images, contain more details than conventional acquisition methods can resolve. LIGHTNING technology in the STELLARIS platform enables more of the information present in images to be revealed. It is fully automated and can be used for imaging of any specimen and for any type of experiment.

LIGHTNING provides an unparalleled extraction of information from images thanks to its adaptive image reconstruction, which works in (near) real time and 5D. While the usual image post-processing follows a one-way-fits-all strategy, LIGHTNING determines dynamically the quintessential information for every location in the image to achieve superior results.

Confocal images acquired with LIGHTNING appear as if a layer of noise has been removed from them compared to what has been the standard so far. The resulting images reveal a clear and unobstructed view of the relevant information. In addition, LIGHTNING image information extraction extends the resolution of the STELLARIS confocal platform into super-resolution territory where nanostructures as small as 120 nm can be resolved.

STELLARIS DLS Digital Light Sheet Microscope

As discussed previously, the complex 3D structure and volumetric size of structures such as spheroids represents a challenge in conventional light microscopy. This can be overcome by light sheet-based microscopy, which allows tracking of sub-cellular changes in large samples within reasonable time.

SEE STELLARIS DLS IN ACTION ...

DLS microscopy to investigate tumor spheroids

An example of how 3D cell biology workflows can be improved with LightSheet microscopy can be seen in this article, which shows how cancer development can be elucidated on a sub-cellular level using *in vivo* like tumor spheroid models.

ARTICLE

Improve 3D Cell Biology Workflow with Light Sheet Microscopy

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Figure 9: STELLARIS DLS Digital Light Sheet Microscope

STELLARIS 5 and STELLARIS 8 Digital LightSheet (DLS) microscopes unite in one place a confocal system and a light sheet microscope – a unique combination aimed at making research more versatile. The exclusive vertical design of DLS, enabled by Leica Microsystems' proprietary TwinFlect mirrors, allows users to combine confocal and light sheet imaging in the same system, so the microscopy method can be easily adapted to different experimental needs.

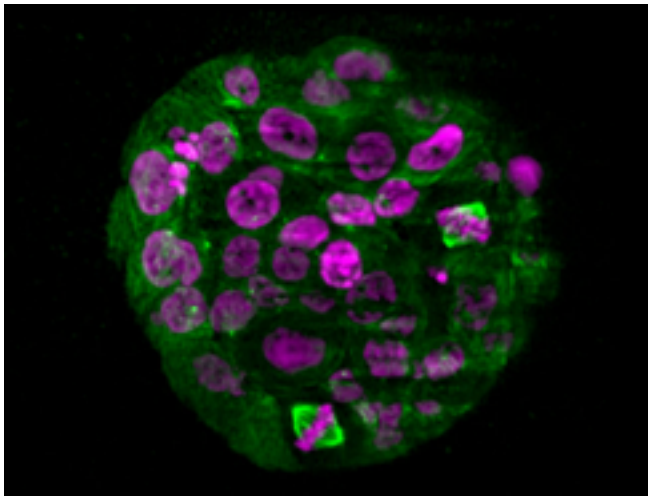

Figure 10: Light sheet experiments of organoids or spheroids with DLS allow you to image >100 µm in depth. Live mammary epithelial spheroid: green nuclei, (MCF10A H2B-GFP); red tubulin cytoskeleton (SiR-tubulin); DLS data processed with LIGHTNING. Courtesy of B. Eismann and C. Conrad, at BioQuant/DKFZ Heidelberg, Germany.

DLS brings flexibility to research with the capacity to image different types of samples, such as organoids, and the ability to take advantage of the full excitation spectrum. This flexibility is possible thanks to the STELLARIS White Light Laser and the ability to perform multi-position light sheet experiments whilst using standard glass bottom plates.

Aivia AI Image Analysis Software

Subjectivity of analysis and poor reproducibility are key hurdles to be overcome for biological image analysis. Standard segmentation can lead to sub-standard results and require substantial manual curation which is subject to human error. Using state-of-the-art, AI-first software architecture, Aivia is a uniquely innovative and complete 2-to-5D image visualization, analysis and interpretation platform designed for the reliable processing and reconstruction of highly complex images in just minutes.

THUNDER and Aivia

For scientists that want to rapidly capture, accurately segment and analyse their image data, even when a low light dosage and high temporal resolution is required, the unique combination of THUNDER imaging systems with Aivia AI Image Analysis Software is a powerful solution.

One of the challenges when using physiological conditions is that low incident light dosages result in low signal levels leading to a drop in contrast. THUNDER increases contrast significantly by removing the blur and haze seen with conventional widefield imagers, allowing clear marking of signal and background. Aivia's machine learning based object detection can then expertly process the images acquired using physiological light conditions. This combination improves speed and accuracy in image segmentation and allows for reliable reconstructions of complex samples such as organoids and spheroids.

FURTHER INFORMATION

STELLARIS DLS Digital Light Sheet Microscope

FIND OUT MORE

SEE AIVIA IN ACTION ...

Using THUNDER and Aivia to analyze immune cell populations

An example of how to remove out-of-focus blur, improve segmentation accuracy, and leverage the benefits of AI for image analysis can be found in this article.

ARTICLE

Accurately Analyze Fluorescent Widefield Images

READ NOW

STELLARIS and Aivia

Localization and selective imaging of rare events is key for the investigation of many processes in biological samples. Yet, due to time constraints and complexity, some experiments are not feasible which limits the horizon for new discoveries.

A rare event detection workflow based on AI-powered microscopy offers the potential to overcome these limitations by the synergistic fusion of an intelligent sample navigation, image-acquisition tool, and AI-powered image analysis. Autonomous Microscopy powered by Aivia for STELLARIS allows a highly economical operation for daily laboratory work that includes performing advanced experiments that would not be possible without automated procedures or considerable manual effort.

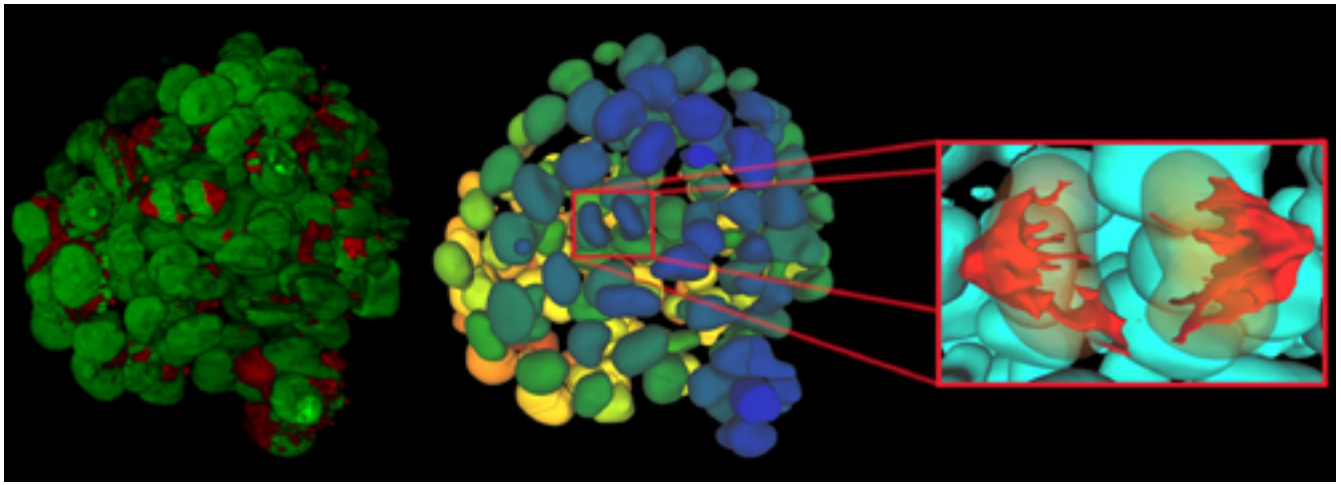

Figure 11: Aivia AI Image Analysis Software

This AI-based detection workflow for confocal microscopy automates the detection of rare events. It follows what the user has defined as the objects of interest that will trigger the rare event scan. Users benefit from the potential to discover more by automatically detecting up to 90% of rare events during an experiment. By focusing on the data that matters during the acquisition process itself, time to result can be reduced by up to 70%. The Aivia-powered workflow reduces time spent at the microscope, leading to increased productivity to do more! ■

In the following case studies, we show practical examples of how scientists have used Leica's imaging platforms to overcome some of the challenges of imaging organoids and spheroids.

SEE AIVIA IN ACTION ...

Autonomous microscopy powered by Aivia

An example of an AI-based workflow for fast rare event detection in living biological samples.

ARTICLE

AI Microscopy Enables the Efficient Detection of Rare Events

READ NOW

CASE STUDY

STUDYING HUMAN BRAIN DEVELOPMENT AND DISEASE

In this case study, researchers investigated the therapeutic and regenerative potential of lung organoids. They aimed to understand the transdifferentiation of lung cells and develop personalized therapies for lung diseases. The cultivation and imaging of lung organoids posed unique challenges as these cells required an air-liquid interface for proper growth. Previous imaging techniques often led to sample damage or loss during removal from the cultivation environment. However, by utilizing Leica THUNDER imaging technology, the researchers were able to overcome these challenges and obtain high-quality images of the lung organoids, enabling them to visualize cellular details and analyze tissue architecture.

Introduction

Studying human brain development and neurological disorders requires access to intact and functioning human brain tissue, which is often challenging.[1] However, recent advancements in 3D neural cultures derived from human induced pluripotent stem cells (hiPSCs) have provided valuable tools for modelling brain development and understanding the underlying pathological mechanisms of neurological disorders.[1,2] In this case study, the effectiveness of THUNDER imaging technology in obtaining sharp, high-resolution images of fluorescently labelled cortical spheroids was demonstrated, enabling improved visualization and analysis of these complex 3D structures.

The challenge

Imaging thick 3D biological specimens, such as neural spheroids, poses significant challenges. Conventional widefield camera-based fluorescence microscopy, while easy to use and fast, often results in images with an out-of-focus blur or haze when applied to visualize thick specimens. This blur reduces image contrast and hampers accurate analysis.

Methods

To address these challenges, live cortical spheroids with a diameter of 1.7 mm were imaged using the THUNDER Imager Model Organism and THUNDER Imager Tissue. The THUNDER Imager Model Organism utilized a 2x, 0.15 NA objective and a 3.4x zoom factor, while the THUNDER Imager Tissue employed a 10x 0.32 NA PL FLUOTAR air objective. Both imaging systems utilized Large Volume Computational Clearing (LVCC) to improve contrast and resolution and eliminate the out-of-focus blur.[3]

Results

THUNDER imaging technology proved to be a time-efficient solution for imaging and screening neural spheroids. The THUNDER Imagers offer the speed of widefield microscopy combined with the power of computational clearing. The cleared cortical spheroid images obtained with both the THUNDER Imager Model Organism (**Figure 12**) and THUNDER Imager Tissue showcased the successful capture of virally labelled neurons and astrocytes in high contrast and resolution. The THUNDER Imager Model Organism enabled observation of whole spheroids and distinct regions, facilitating rapid specimen screening.

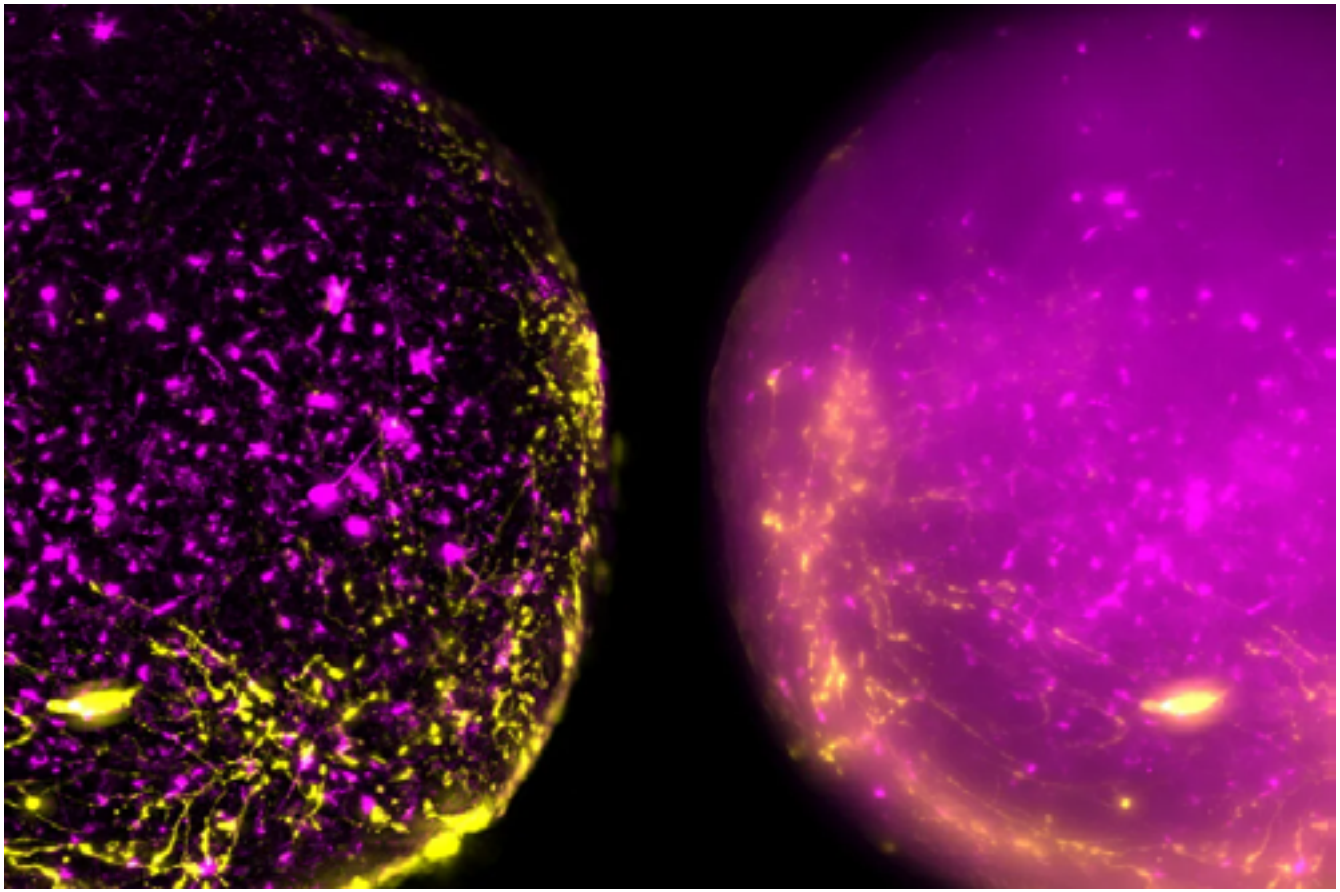

Figure 12: Virally labeled neurons (magenta) and astrocytes (yellow) in a cortical spheroid derived from human induced pluripotent stem cells (iPSCs). A THUNDER Model Organism Imager with a 2x 0.15 NA objective at 3.4x zoom was used to produce this 425 µm Z-stack (26 positions), which is presented here as an Extended Depth of Field (EDoF) projection. Shown are both the raw widefield image and the same image after Large Volume Computational Clearing (LVCC) is applied. Images courtesy of Dr. Fikri Birey from the Pasca laboratory at Stanford University.

Conclusion

By utilizing THUNDER imaging technology and LVCC, sharp and clear images of cortical spheroids were achieved, overcoming the out-of-focus blur commonly encountered with widefield systems. THUNDER Imagers provided enhanced contrast and sharper details throughout the 3D image reconstruction process, allowing for improved visualization and analysis of cortical spheroids. The THUNDER Imager Model Organism, in particular, enabled efficient screening of specimens by offering the ability to freely zoom in and out, facilitating observation of whole spheroids and specific regions. This combination of THUNDER imaging and computational clearing technology offers a valuable tool for studying human brain development, neurodevelopmental disorders and related research areas. ■

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CASE STUDY

EXAMINING "BRAINS-IN-A-DISH" FROM INDUCED PLURIPOTENT STEM CELLS (iPSCS)

This second case study focuses on studying human brain development and disease using 3D neural cultures derived from human induced pluripotent stem cells (hiPSCs). Imaging thick 3D biological specimens, such as neural spheroids, posed significant challenges. Conventional widefield fluorescence microscopy often resulted in images with an out-of-focus blur or haze, reducing image contrast and hampering accurate analysis. The researchers utilized Leica THUNDER imaging technology to address these challenges and obtain sharp, high-resolution images of fluorescently labeled cortical spheroids. This improved visualization and analysis of the complex 3D structures within the spheroids, enabling a deeper understanding of human brain development and neurological disorders.

Introduction

Glia are crucial components of the central nervous system (CNS) and play vital roles in regulating its function.[1] However, studying human glia and their contributions to CNS development and disease has been challenging due to limited access to primary samples at critical developmental stages.[1] To overcome this limitation, advancements in human stem cell differentiation protocols have allowed the generation of iPSC-derived cortical brain organoids, serving as functional 3D "brains-in-a-dish". These organoids offer a model system to investigate glial development and its implications for neurodevelopmental disorders such as autism-spectrum disorders.[1] To address research questions using *in vitro* systems, it is essential to perform multi-channel fluorescence imaging of various cell types across a large 3D volume and over extended periods.

The Challenge

Imaging thick biological specimens, such as organoids, requires an imaging solution that enables rapid screening over a large area while ensuring high-magnification imaging with excellent contrast deep inside the specimen. Widefield fluorescence microscopy is widely used for its ease of use, speed and detection sensitivity. However, imaging thick specimens often results in blurred or hazy images, reducing contrast. This haze is caused by fluorescence signals detected from out-of-focus planes within the specimen.[2]

Methods

Human cortical organoids, resembling 3D brains-in-a-dish, were generated from iPSCs derived non-invasively.[1] The iPSCs were infected with the pAAV-hSyn-EGFP virus, which uses the human synapsin promoter to fluorescently label neurons (green), and the pLX-hGFAP-mCherry virus, which uses the human glial fibrillary acidic protein promoter to fluorescently label astrocytes (red).[1] To image deep inside the 3mm-thick organoids, the researchers employed the THUNDER Imager 3D Cell Culture. To enhance image clarity, they utilized the Large Volume Computational Clearing (LVCC) method developed by Leica.[2]

Results

The researchers successfully captured images of a thick brain organoid derived from iPSCs using the THUNDER Imager 3D Cell Culture. Figure 13 presents the 36th plane cropped from a 53-plane Z-stack volume, showcasing both the raw widefield image (A) and the same image after applying Large Volume Computational Clearing (LVCC) (B). Neurons are labelled in green, while astrocytes are labelled in red.

Conclusion

The results demonstrate the effectiveness of the THUNDER Imager 3D Cell Culture combined with LVCC in eliminating the out-of-focus blur or haze commonly encountered when imaging thick cortical organoids. ■

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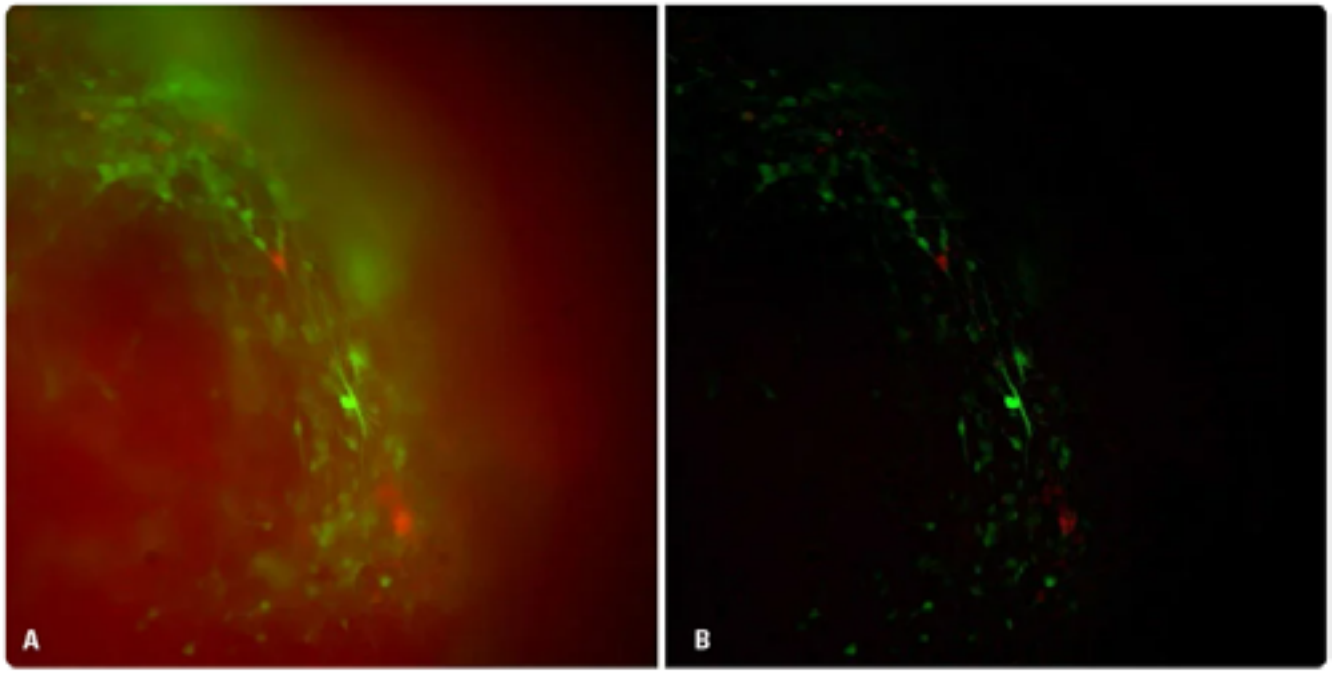

Figure 13: Images of a brain organoid derived from iPSC cells acquired with a THUNDER Imager 3D Cell Culture. The cells were infected with the pAAV-hSyn-EGFP and pLX-hGFAP-mCherry virus. The image is the 36th plane cropped out of a 53 plane Z-stack volume. Shown are both the A) raw widefield image and B) the same image after Large Volume Computation Clearing (LVCC). Neurons are labeled in green and astrocytes in red.

CASE STUDY

OBSERVING 3D CELL CULTURES DURING DEVELOPMENT

In this case study, researchers in Germany cultured lung organoids from transgenic mice, in order to study potential new therapeutic approaches for lung diseases such as influenza. The cultivation of these organoids can be very complex as they only generate at an air-liquid interface, similar to the human body, and removing them for microscopy evaluation can damage the sample. In the past, a widefield microscope could only be used to check if the fluorescent transgenes were expressed at all. Initial tests with a THUNDER Imager showed the formation of organoids down to the level of individual cells.

Introduction

Traditional experimental techniques for imaging organoids or spheroids typically included imaging at the end of the workflow, as the samples would need to be removed from the cultivation environment.

This physical manipulation often resulted in a loss of sample material or malformation of 3D structure. This meant that 3D cell cultures could only be observed when they had been fully developed. Imaging as an endpoint measurement on potentially damaged samples can be a frustrating experience. Handling a sample gently to retain its natural growth and cellular interactions is immensely important to understand the mechanisms of disease. To observe 3D cell cultures during growth, the microscope must adapt to the sample and provide an environment suitable for cultivation, and imaging should not influence or interfere with further development. In addition to low mechanical stress during sample handling, this requires low-light stress by using low-light intensities and short exposure times. With the THUNDER Imager systems, Leica Microsystems provides solutions that retain the favorable characteristics of widefield microscopes for sample handling, speed and low phototoxicity.

Part 1: The therapeutic, regenerative potential of lung organoids

Research is being carried out to investigate the therapeutic potential of progenitor cells of different cell types in the case of acute and chronic lung diseases, such as influenza. Based on established transgenic mouse models, special cells were isolated and co-cultured (a complex model system for the simultaneous cultivation of different cell types) with bone marrow-derived cells. The focus was on characterizing cell-cell communication at the molecular level, as well as transcriptional changes. The aim of this study was to identify key molecules that cause transdifferentiation of pneumocytes from type 2 to type 1 (mainly responsible for gas exchange in the lung).

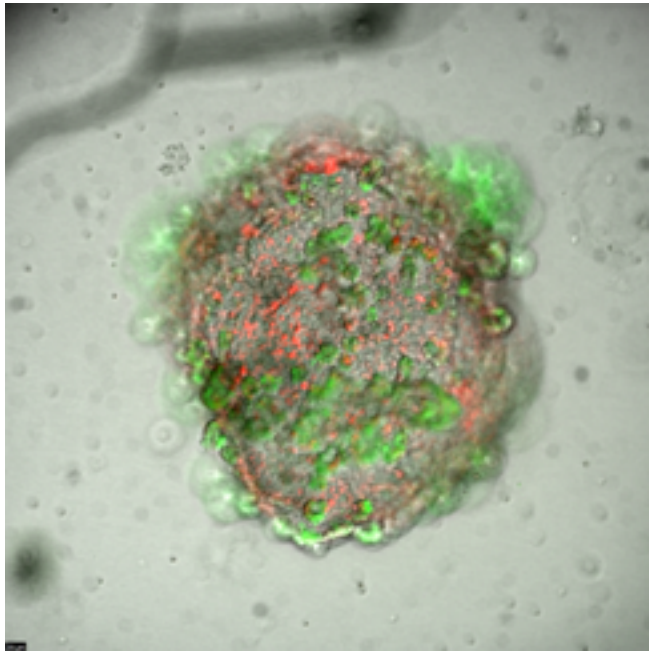

Figure 14: Lung organoid taken at the "liquid-air interface" with a THUNDER Imager 3D Cell Culture. The cells originate from transgenic mice, so that the different fluorescence represents the degree of differentiation of the respective cell (superposition). The image acquisition was performed on day 21 after the start of the culture. Courtesy of P. Kanrai, MPI-HLR Bad Nauheim.

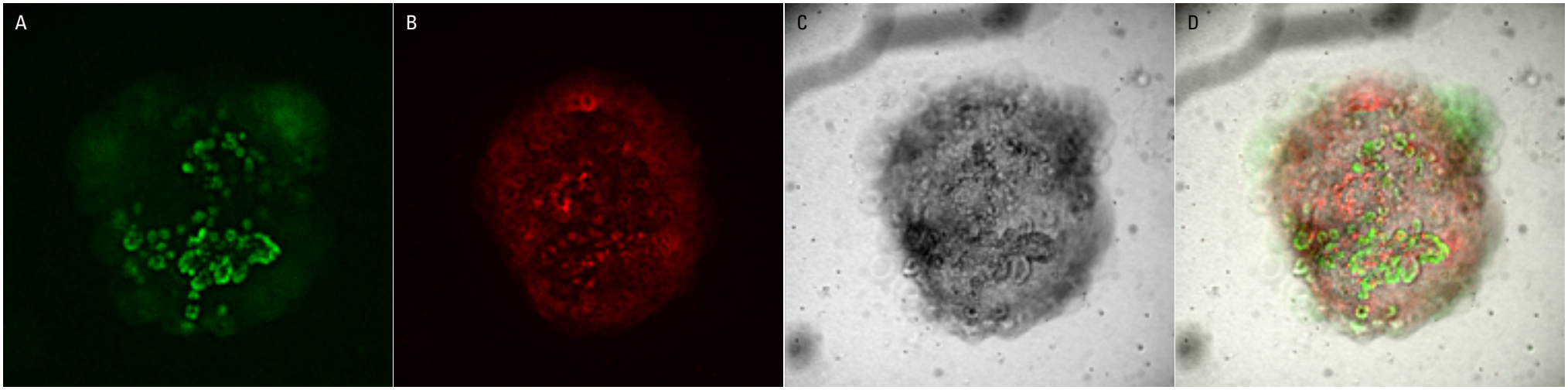

Figure 15: The images show a mature lung organoid taken at the "liquid-air interface" with a THUNDER Imager 3D Cell Culture. The cells in these systems originate from transgenic mice, so that the different fluorescence represents the degree of differentiation of the respective cell (A is YFP, B is mCherry, C is BF, and D is a superposition image). The image acquisition was performed on day 21 after the start of the culture. Images courtesy of P. Kanrai, MPI-HLR Bad Nauheim.

This mechanism is often triggered by infections, but it is not yet understood in detail. In the long term, the research should make it possible to develop a personalized form of therapy tailored to help a patient after a lung infection, for example, and allows the damaged lung tissue to be regenerated.

The Challenge

The cultivation of lung organoids can be complex. Lung cells only generate at an air-liquid interface – similar to the human body. For this research, a 12-well plate holding nutrient medium and a porous membrane insert was used. On these inserts, the desired 3D cell cultures develop at the transition from matrix to air (Figure 14). Owing to the different media and refractive indices, imaging is a challenge with this setup. It is not possible to remove the cells from the cultivation environment and return them for microscopic evaluation without damaging the valuable specimen. In the past, it was only possible to use a widefield microscope to check whether the fluorescent transgenes were expressed at all.

Results

Initial tests with a THUNDER Imager have already shown the formation of organoids down to the level of individual cells (Figure 15).

Part 2: Understanding brain development

Another important area of research for this group focuses on achieving an improved understanding of the development and folding of the brain. To conduct this work, ferrets are used as model organisms. In contrast to the brain of mice, the much larger ferret brain has as many folds as the human brain and, therefore, is much better suited for these types of biological investigation due to its greater similarity to the human brain.

To understand normal brain development in ferrets, human genes were introduced into the ferret brain. Brains were genetically modified so that certain proteins could be localized during imaging. In the future, mutations could also be systematically investigated. The goal is to understand the patterns of brain folding, which are large macrostructures.

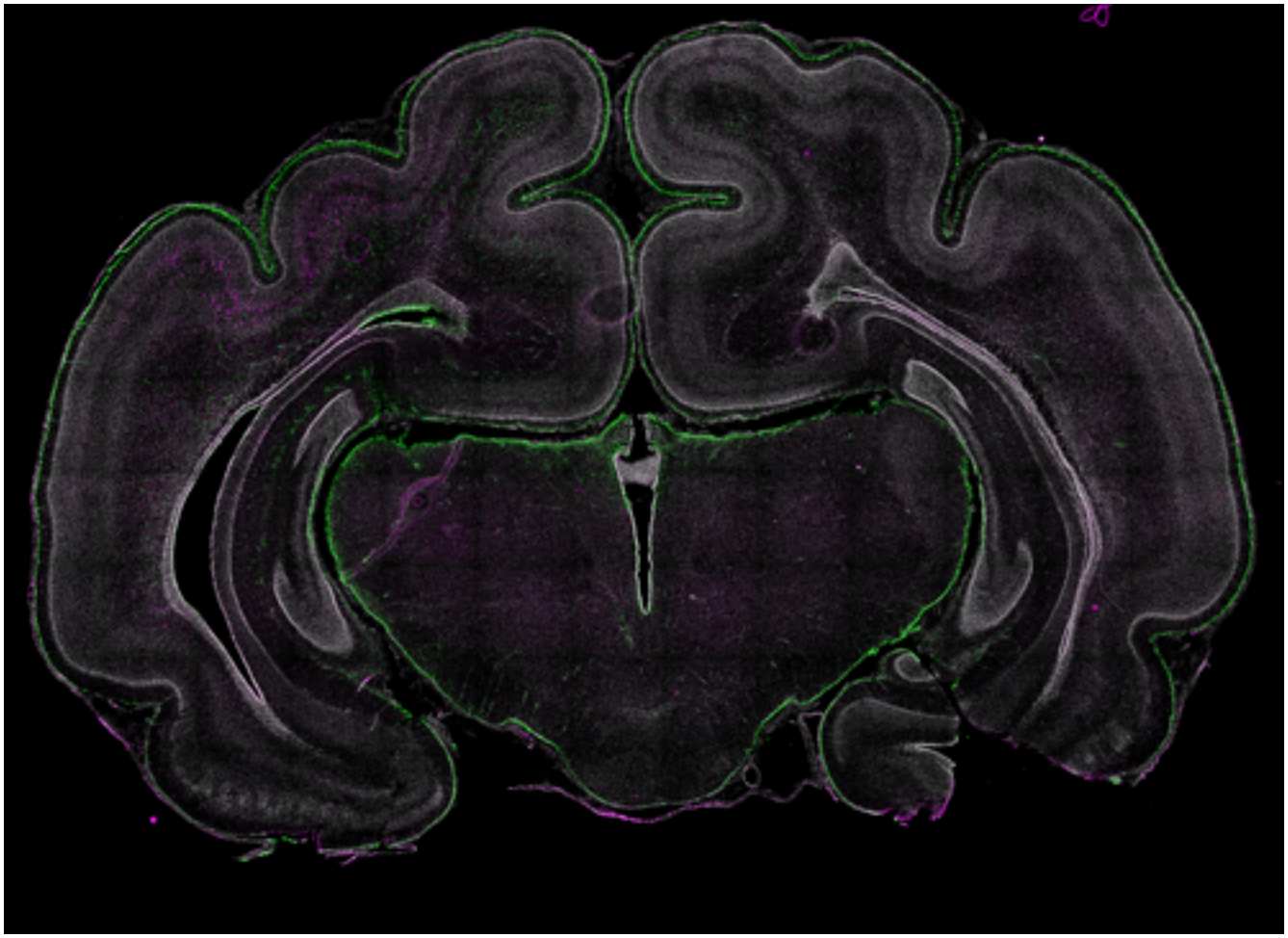

Figure 16: An overview of the developing ferret brain recorded on a THUNDER imager with Computational Clearing applied. Marked here are star-shaped glial cells (astrocytes in green) as well as the various developing neuron species (MCherry, magenta, marked using *in utero* electroporation).

The Challenge

In order to capture the entire sample context, the imaging focus is on a large tissue area. To quantify relevant parameters, individual cells must be recognizable within the same image. This requires a very large image field with sufficient resolution and clear imaging. Confocal microscopy is used in a further step to examine certain regions in detail on the subcellular level.

Results

The size of a brain section at the stage of development that is relevant for this examination is approximately 2cm^2. For such large specimens, a THUNDER Imager with a large image field and clear image of the sections enables an efficient workflow (**Figure 16**).

The resolution and contrast improvements provided by computational clearing allow cell nuclei to be easily distinguished and is the basis for meaningful and reliable results in terms of quantification. Until now, the same tissue sections had to be visualized with multiple imaging systems to achieve a proper post-processing of the images. Once acquired, THUNDERed images are almost ready for publication, which is a great advantage as researchers with less experience in image processing can quickly produce meaningful results.

Conclusion

Thick 3D cultured cell specimens provide data with more physiological relevance in terms of gene expression and morphology. When creating and maintaining 3D cell cultures, it is advantageous for scientists to have practical ways to easily image and analyze their results. The structure of organoids and spheroids can be difficult to image, so care must be taken to be gentle with the specimen.

THUNDER Imagers provide a fast, gentle, accurate and reproducible way to image organoids and spheroids, combining the speed and high-throughput capabilities of widefield microscopes with the power of computational clearing. Upright-, stereo- and inverted-microscope-based THUNDER Imagers allow intuitive imaging for various applications, such as neurospheres, lung organoids or tumor spheroids. THUNDER Imagers provide improved contrast and resolution in comparison to conventional widefield imaging systems, enabling researchers to gather insights into their research more efficiently and to expand their range of applications. ■

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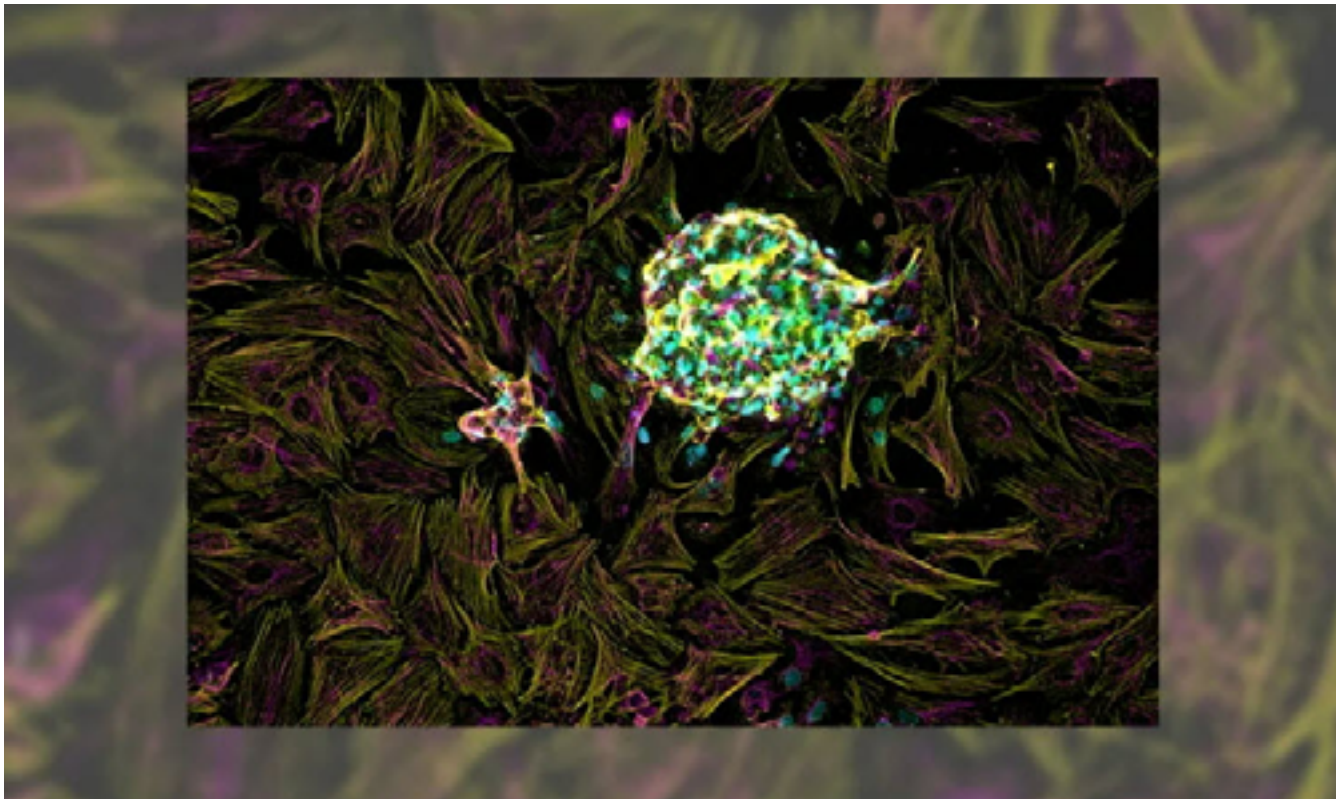
CASE STUDY

DEVELOPING HEART PACEMAKER CELLS FROM CARDIAC SPHEROIDS

In the past decade, 3D cell cultures have emerged as a superior model compared to traditional 2D culture systems. By forming miniature 3D structures called spheroids, cells can mimic organ function and development more realistically. This makes them invaluable for studying diseases *in vitro* and holds the potential for producing implantable "spare parts" to cure diseases.



Introduction

Arrhythmias, affecting millions of people, are characterized by irregular heartbeats. Typically managed with artificial pacemakers, which can impact the patient's quality of life, arrhythmias pose a challenge for children due to the size of these electronic devices. To address this issue, one research laboratory in the Department of Biomedical Engineering at Georgia Tech and Emory University aims to develop functional pacemaker cells from ordinary heart muscle, eliminating the need for hardware.[1] Such research requires the utilization of 3D cell culture and spheroids, presenting challenges for high-resolution microscopy observations.[2]

The Challenge

While widefield microscopy offers advantages in terms of speed, viability and cost, imaging large spheroids with multiple fluorescent channels using traditional light microscopy methods results in significant out-of-focus haze. This limitation hampers the documentation of spheroid growth and function.

Methods

Spheroids were derived from neonatal rat ventricular myocytes (NRVM) cultivated on specialized plates. A monolayer cell culture was then applied to cover the spheroids.[1] Staining with alpha-actin and vimentin antibodies followed. Utilizing the THUNDER Imager Live Cell, a Z-stack of approximately 60 µm was acquired, incorporating the opto-digital technique known as Instant Computational Clearing (ICC).[3] A maximum projection of raw widefield and THUNDER ICC images was generated for comparison.

Results

The THUNDER images, processed with computational clearing, offer enhanced details of the spheroids compared to classical widefield images. Figure 17 demonstrates the ability to explore the dynamics between the monolayer cells and those within the spheroid more effectively. ■

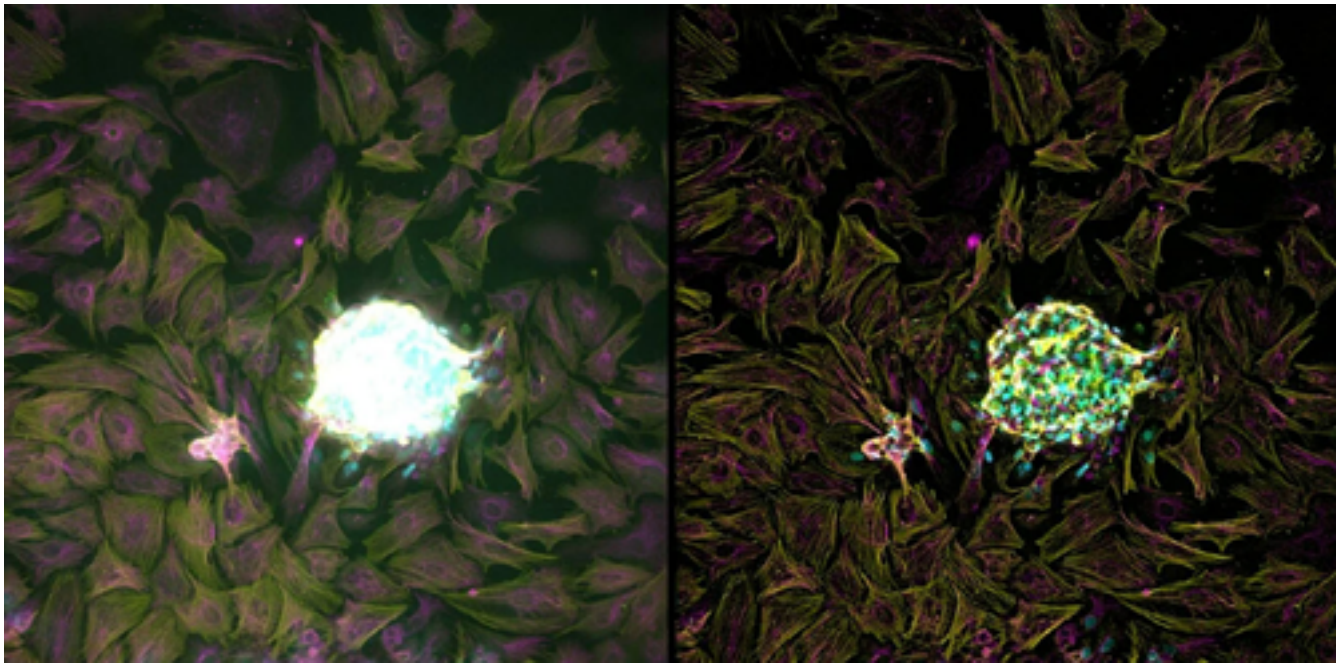


Figure 17: Spheroid shown here as a maximum projection of the raw widefield image data (left) and THUNDER image after Instant Computational Clearing (right). The images are derived from approximately 60 µm Z stacks. Different stains (alpha actin and vimentin) are used to help identify the various cell types. Images courtesy of Sandra Grijalva, Wallace H. Coulter Department of Biomedical Engineering, Georgia Tech and Emory University, Atlanta, USA.

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CASE STUDY

IMAGING ANTI-CANCER DRUG UPTAKE IN SPHEROIDS USING DLS MICROSCOPY

Spheroid 3D cell culture models are a useful tool to study tumor morphology and screen anti-cancer drugs. The drug AZD2014 is a recognized inhibitor of the mammalian Target Of Rapamycin (mTOR) pathway.[1] Aberrant activation of mTOR promotes tumor growth and metastasis and has led to AZD2014 clinical trials as an anti-cancer molecule. However, the anti-tumor mechanism of AZD2014 is not currently well understood. In this study, Ahmed *et al.*[1] presented imaging data from the AZD2014 treated spheroids using Digital LightSheet (DLS) microscopy and shed light on how this small molecule elicits it anti-tumor activity.

Introduction

The mTOR pathway co-ordinates the availability of nutrients, growth factors, and the energy status of the cell with the activation of its downstream target proteins via phosphorylation events.[2] In this cascade signaling pathway, these downstream substrates are responsible for regulating functions, such as cell proliferation, protein synthesis, autophagy, senescence, and apoptosis. The mTOR protein exists in two complexes; Complex 1 (mTORC1) and Complex 2 (mTORC2). The broad functionality of the mTOR protein makes it an attractive drug target in diseases closely associated with dysfunctional or hyper-activated mTOR signaling such as cancer.

First generation mTOR inhibitors, such as rapamycin and clinically approved analogues (rapalogs), even though they initially showed effectiveness in previous preclinical models, turned out to only partially inhibit mTOR by targeting Complex 1. Recent studies have also shown that tumors develop rapalog resistance to first generation mTOR inhibitors and exhibit effectiveness in few types of cancer. As mTORC2 directly phosphorylates Akt, an important protein involved in cell survival, there is a likely need to inhibit both complexes for effective cancer treatments.

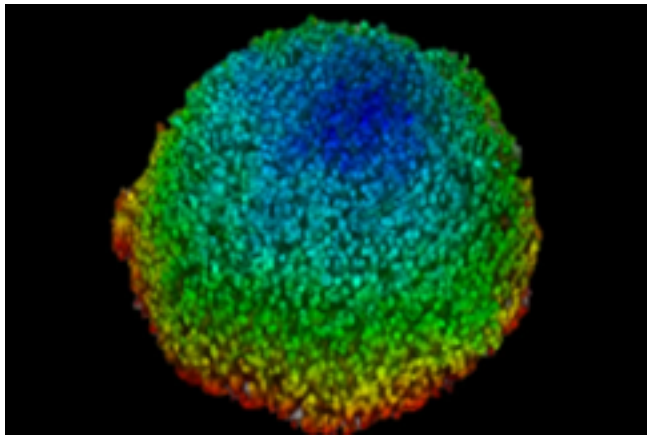

Figure 18: 3D volume rendered light sheet microscope image of a spheroid showing depth coding in different colors.

Novel second-generation ATP-competitive inhibitors, such as AZD2014, provide improved antitumor activity by targeting both mTORC1 and mTORC2[3]. Although AZD2014 is currently undergoing active clinical trials, its mode of action is unknown. To investigate the mechanism of AZD2014, DLS microscopy was used to observe the cellular uptake of AZD2014 within a living 3D cell culture model environment, taking advantage of its naturally occurring fluorescence properties and its localization within living spheroids.[5]

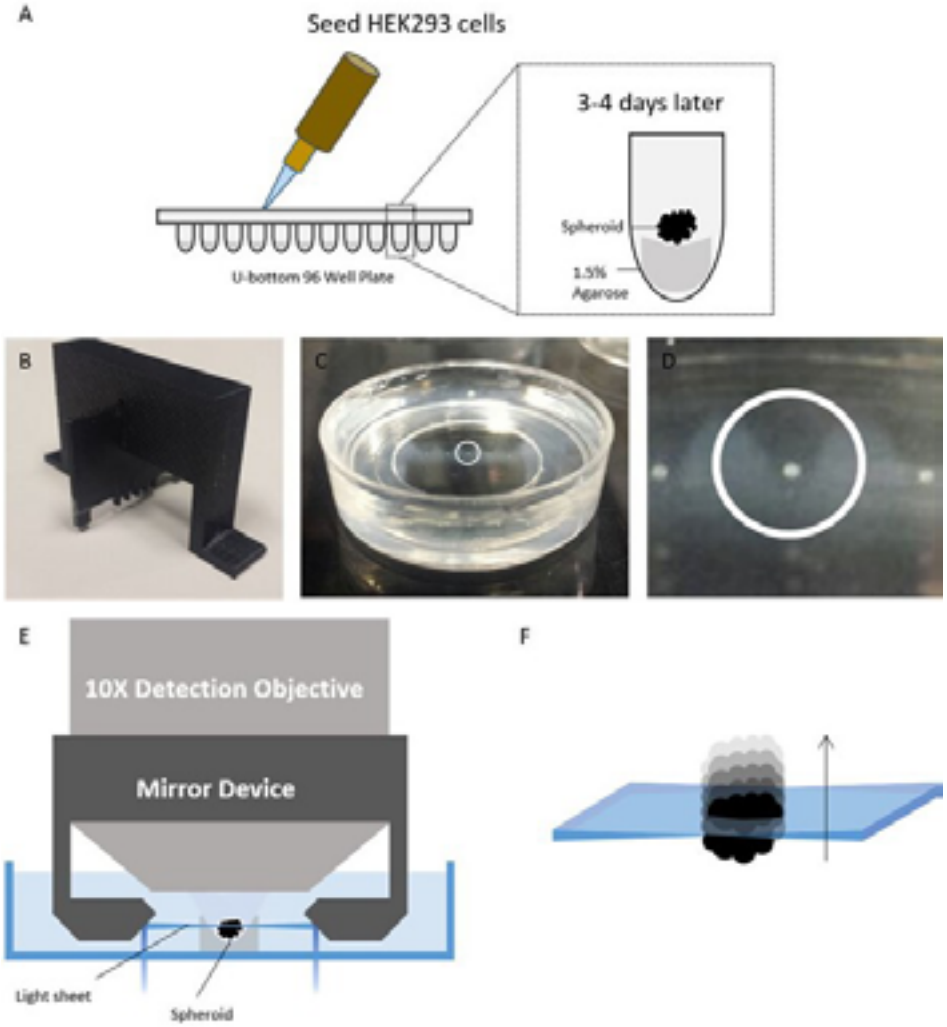

Figure 19: Spheroid seeding, mounting, and imaging procedure. (A) HEK293 cells were seeded into a 96-well round (U-well) bottom plate pre-cast with 100 µl of 1.5% agarose at a cell density of 10,000 cells per well with a final volume of 200 µl in complete growth media. (B) A 3D-printed comb used to create wells in agar to hold the spheroids during the imaging experiment. (C) The spheroids were then transferred to the wells. The white circle shows a single spheroid in a well. (D) Zoomed-in view of the circled spheroid in the panel. (E) Principle of the Leica DLS fluorescence microscope with the light sheet created between two mirrors where the spheroid is placed. (F) The spheroid is moved through the light sheet and is optically sectioned. Figure taken from Ahmed *et al.*[1]

Methods

To investigate AZD2014 within the spheroid environment, HEK293 cells were cultured in 96-well round-bottom plates (U-well). After growing the cells for 72 hours at 37˚C and 5% CO_2, the HEK293 cells self-assemble into spheroids. The spheroids were then transferred to low-melting-point-agarose holders sitting in 35-mm glass-bottom dishes, each dish containing wells capable of holding up to 5 spheroids. Once all 5 spheroids were loaded, the glass-bottom dish was filled with complete growth media and subsequently placed onto the microscope (**Figure 19**).

After adding AZD2014 (to a final concentration of 7 µM), the samples were imaged at 37 °C and 5% CO_2 using a DLS microscope to acquire 3D time-lapse images of the spheroids (**Figure 20**). To monitor the uptake of the drug into spheroids, 44 planes were recorded every 15 seconds for a total of 2 hours. The dimensions of the xyz stacks were 780 µm × 780 µm × 300 µm.

Results

This study examined the effects of AZD2014 in a tumor environment by first characterizing its uptake within a spheroid 3D cell culture model. After addition of AZD2014, a clear increase in AZD2014 intrinsic fluorescence could be observed. The outer layers of the spheroids showed a faster rate of uptake than the inner core of the spheroid (**Figure 20**). This became evident by looking at the different uptake rates at three selected depths relative to the spheroid surface [0 µm (surface), 100 µm (inner layer), and 200 µm (deeper layer)]. The corresponding rates were 80, 200, and 318 seconds after addition.

Furthermore, a 25% (std = ±0.019%) increase in the average radius of the spheroids could be seen after 30 minutes exposure to AZD2014 (7 µM). This increase in size occurred only when both the drug and 405 nm illumination were present on the samples. The latter finding suggests a unique, previously unknown, photoactivable property of this molecule.

The data suggests that AZD2014 may have the potential to act as a photoactivable drug that could be used in anti-cancer photodynamic therapies.

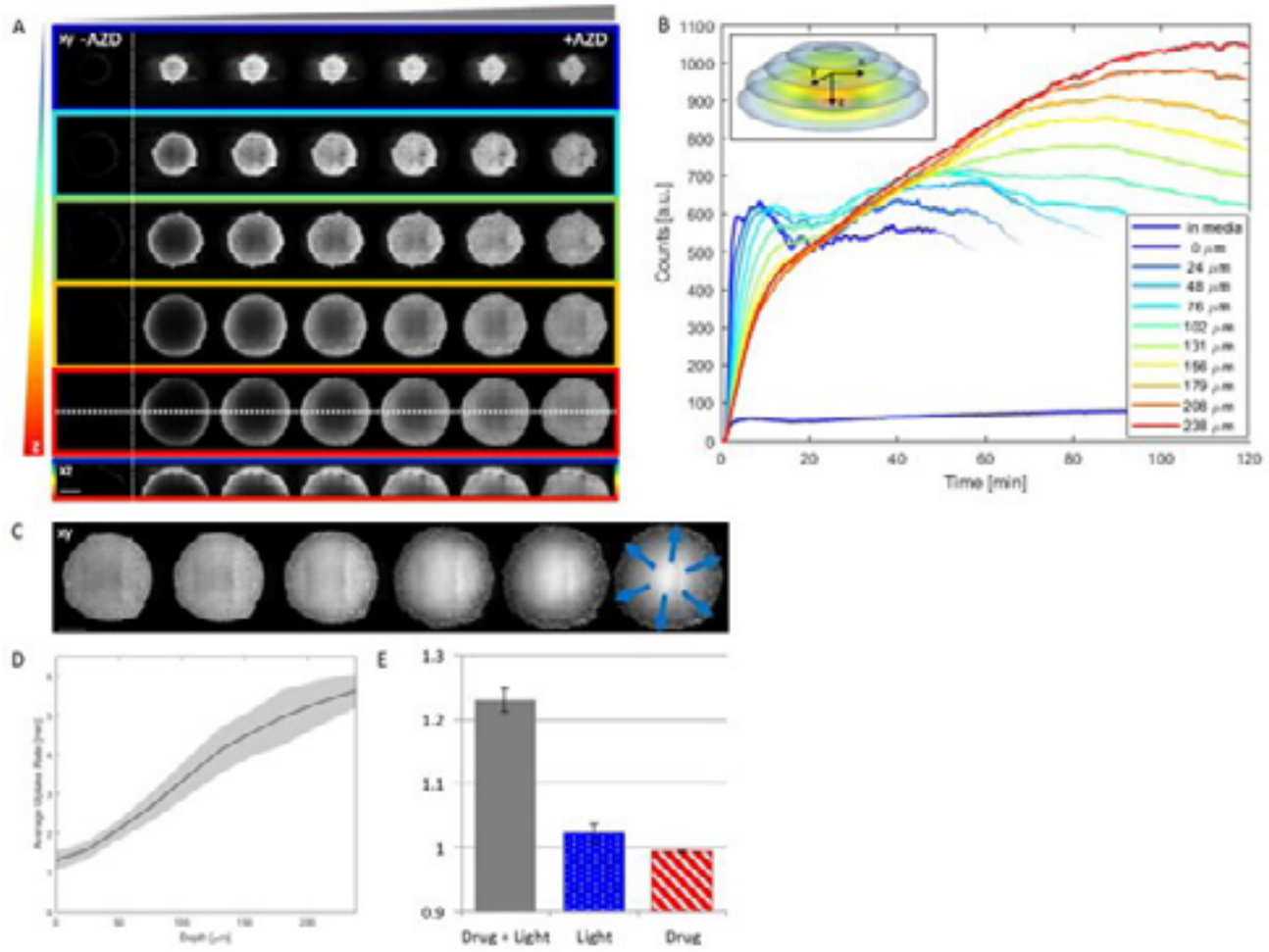

Figure 20: Uptake of AZD2014 in 3D multi-layered spheroids. (A) AZD2014 administration and imaging of HEK293 spheroid. Different xy planes or depths (rows) are shown as well as an orthogonal projection (xz plane). (B) Uptake rates of AZD2014 fluorescence in spheroids over a 2-hour time-lapse were studied at different depths from the surface. (C) Spheroid radius increase during imaging with AZD2014 administration. Image planes at 250 µm depth is shown at different time points from 30 min to 2 hours after administration. (D) Mean rates of AZD2014 uptake at different depths. (E) Relative increase in spheroid radius vs AZD2014 + 405-nm illumination, only 405-nm illumination, or AZD2014 only. Figure taken from Ahmed *et al.*[1]

Conclusion

The ability to non-invasively monitor drug localization and behavior within living cancer cells in a 3D environment is a powerful way to gain a better understanding of tumor biology and improve cancer drug screening and development. Imaging with the TCS SP8 Digital LightSheet microscope in this study enabled the visualization of dynamic interactions of drug-administered cells within 3D tumor spheroid models by:

- Giving the user unrestricted access to their samples due to its set-up, which allows easy and effective delivery of the drug or compound of interest during the workflow described here
- Combining gentle and fast light sheet imaging with good subcellular resolution, allowing the study of dynamic processes like drug uptake or response in living samples
- Facilitating good penetration and image quality of 3D samples like spheroids due to dual-sided light sheet illumination and system-specific deconvolution algorithms
- Allowing imaging of multiple spheroids within one round of imaging and therewith provides a workflow that delivers more data for quantitative evaluation during a single experiment. ■

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GOING DEEPER INTO 3D WITH CORRELATIVE LIGHT AND ELECTRON MICROSCOPY

When it comes to obtaining information on nanometer, or even subnanometer, characteristics within cells, scientists turn to electron microscopy (EM) techniques such as scanning or transmission electron microscopy (SEM or TEM respectively), or electron tomography (ET), which allow high-resolution images of biological specimens to be obtained. The art of obtaining a great image lies in precise sample preparation: ensuring the nanoscale cellular feature is identified, and "frozen" in time so it can be imaged with EM. This is enabled by correlative light and electron microscopy (CLEM) which combines the versatility of the types of light microscopy techniques we have discussed previously in this eBook, with the sub-nanometer resolution power of EM (**Figure 21**).

Techniques such as Array Tomography (AT) can offer time-saving advantages for specimen preparation when doing CLEM. Furthermore, advances in correlative systems, including cryo-CLEM and 3D volume imaging have led to breakthroughs in cellular imaging.

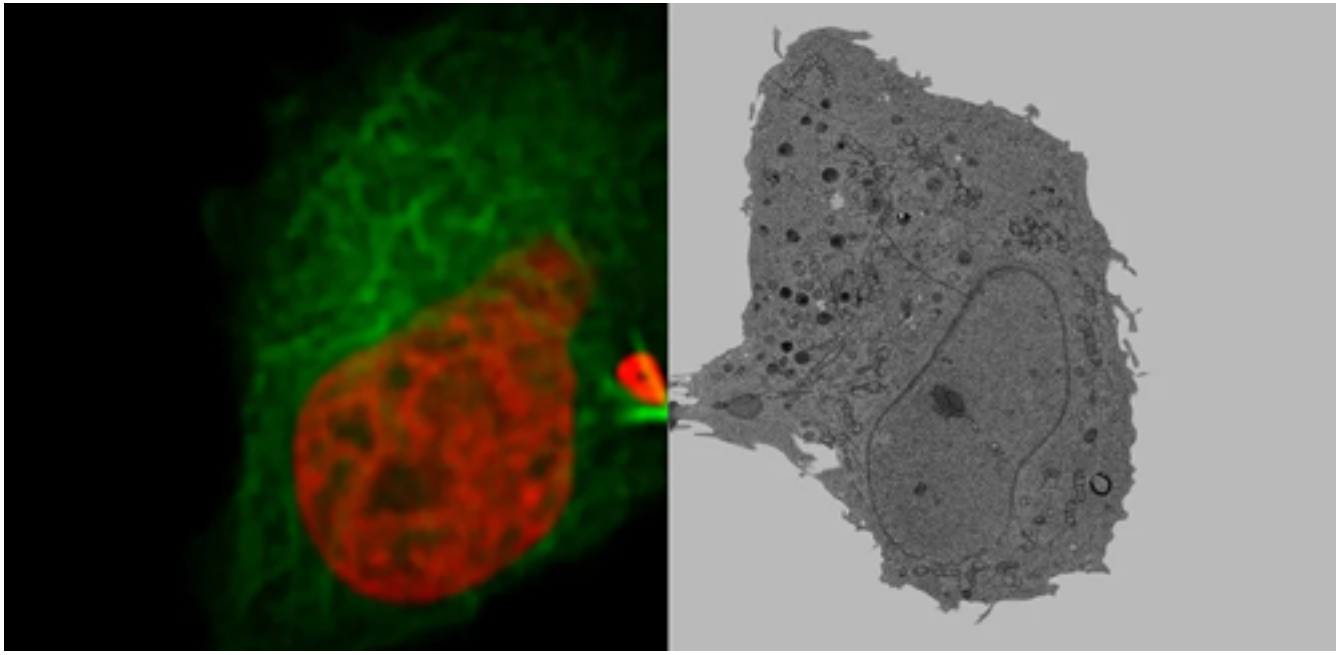

Figure 21: HeLa cells expressing H2B-mCherry and alphaTubulin-mEGFP to visualize microtubules and DNA. Left: Widefield imaging using THUNDER. Right: Sample after correlated EM imaging using Leica Microsystems Coral Life workflow.

Live Cell CLEM

Traditional CLEM workflows are not ideal for studying fast-moving processes or precisely localizing events because there is a time-delay between the live-cell imaging and EM due to the need to fix the sample and transfer to EM. However, correlative microscopy workflows have evolved to address these challenges, enabling the study of fast-moving cellular processes and precise event localization. Unlike conventional CLEM, which involves fixing and processing the sample before EM imaging, live cell CLEM aims to bridge the temporal gap by directly transitioning from live cell imaging to EM while preserving cellular dynamics.

Live Cell CLEM can be achieved using workflows such as High Pressure Freezing (using Leica's EM ICE), which rapidly freezes cellular dynamics to enable subsequent high-resolution EM imaging. This allows the visualization of ultrastructural details of cells, such as the morphology of organelles, membranes, and other cellular components. Furthermore, capturing marker coordinates of target regions of interest (ROIs) observed during the light microscopy imaging step, facilitates the alignment and correlation of the light microscopy and EM images. This correlation enables accurate mapping of structures observed under the light microscope onto the high-resolution electron microscopy data. 3D reconstruction techniques are then applied to merge the information obtained from both modalities, resulting in a comprehensive 3D representation of cellular structures and their dynamic changes. The correlated 3D images provide insights into the spatial organization, interactions, and dynamics of cellular structures and components. Processes such as endocytosis, exocytosis, organelle movement, and much more, can be studied with a level of detail not achievable using either light microscopy or EM alone.

SEE IT IN ACTION ...

How to Successfully Perform Live cell CLEM

Practical guidance on how to effectively conduct live cell CLEM for dynamic cellular imaging, using a workflow from Leica Microsystems called Coral Life, can be found in this article. In this example, researchers wanted to study the final separation of two mitotic cells in a process known as abscission. After cytokinesis, the two dividing cells are only connected by an intercellular bridge which needs to be resolved. Because of its size, the mechanism of how intercellular bridges are resolve, leading to final cell separation, cannot be fully understood by live cell imaging. Therefore, ultrastructural analysis is needed.

ARTICLE

The Coral Life Workflow

READ NOW

Array Tomography

AT is a 3D image reconstruction technique for biological specimens. It involves the serial acquisition of ultrathin sample sections using a specialised microtome, subsequent imaging with SEM, and then reconstruction in 3D, thus enabling high resolution images of sample volumes. AT offers high resolution of EM in a spatial context and can reveal additional information about cellular structures to clarify poorly understood or unresolved features. The workflow for AT is usually facilitated by using an automated ultramicrotome that can achieve uniform, ultrathin serial sections for orderly array image acquisition with the SEM, such as the ARTOS 3D from Leica Microsystems. The following case study on page 30 examines how faster 3D imaging of cellular structures can be achieved using AT.

Cryo-ET and 3D Targeting

Cryo-ET is a dedicated TEM technique where a 3D volume of the observed area can be reconstructed. With state-of-the art cryo-EM, resolutions down to an incredible sub-nanometer range can be achieved. This enables biomolecules to be investigated in their context within the cell, thus allowing previously unknown molecular mechanisms to be revealed. As cells and tissue must be thin enough to allow electron transmission, samples must be prepared to obtain a thin sample volume (lamella). Beside cryo ultramicrotomy, focused ion beam (FIB) milling, using a dedicated or multi-modal cryo scanning electron microscope is the method of choice for this.

Imaging at sub-nanometer resolution comes with a major challenge in that sites of interest need to be located and precisely targeted. For correlative workflows, it is crucial to retrieve the exact target coordinates identified in the cryo light microscope because fluorescence signals are not visible in subsequent electron microscope steps. Cryo-confocal microscopy is therefore an essential tool for identifying target sites in the samples precisely in 3D.

SEE IT IN ACTION ...

How to target fluorescent structures in 3D for cryo-FIB

This whitepaper describes the main steps of the cryo-ET workflow and how targets are identified by cryo-confocal microscopy and provided for focused ion beam (FIB) milling to improve the reliability of the workflow.

ARTICLE

Cryo-confocal microscopy and its benefits for cryo-electron tomography

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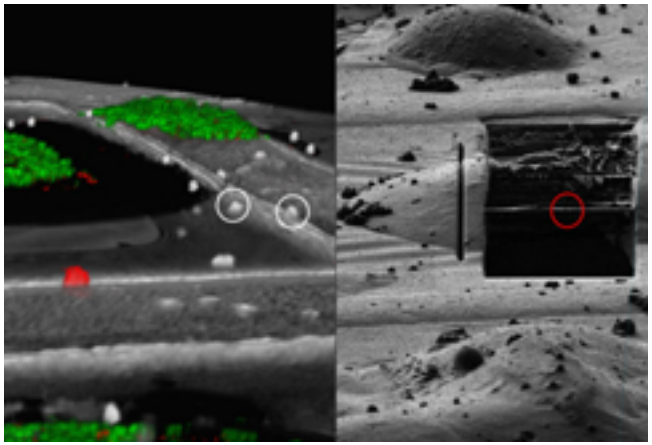

Figure 22: Correlation of markers in the light microscope (left) and FIB image (right). Beads surrounding a structure-of-interest are selected and the center of mass definition is performed in 3D (green dots in white circles). The calculated positions are then projected onto the FIB image. According to the bead markers, the position of the structure of interest is calculated and marked as well in the FIB image (red dot within the red circle). Ion beam images provided by Herman K. H. Fung, Mahamid Group, EMBL-Heidelberg, Germany. Scale bar: 20 µm.

The STELLARIS 5 Cryo from Leica Microsystems is a confocal light microscope system that helps scientists target areas of interest and obtain coordinates of regions within the volume of the confocal stack for Cryo-ET. It is part of a dedicated workflow solution called Coral Cryo 3D Cryo-ET which ensures sample viability, quality checks and most of all, a precise and reliable 3D targeting mechanism. ■

FURTHER INFORMATION

STELLARIS 5 Cryo Confocal Light Microscope

FIND OUT MORE

CASE STUDY

FASTER 3D IMAGING OF CELLULAR AND PROTEIN STRUCTURES

This case study describes the optimization of high resolution 3D sub-cellular structure analysis with array tomography (AT) using an automated serial sectioning solution to achieve a high section density on the carrier substrate. AT is a 3D image reconstruction technique for biological specimens. It involves the imaging of ordered arrays of ultrathin serial sections with scanning electron microscopy (SEM) and enables high resolution, quantitative analysis of biological structures. AT offers higher spatial resolution than conventional confocal microscopy. It can reveal additional information about cellular and protein structures to clarify poorly understood or unresolved features. The workflow for AT can be optimized when using an automated ultramicrotome that can achieve uniform, ultrathin serial sections for orderly array image acquisition with SEM. A high section quality is preserved by the elimination of artifacts that may occur during the section ribbon collection process due to manual handling.

Introduction

AT is a high resolution 3D image reconstruction method for cellular and protein structure analysis. It exploits SEM imaging of ordered arrays of ultrathin (>20 nm), resin-embedded serial sections of biological specimens collected on silicon substrate carriers.[1] AT allows for quantitative, volumetric structural analysis of many different types of tissues and proteins. As it has better lateral and spatial resolution than conventional confocal microscopy, it can help to better visualize and understand cellular and protein structure. In addition, it allows cellular and protein structure to be examined in a partially automated manner leading, to higher throughput.

Lymph nodes are kidney-shaped organs of the lymphatic system located throughout an animal or human body. They are linked by lymphatic vessels and trap foreign bodies from surrounding tissues, helping to initiate the immune response. Lymph nodes are ideal for studying the interactions of immune cells (lymphocytes, dendritic cells, and macrophages), as well as the spread of cancer (tumor metastasis).[2,3]

The Challenge

Precise examination of lymph nodes is critical for these types of immune and cancer studies. In this study, lymph nodes from mice were investigated with AT using an automated serial sectioning solution.

Serial sectioning with an automated ultramicrotome capable of producing uniform, ultrathin, high quality sections in an aligned array makes the overall workflow for AT (3D image data acquisition) more efficient (**Figure 23**). The ARTOS 3D ultramicrotome from Leica Microsystems automatically produces hundreds of uniform serial sections and avoids wrinkling of the section ribbons during collection due to a specially designed diamond knife. Furthermore, the high section density achieved during collection onto the substrate carrier allows time to be saved during SEM setup. Additionally, AT offers time-saving advantages for specimen preparation when doing correlative light and electron microscopy (CLEM)[4] studies with a transparent section carrier made out of a glass substrate.

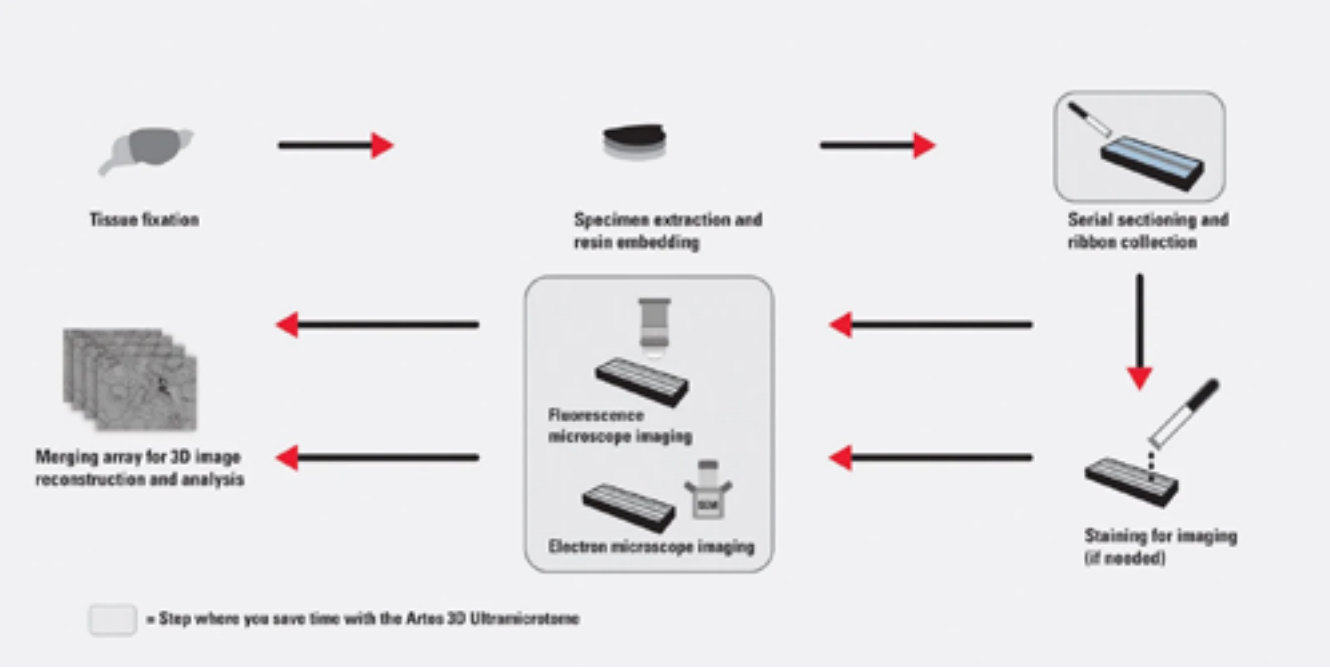

Figure 23: A typical workflow for the AT method. The steps where the ARTOS 3D solution can be used are indicated.

Methods

Lymph node isolation

Popliteal lymph nodes were harvested from 6–10 week old C57BL/6 mice. Specimen preparation was done following the OTO (osmium-thiocarbohydrazide-osmium) and ferrocyanide reduced osmium tetroxide fixation protocol of Deerinck *et al.*[5,6] to enhance the contrast of the tissue for SEM imaging.

The mice were anesthetized with an intraperitoneal injection of 100 mg/kg (wt/wt) ketamin, 10 mg/kg (wt/wt) xylazine, and 3 mg/kg (wt/wt) acepromazin. They were then perfused transcardially with phosphate buffered saline (PBS) [0.025 M phosphate buffer, 0.9% (wt/vol) NaCl, pH 7.4] for 2 min. Afterwards, the PBS was replaced via perfusion for 5–10 min with a fixative 0.1 M phosphate buffer (PB) solution having 2% (vol/vol) paraformaldehyde and 2.5% (vol/vol) glutaraldehyde. Immediately after perfusion, the lymph nodes were harvested and post-fixed for another 2 h at room temperature (RT).

Fixation and contrast enhancement

After harvesting and post-fixation, the lymph node specimens were washed in 0.1 M PB (pH 7.4). Then, to enhance contrast during AT, the specimens were immersed for 1 hour in 0.1 M PB with 2% (wt/vol) osmium tetroxide (aqueous) and 1.5% (wt/vol) potassium ferrocyanide on ice and in the dark. Afterwards, they were washed with MilliQ water (5 × 3 min at RT), transferred into filtered thiocarbohydrazide for 20 min at RT, and then again washed with MilliQ water (5 × 3 min at RT). The specimens were placed in 2% (wt/vol) osmium tetroxide (aqueous) for 30 min at RT in the dark and then again washed with MilliQ water (5 × 3 min at RT). They were incubated in 1% (wt/vol) uranyl acetate (aqueous) and placed in the refrigerator at 4°C overnight. The next day, the specimens were washed in MilliQ water (5 × 3 min at RT), incubated in a Walton's lead aspartate solution at 60°C for 30 min, and washed in MilliQ water (5 × 3 min at RT). They were dehydrated using a graded ethanol series (50%, 70%, 90%, 96%, and 100%) for 5–10 min each and then washed in anhydrous acetone (2 × 10 min at RT).

Resin embedding

The specimens were infiltrated with resin by immersing them in a graded series of Durcupan™ ACM hard resins dissolved in acetone (25%, 50%, and 75%) for 1–2 hours each. Then, they were placed in 100% Durcupan resin overnight and transferred to BEEM capsules filled with fresh resin. The recipe used for the Durcupan™ ACM resin (Sigma-Aldrich) in terms of its 4 components (A–D) was: A) 11.4 g, B) 10 g, C) 0.3 g, and D) 0.1 g. Finally, for polymerization of the resin-embedded specimens, they were kept in an oven at 60°C for 3 days.

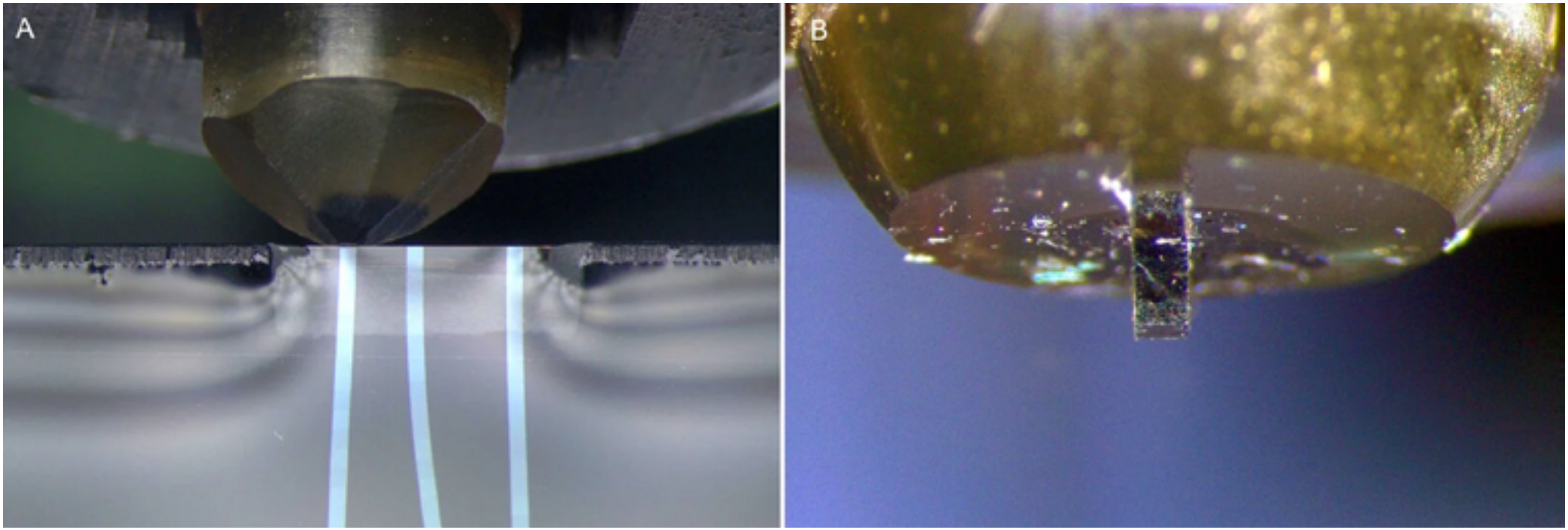

Figure 24: Features of the ARTOS 3D solution which lead to a more efficient workflow include: A) automated serial sectioning with seamless transition to the following ribbon without thickness variation and B) auto-trimmed block face of a resin-embedded specimen (size 0.5 x 0.5 x 1 mm).

Specimen trimming

The resin-embedded mouse lymph node specimens were trimmed with a trimming device from Leica Microsystems. For straight ribbon formation to occur during the sectioning process, the specimen must have parallel edges. Normally, the specimen is trimmed to this shape with a high-speed milling system, such as the Leica EM Rapid.

Specimen sectioning

The trimmed, resin-embedded mouse lymph node specimens were sectioned with the ARTOS 3D ultramicrotome. Additional reasons why an efficient sectioning workflow is possible with the ARTOS 3D solution include:

- Fully motorized movement of the knife stage and auto-transition between section ribbons (**Figure 24A**)
- Library of sectioning protocols available for use with new samples
- Specimen autotrim mode for practical sectioning when preparing large volumes of 3D image data (**Figure 24B**)
- Digital East-West measuring function; and Automatic approach of the selected knife segment

The automation of serial sectioning achieved with the ARTOS 3D solution enables greater workflow efficiency compared to a conventional ultramicrotome.

Imaging/Array tomography (AT)

After sectioning, the lymph node arrays were transferred to a silicon substrate carrier. Then, AT was performed with SEM to study the targeted proteins in-depth (**Figure 25**). High resolution images of the lymph node sections were acquired with AT using a field emission SEM (FE-SEM). Secondary electron detection was performed at 5 kV.

Image reconstruction

Image reconstructions in 3D were performed as follows:

- Section image dataset was aligned and annotations marking the sub-cellular structures manually made using the TrakEM2 software plugin[7] for the FIJI platform[8] and
- Binary exports of segmented cellular structures were imported into the software Imaris (version 9 Bitplane)[9] for 3D reconstruction and visualization of the iso-surfaces of the T-cells.

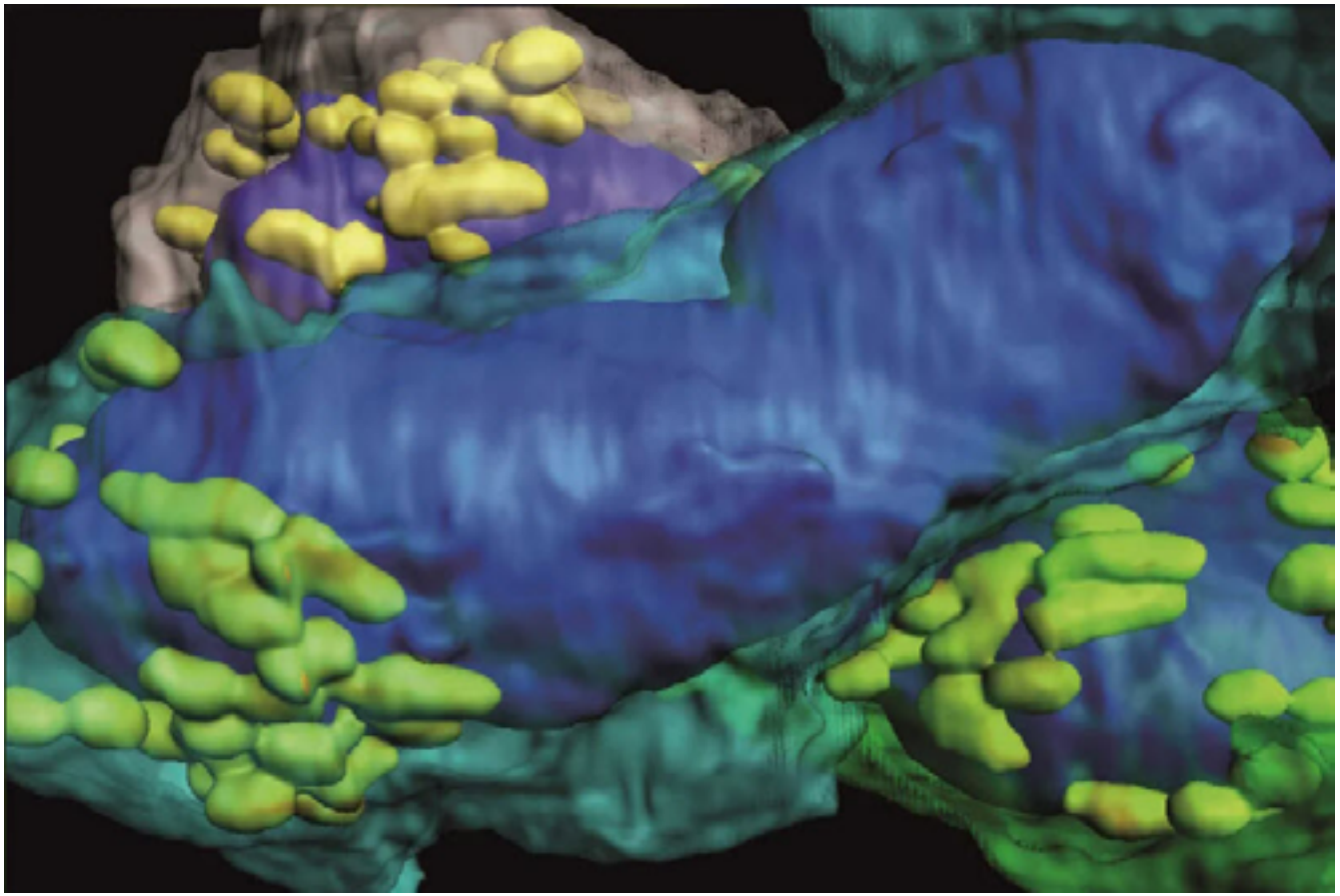

Figure 25: Array tomography image of T-cells in mouse lymph nodes.

Results

The goal of this mouse lymph node study was to elucidate the complex 3D organization of its paracortical conduit system which transports filtered lymph (containing small soluble antigens) from the periphery towards the venous circulatory system.[10] High resolution 3D image reconstruction of the lymph node ultimately should show how the stromal, myeloid and lymphoid cells spatially interact and have access to the lymph node conduit system.

3D images of the paracortex of a mouse popliteal lymph node were reconstructed using the array topography method (**Figure 26**). The images show the 3D volume and shape of the T-cells migrating within the lymph node cells, plus their sub-cellular structures, such as the nucleus, mitochondria, and cytoplasm.

Conclusion

The AT method has paved the way for high resolution, reproducible 3D image reconstruction results which allow significant advances in cell biology.

The ARTOS 3D ultramicrotome renders specimen preparation and section alignment for AT more simple leading to a more efficient overall workflow. Its wafer section carrier is capable of a high ribbon density and is small enough for optimal loading of multiple carriers into a SEM at the same time. Thus, hundreds of sections can be imaged via SEM without reloading. Transparent materials can also be used for the section carrier, so the ARTOS 3D ultramicrotome is also well suited for CLEM.[4] ■

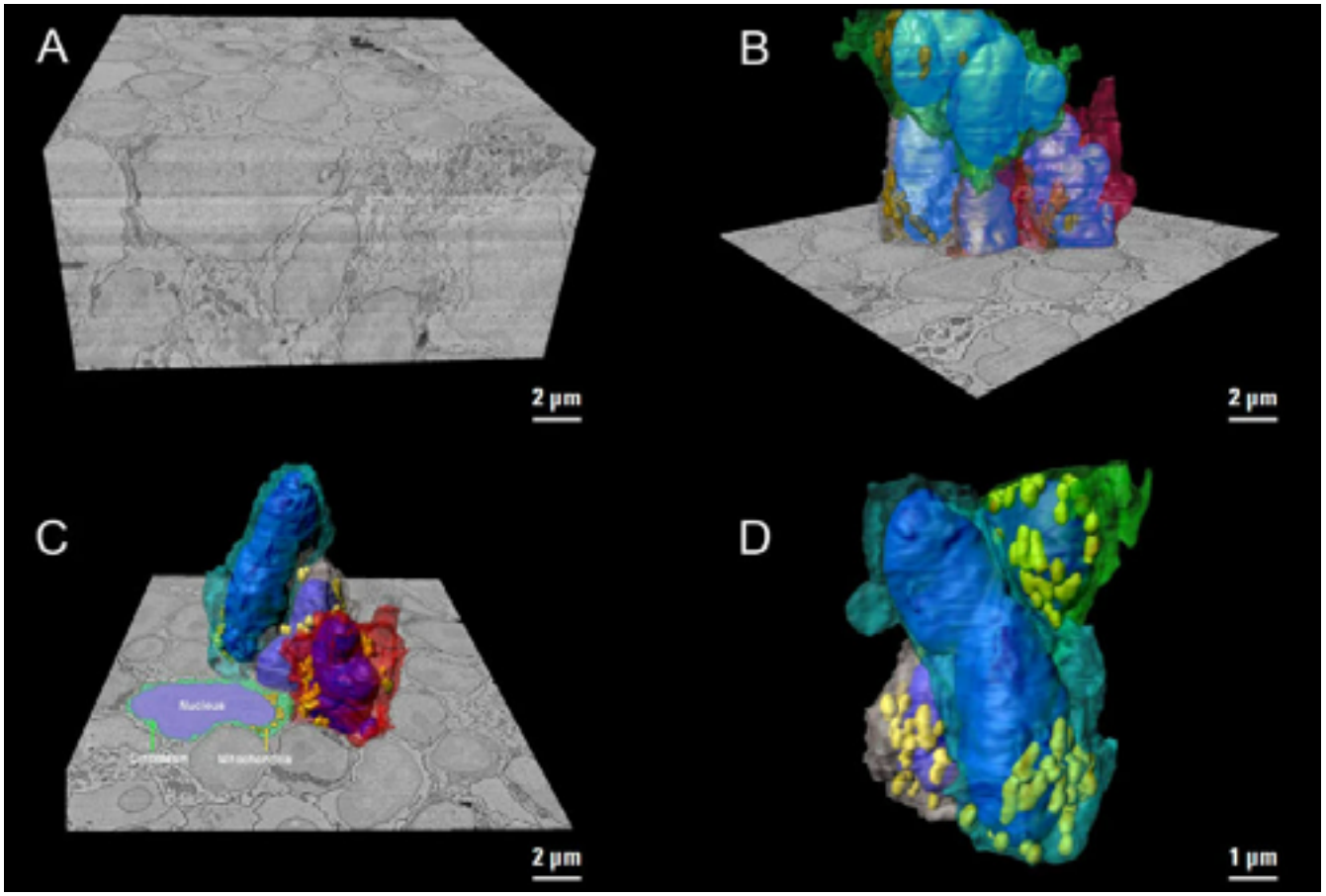

Figure 26: 3D image reconstructions of mouse lymph nodes acquired with array topography: A) 3D SEM image stack of the trimmed, resin-embedded nodes consisting of 150 sections; B) side view showing 3 T-cells within the densely packed lymph node paracortex in 3D; C) top view where the cytoplasm (green), nucleus (blue), and mitochondria (gold) of the T-cells are visible; and D) zoom-in on the 3D image of the T-cells. Courtesy Frank Assen, Ludek Lovicar, Vanessa Zheden, and Michael Sixt, Institute of Science and Technology (IST) Austria, Klosterneuburg.

Acknowledgments

Thanks to Frank Assen, Ludek Lovicar, Vanessa Zheden, and Michael Sixt of the Institute of Science and Technology (IST), Austria, for providing the AT image data shown here.

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CONCLUSION

The transition from 2D to 3D cell culture has revolutionized drug discovery and development processes by providing more physiologically relevant data that could enable significant improvements in future for the treatment of human diseases. Organoids and spheroids have become indispensable tools for studying complex biological phenomena, disease modeling, and identifying and evaluating potential drug candidates. However, practical imaging solutions are crucial if deeper insights are to be gained. Leica Microsystems has developed a number of solutions that deliver high performance for imaging 3D models. These are empowering scientists to capture dynamic processes in organoids and spheroids in real-time, thus facilitating detailed observations at the cellular and subcellular levels. The case studies presented in this eBook demonstrate how these innovative imaging solutions can address many of the challenges encountered when imaging 3D samples.

Leica Microsystems' THUNDER Imagers have been shown to offer fast, gentle, and accurate imaging of organoids and spheroids. They have proven invaluable in studying the therapeutic and regenerative potential of lung organoids, as well as investigating brain development in ferrets. The advanced widefield microscopy capabilities of THUNDER Imagers, coupled with Computational Clearing technology have allowed scientists to obtain sharper and more detailed images, enabling efficient analysis and gathering deeper insights. This technology is also present in the Mica imaging Microhub, and both Mica and THUNDER Live Cell & 3D Assay offer an incubating environment for maintaining samples at near physiological conditions.

The simplified workflow in Mica, with automatic selection and optimization of imaging settings, means that anyone can go from set up to beautifully visualized results efficiently, accurately and confidently – without all the training. This could be especially useful in biopharma where specialist microscopy expertise is not widespread, allowing a greater number of scientists to be able to study the development and behavior of organoids and spheroids.

Using the STELLARIS confocal microscopy platform, it is possible to go deeper into 3D samples, with precise imaging of subcellular structures and dynamic processes. When LIGHTNING technology is applied, the resolution of STELLARIS can be extended further still, resolving nanostructures as small as 120 nm. For more versatility in experimental design, confocal microscopy can be combined with light sheet microscopy, using Leica's STELLARIS 5 and STELLARIS 8 Digital LightSheet (DLS) microscopes. This offers the ability to perform fast and gentle volumetric light sheet imaging and to improve live cell imaging applications by increasing cell viability.

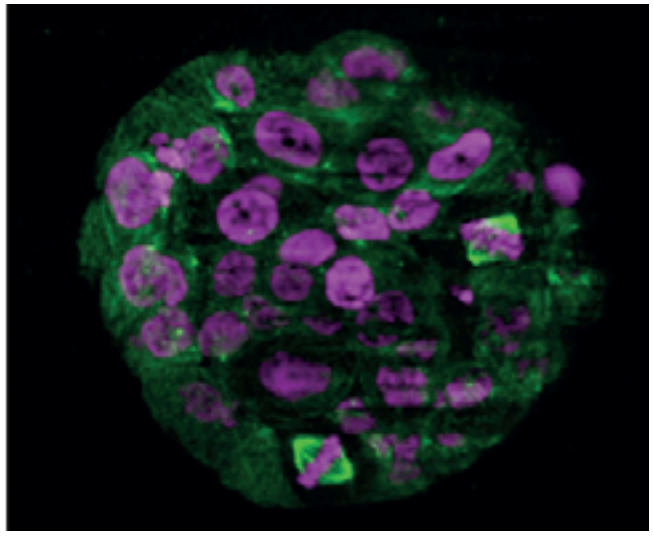

Figure 27: Live mammary epithelial spheroid acquired using DLS microscopy. Courtesy of B. Eismann and C. Conrad, BioQuant/DKFZ Heidelberg, Germany.

To further improve accuracy, streamline workflows, and enhance data exploration, the Aivia AI Image Analysis Software offers exciting new possibilities for analysis with both THUNDER and STELLARIS, including autonomous microscopy with rare-event detection.

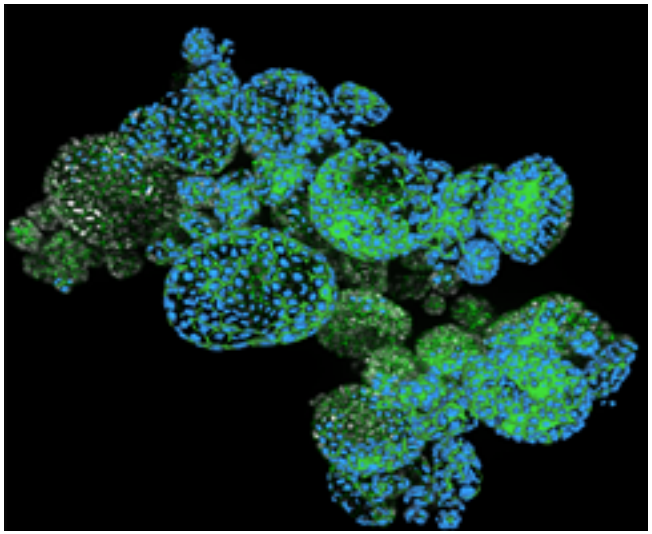

Figure 28: Organoid cluster acquired using THUNDER and analyzed with Aivia. Courtesy of Dana Krauß, Cancer Research Institue, Medical University of Vienna.

Bridging light microscopy and EM, Leica's Coral Life workflow for Live Cell CLEM enables high-resolution EM while preserving dynamics. High Pressure Freezing with the EM ICE can also aid visualization, mapping light microscopy observations onto EM data for 3D insights. Array Tomography with the ARTOS 3D enhances 3D volume imaging of biological samples with EM, reconstructing ultrathin sections for higher resolution and spatial context. Automated serial sectioning optimizes array image acquisition, advancing cellular structure understanding. Furthermore, for Cryo-ET workflows, Leica's Coral Cryo enables targeting through Stellaris 5 Cryo, comprising sample preparation via FIB milling and subsequent imaging by Cryo-ET. ■

By allowing scientists to visualize cellular details more clearly, Leica's imaging solutions can help push the boundaries of 3D culture research and enable translatable insights in fields such as cancer research, neuroscience and immunology.

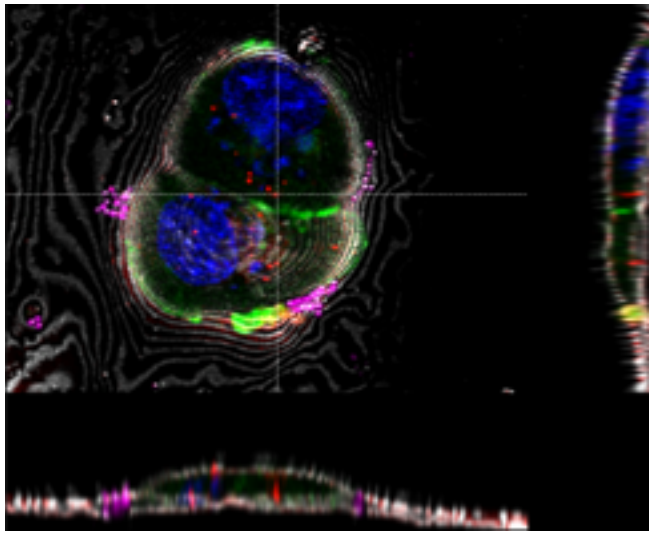

Figure 29: LAS X Coral Cryo: Interpolation based targeting in 3D using sections through the z-stack in x and y directions.

Front cover image: Murine esophageal organoids (DAPI, Integrin26-AF 488, SOX2-AF568) imaged with THUNDER Imager 3D Cell Culture.
Courtesy of Dr. F.T. Arroso Martins, Tamere University, Finland.

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Leica Microsystems GmbH | Ernst-Leitz-Strasse 17–37 | D-35578 Wetzlar (Germany)
Tel. +49 (0) 6441 29-4000 | F +49 (0) 6441 29-4155

www.leica-microsystems.com