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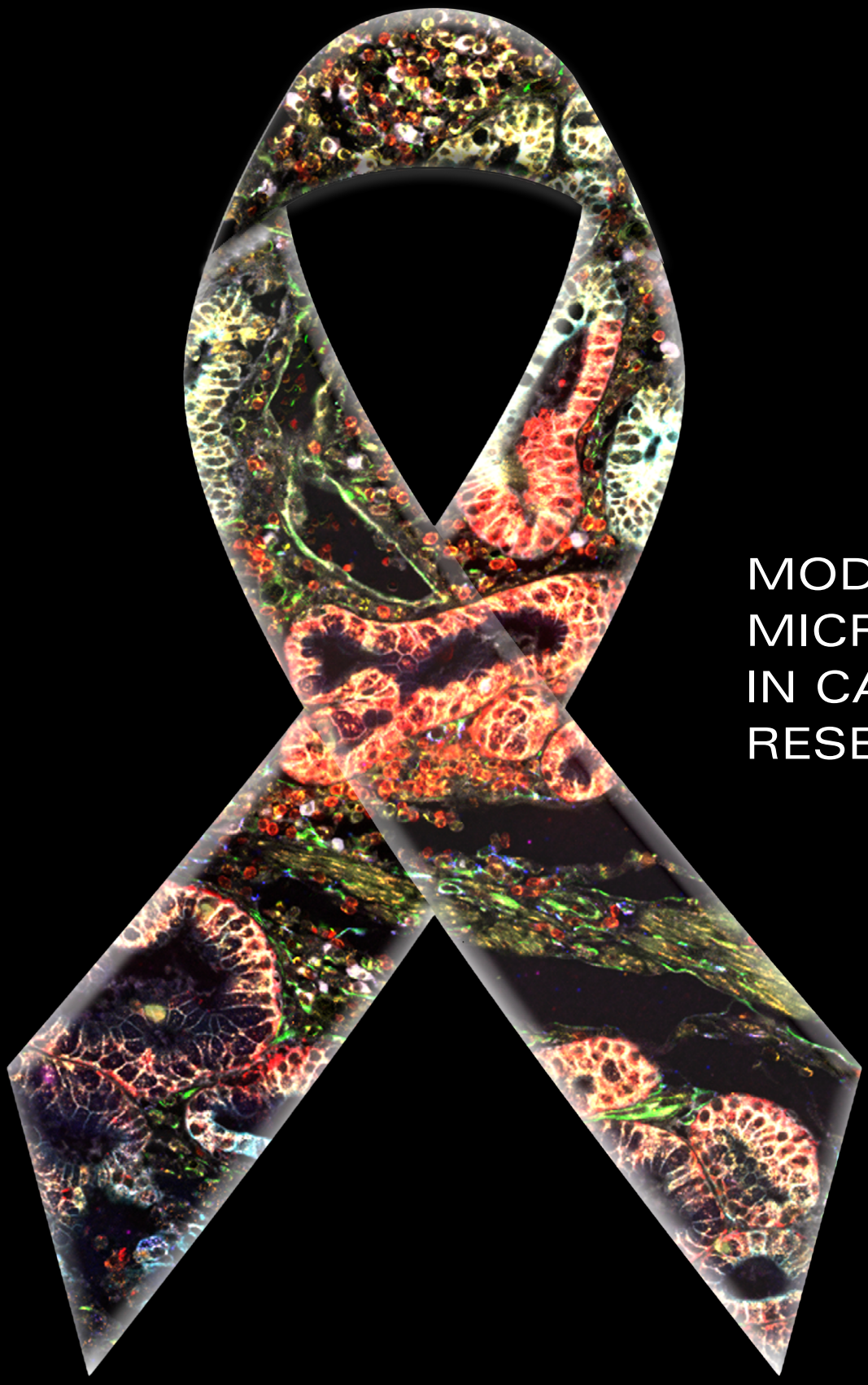

MODERN MICROSCOPY IN CANCER RESEARCH

Index

Chapter 1: History and Development of Microscopy in Cancer Research

Cancer is a global disease, with 18 million new cases diagnosed and 10 million cancer-related deaths worldwide in 2020 (1). This burden is set to increase, with a projected increase in cases of ~55% by 2040 (1). Research into understanding cancer initiation, development and to develop new diagnostic tools and therapeutic approaches is vital.

Microscopy has and continues to play a crucial role in cancer research, increasing our understanding of how cancer develops and progresses, offering ways to diagnose cancers, and allowing research into effective treatments.

The Benefits of Microscopy in Cancer Research

Cancerous cells are cells that contain genetic mutations resulting in aberrant behaviors, including increased cell growth and division, evasion of programmed cell death (apoptosis), impaired self-repair, and abnormal cell attachment (2).

They can be distinguished from normal cells as they have visually distinct characteristics, including differences in size, shape, metabolism, and nuclear organization.

Morphology-based Identification of Cancer Cells

From the early days, microscopy has helped illuminate how cancerous cells differ in size and shape from normal cells, providing insight into the underlying pathophysiology of cancer and offering ways to identify and diagnose cancers. Morphology-based pathology is still considered the gold standard for identifying and distinguishing cancer types (3).

The usefulness of morphological and histological techniques is not limited to clinical settings and is still heavily used in cancer research.

High-resolution Imaging and Fluorescence Microscopy

While traditional brightfield imaging is still widely used in both clinical and research settings, the desire to understand the molecular mechanisms underlying cancer initiation and progression requires imaging techniques capable of visualizing proteins and molecules within cells. Fluorescent imaging offers a way to do this. It allows an understanding of the intracellular distribution of proteins and other molecules and how these change in cancer.

Fluorescent imaging of proteins and molecules can be achieved in multiple ways:

- Tagging with fluorescent proteins such as green fluorescent protein (GFP)
- Protein self labelling with HaloTag®, SNAP-tag® and CLIP-tag™
- Using fluorescently tagged antibodies that bind to targets of interest
- Direct fluorescent labels (e.g., DAPI, FITC, Alexa Fluor®)

Out-of-focus light impacts the resolution of fluorescent imaging, adding blur to images and making it more challenging to distinguish molecules and proteins of interest. Luckily, a wealth of imaging techniques have been developed that overcome this issue.

Confocal microscopy blocks out-of-focus light with the introduction of a pinhole, thereby achieving optical sectioning through the specimen under observation (Figure 1). Complete high-resolution images are created by moving this spot across the entire sample, and 3D images can be reconstructed from the high-resolution image stacks allowing us to visualize processes in three dimensions (4).

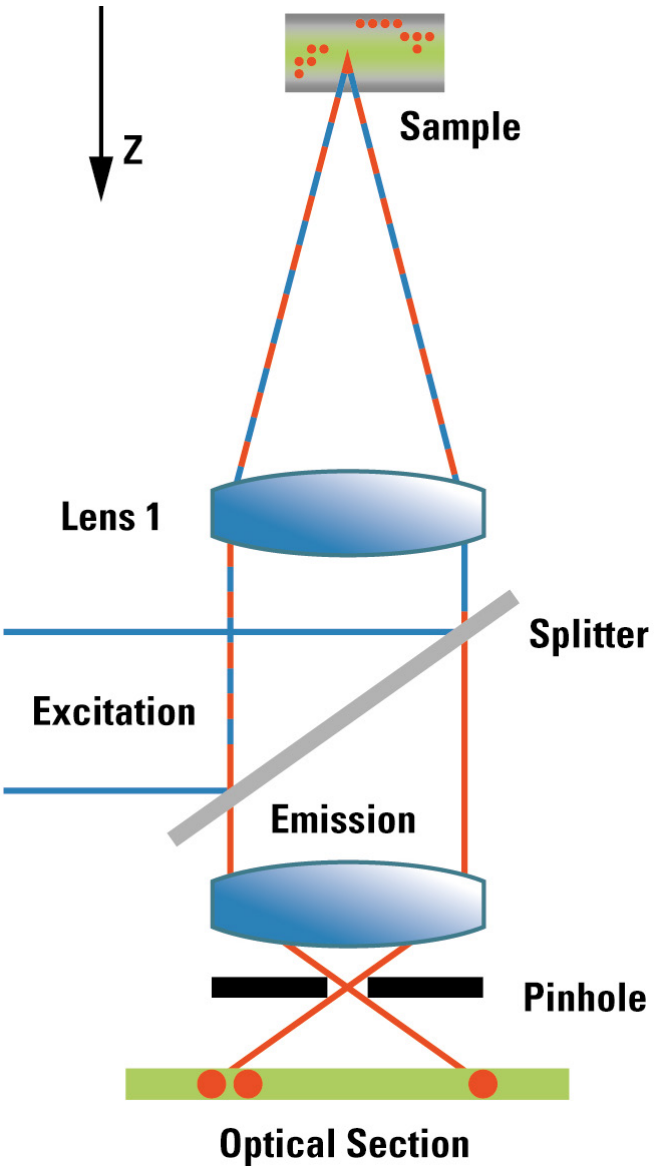

Figure 1: Schematic diagram of confocal scanning microscope, showing how the pinhole reduces out-of-focus light. The excitation light (blue) is coupled into the microscope by a splitter for incident illumination and focused to a diffraction-limited spot by the objective lens 1. Emission passes the splitter and is filtered spatially by the pinhole. Scanning the spot in x and y direction generates an image, the optical section.

Live Cell Imaging in Cancer Research

The need to preserve clinical samples to prevent physiological and molecular changes is critical since it is not always easy or possible to collect further samples from a patient. Such static, fixed samples can provide a wealth of information. Fixed samples, such as formalin-fixed paraffin-embedded (FFPE) tissues, allow researchers to study relevant samples.

The information available from fixed samples has limitations, the most notable being that they only provide a single snapshot in time. This restricts the information we can gain about dynamic cellular processes, including metastasis and cell migration (5).

Live cell imaging enables researchers to visualize such processes in real time, providing a greater understanding of them and their role in cancer. Fluorescence and live cell imaging offer a potent combination that can help better understand cancer initiation, progression, and treatment.

Several imaging technologies can visualize and record dynamic cell processes in various sample types, from cultured cells to organoids and even whole organisms. To gain meaningful results, it is vital to maintain cellular viability, meaning samples must be kept at appropriate temperature, humidity, and CO_2 levels.

Depending on the research needs, multiple technologies can be used to reliably and reproducibly image live cells, including widefield microscopy, confocal microscopy, light-sheet microscopy, multiphoton microscopy, and stimulated emission depletion (STED) microscopy.

The Mica Microhub from Leica Microsystems combines widefield fluorescence, confocal, and transmitted light microscopy, enabling reliable, high-resolution imaging of live cells. The advanced incubation chamber allows users to better mimic physiological conditions by regulating temperature, humidity, and CO_2, ensuring cells remain viable, and data is more relevant.

Discover the Range of Imaging Possibilities

The Leica Cancer Research Image Gallery reveals the possibilities cancer imaging offers and the insights to be gained. From label-free imaging to deep *in vivo* imaging (Figure 2), the latest microscopy technologies offer incredible insights into cancer.

The Cancer Research Solution page offers information on the broad range of technologies available to assist in cancer research.

In the next chapter, we discuss cancer imaging solutions for spheroid and organoid samples, including additional considerations, challenges, and case studies.

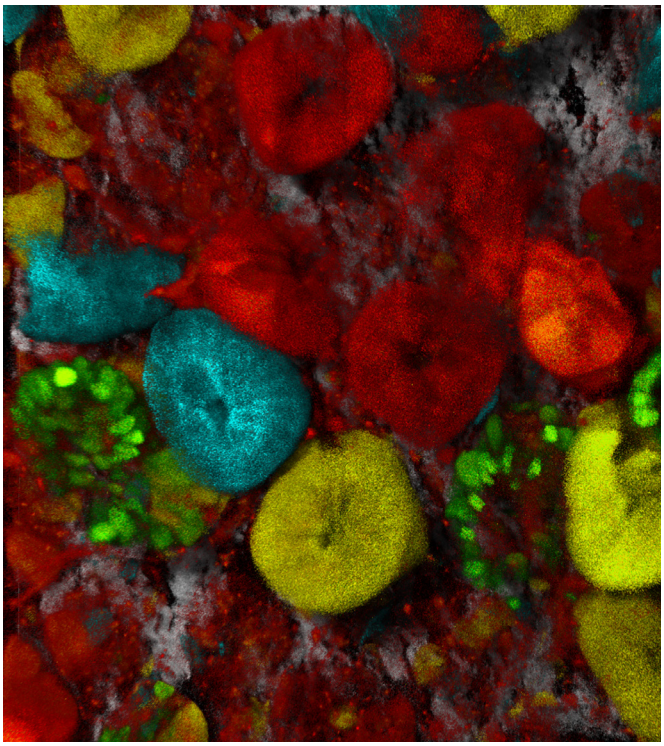

Figure 2: Confetti mouse small intestines. Type 1 collagen is shown in gray [second harmonic generation (SHG)], and lineage-traced stem cells are shown in cyan, green, yellow, and red. Stem cells play an important role in the spread of cancer within organisms. Sample courtesy of Jacco van Rheenen, Netherlands Cancer Institute, NL.

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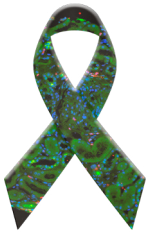

Chapter 2: Gentle 3D Imaging of Spheroids and Organoids

Cancer is a heterogenous, multifaceted disease that is influenced not only by the genetic and physiological changes of the cancer cells but also by the microenvironment and neighboring cells. Studying cancer *in vitro* is traditionally performed using cancer cell lines. However, these cell lines often lose the heterogeneity and lack the tissue architecture and microenvironment seen *in vivo* (1).

Organoids and spheroids offer a more robust and realistic model for cancer. These 3D cultures better mimic both the heterogeneity, 3D organization, and microenvironment seen *in vivo*, making the insights gained from these models more reflective of cancer biology and the results more translational (1).

However, imaging spheroids requires additional considerations, as these delicate structures can become easily damaged when moved, and their thickness poses challenges for obtaining clear, high-resolution images.

Observing 3D Cell Cultures During Development

Handling a sample gently to retain its natural growth and cellular interactions is immensely important to understanding 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 (2). In this case, imaging should not influence or interfere with further development. After imaging, the 3D cell culture should continue to develop. In addition to low mechanical stress during sample handling, this requires reducing light stress by using low light intensities and short exposure times.

The evolution of life science research from 2D to 3D cell culture has been an exciting progression that can enable significant improvements in the future for the treatment of human diseases. Thick 3D cultured cell specimens provide data with more physiological relevance in terms of gene expression and cell morphology. Organoids and spheroids allow the modeling of various physiological aspects, including:

- development
- homeostasis
- regeneration
- disease

Organoid cultivation is becoming one of the most important tools for research with implications for personalized medicine in the future. Nevertheless, 3D approaches still face some challenges.

When creating and maintaining 3D cell cultures, it is a big advantage 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 when handling the specimen.

Mica, the imaging Microhub from Leica Microsystems is ideal for imaging spheroids and organoids as the sample-protecting incubator offers control of environmental conditions. Additionally, Mica offers widefield, confocal, THUNDER imaging, LIGHTNING, z-stacks and time-lapse, making it ideally placed for imaging complex 3D cultures over time.

Click here to watch an interview with Organoid Researchers from Technical University Munich to discover how the Mica Microhub can provide a stable environment for imaging organoid models.

Clear Imaging of Organoids and Spheroids

Imaging using widefield microscopes offers great speed, easy handling, and low phototoxicity. However, a typical 'turbidity' in thicker samples impairs the view of detail. The THUNDER Imager systems, whose technology is also included in Mica, provide solutions that retain the favorable characteristics of widefield microscopes in handling, speed, and low phototoxicity, whilst further extending the range of applications to 3D samples.

THUNDER is an opto-digital technology that uses a method called Computational Clearing to effectively remove out-of-focus blur from areas outside the focal plane in real time. Computational clearing results in high-resolution and high-contrast images, revealing the details of life, which can then be easily identified, even at the depths of an intact living sample. By combining flexible illumination technology, high-resolution optics, stable environmental controls, and novel image processing techniques, THUNDER Imagers are intuitive, workflow-oriented 3D cell culture imaging solutions.

As an example, lung organoids were cultured from transgenic mice to study potential new therapeutic approaches for lung diseases such as influenza. The cultivation of these organoids can be 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 have already shown the formation of organoids down to the level of individual cells (Figure 1).

Imaging of Anti-Cancer Drug Uptake in Spheroids Using Digital LightSheet (DLS)

Spheroid 3D cell culture models mimic the physiology and functions of living tissues, making them 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 (3,4). 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 at present.

To investigate the mechanism of AZD2014, Digital LightSheet (DLS) microscopy was used to observe the cellular uptake of AZD2014 within a living 3D-cell-culture-model environment, taking advantage of its intrinsic fluorescence and localization within living spheroids (5).

Using Spheroids to Investigate Mechanism of AZD2014

HEK293 spheroids were placed onto low-melting-point-agarose holders sitting in 35 mm glass-bottom dishes, with 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 2).

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

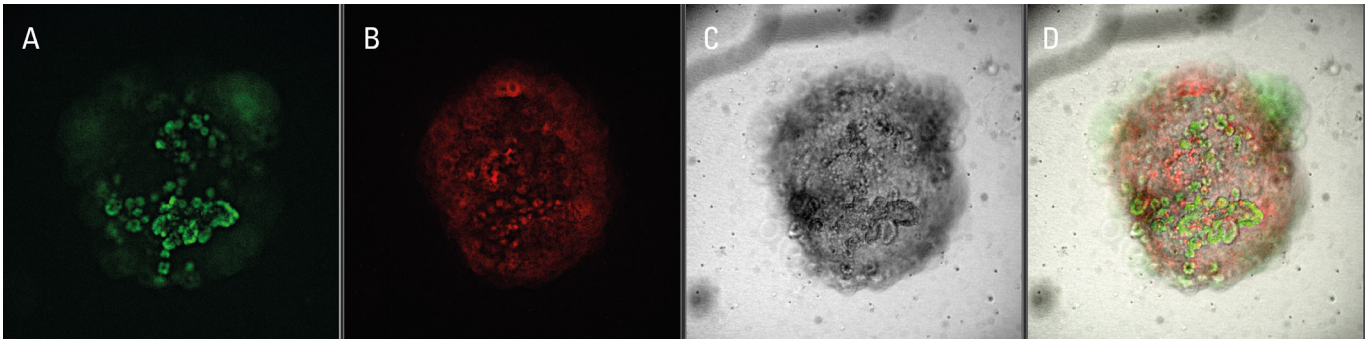

Figure 1: A mature lung organoid taken at the 'liquid-air interface' with a THUNDER Imager 3D Cell Culture. The cells originate from transgenic mice, so the different fluorescence represents the degree of differentiation of the respective cell. (A) Is yellow fluorescent protein (YFP), (B) is mCherry, (C) is brightfield, and (D) is a superposition. The image acquisition was performed 21 days after the start of the culture. Reference: P. Kanrai, MPI-HLR Bad Nauheim.

Potential of AZD2014 as a Photoactivatable Anti-cancer Therapy

Imaging of the spheroids treated with AZD2014 showed uptake of the inhibitor. The outer layers of the spheroids showed a faster rate of uptake than the inner core of the spheroid (Figure 3). Furthermore, a 25% increase in the average radius of the spheroids was seen after 30 minutes of exposure to AZD2014 (7 µM). This increase in size occurred

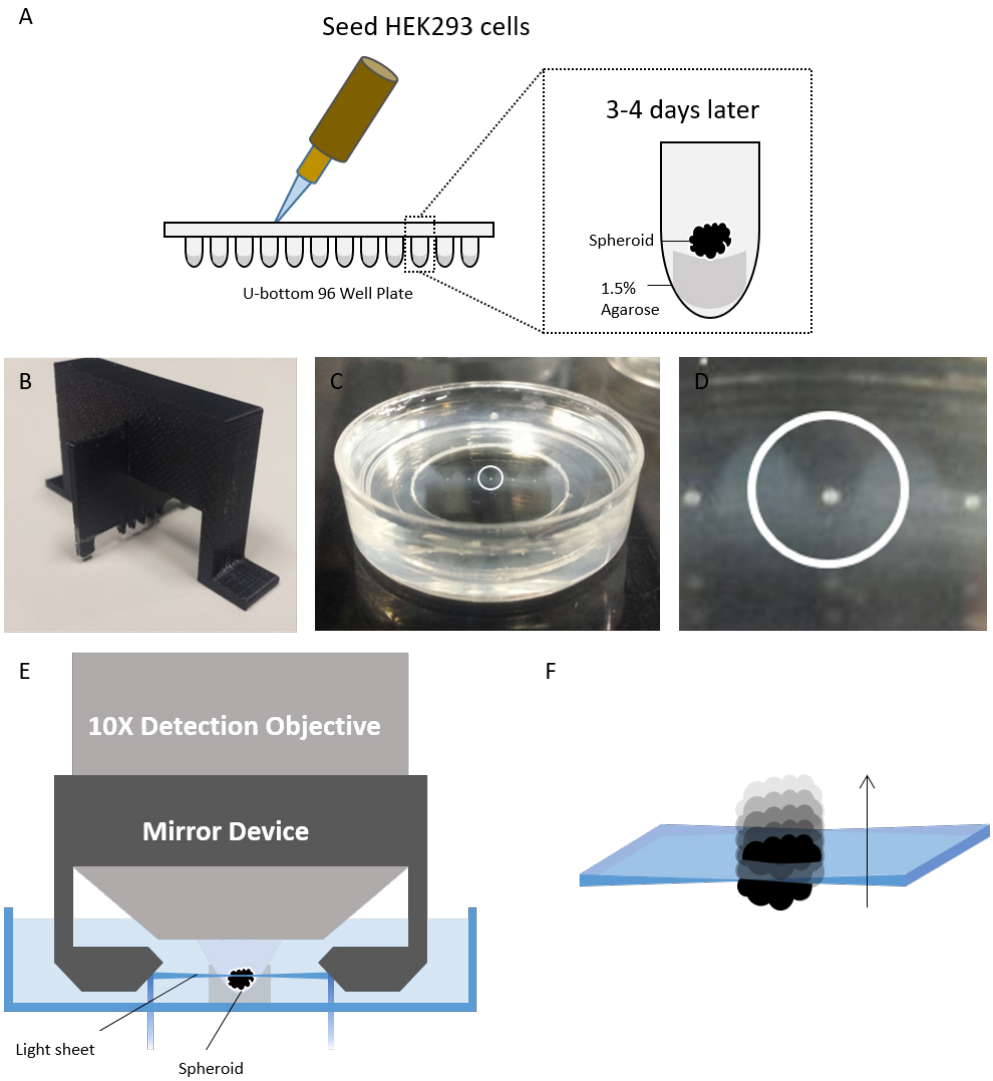

Figure 2: Spheroid seeding, mounting, and imaging procedure. (A) HEK293 cells were seeded into a 96-well round (U-well) bottom plate pre-cast with 100 µl of 1.5% agarose at a cell density of 10,000 cells per well with a final volume of 200 µl in complete growth media. (B) A 3D-printed comb was used to 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 digital-light-sheet-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.* (3).

only when both the drug and 405 nm illumination were present on the samples. The latter finding suggests a unique, previously unknown, photoactivatable property of AZD2014.

The data suggest that AZD2014 may have the potential to act as a photoactivable drug that could be used in anti-cancer photodynamic therapies (Figure 3).

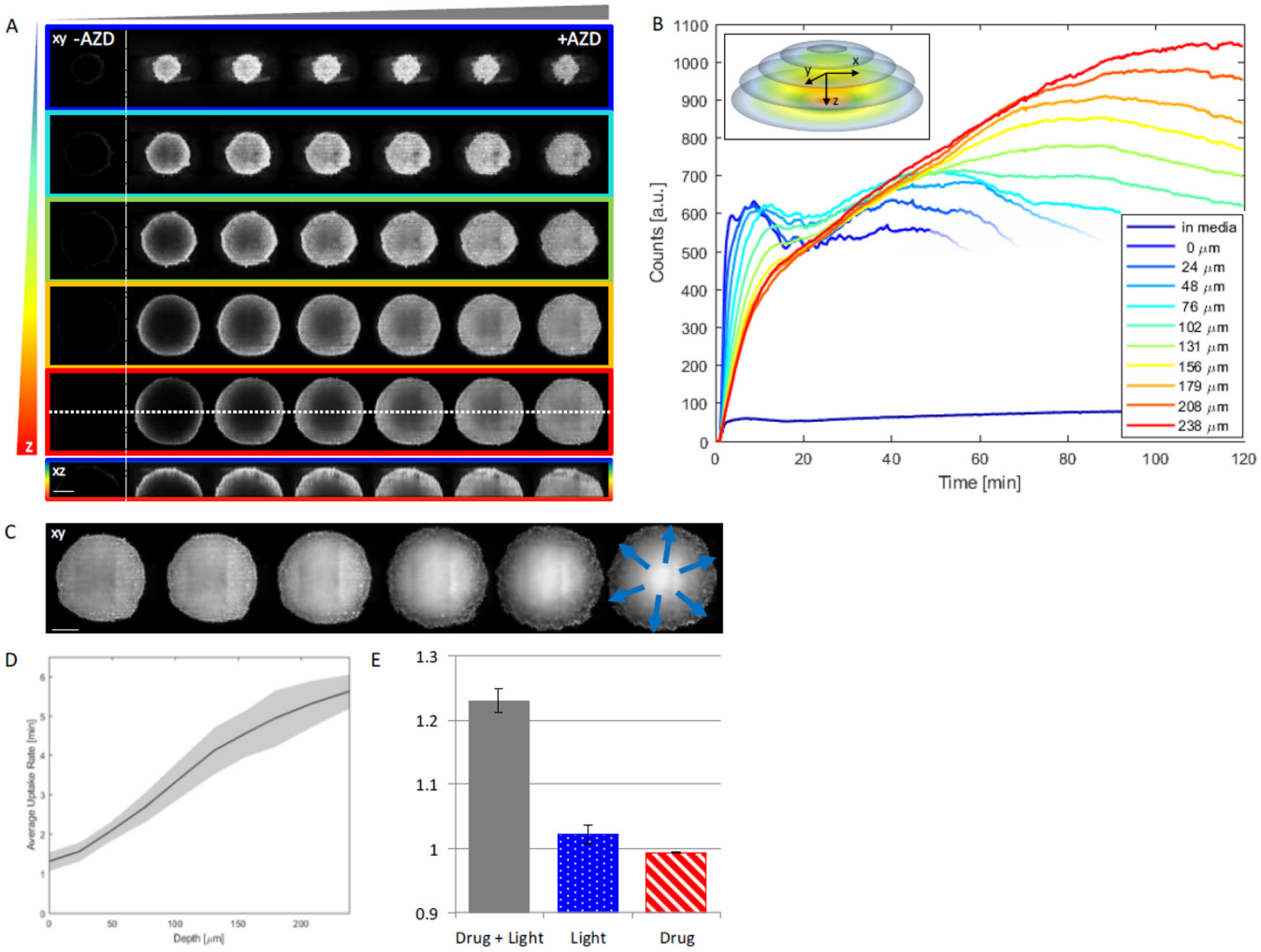

Figure 3: Uptake of AZD2014 in 3D multi-layered spheroids. (A) AZD2014 administration and imaging of HEK293 spheroid. Different xy planes or depths (rows) are shown as well as an orthogonal projection (xz plane). (B) Uptake rates of AZD2014 fluorescence in spheroids over a 2-hour time-lapse were studied at different depths from the surface. (C) Spheroid radius increased during imaging with AZD2014 administration. Image planes at 250 µm depth are 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.* (3).

Visualization of Dynamic Interactions in Spheroids

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 Digital LightSheet enables 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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Chapter 3: Tissue Multiplexed Imaging and Analysis

Cells do not exist in isolation, and imaging tissues is crucial for fully understanding the complex processes underlying cancer. Multiplex imaging in tissues allows multiple pathways and processes to be visualized in concert, giving greater insight into cellular and molecular processes. There are four current approaches for immunofluorescence multiplexing of tissues (1):

1. Fluorescence inactivation involves removing fluorescence signals by various quenching methods
2. Antibody stripping physically detaches the antibodies from the tissue
3. Oligonucleotide conjunction uses DNA-barcoded antibodies that are visualized using multiple rounds of labeling
4. Linear unmixing uses an algorithm to separate overlapping fluorescence spectra that have been imaged together

A patented technology called FluoSync, included in the Mica Microhub, offers a new way to do spectral unmixing that enables simultaneous imaging on the fly. FluoSync is a streamlined approach for simultaneous multiplex fluorescence imaging combined with phasor analysis that enables you to acquire up to 4 fluorophores – at the same time (2). This offers several advantages over more traditional methods for multiplex imaging, including faster imaging workflows due to simultaneous imaging.

Benefits and Challenges of Multiplex Imaging with Widefield Microscopy

In widefield microscopy, the entire sample is exposed to light during imaging. This can make imaging tissues challenging because the thick nature of these samples means out-of-focus blur or hazing can occur using conventional widefield microscopy (3).

Widefield microscopy has in general several benefits:

- Microscope setups are typically affordable
- Faster imaging
- Easy to use

The challenge of out-of-focus blur in widefield microscopy can be overcome using background subtraction methods. These methods can improve image contrast by providing sharper detail to important structures.

Increasing Resolution with Computational Clearing

Computational clearing offers an efficient solution to out-of-focus-blur or haze. It detects and removes unwanted background signal from images, making the signal of interest directly accessible (Figure 1). At the same time, in the in-focus area, edges, and intensity of the specimen features remain (4). THUNDER Imagers offer three computational clearing methods:

1. Instant Computational Clearing (ICC)
2. Small Volume Computational Clearing (SVCC)
3. Large Volume Computational Clearing (LVCC)

In this chapter, we share two case studies that showcase the benefits of multiplexed imaging and how computational clearing can aid in obtaining clear, focused images.

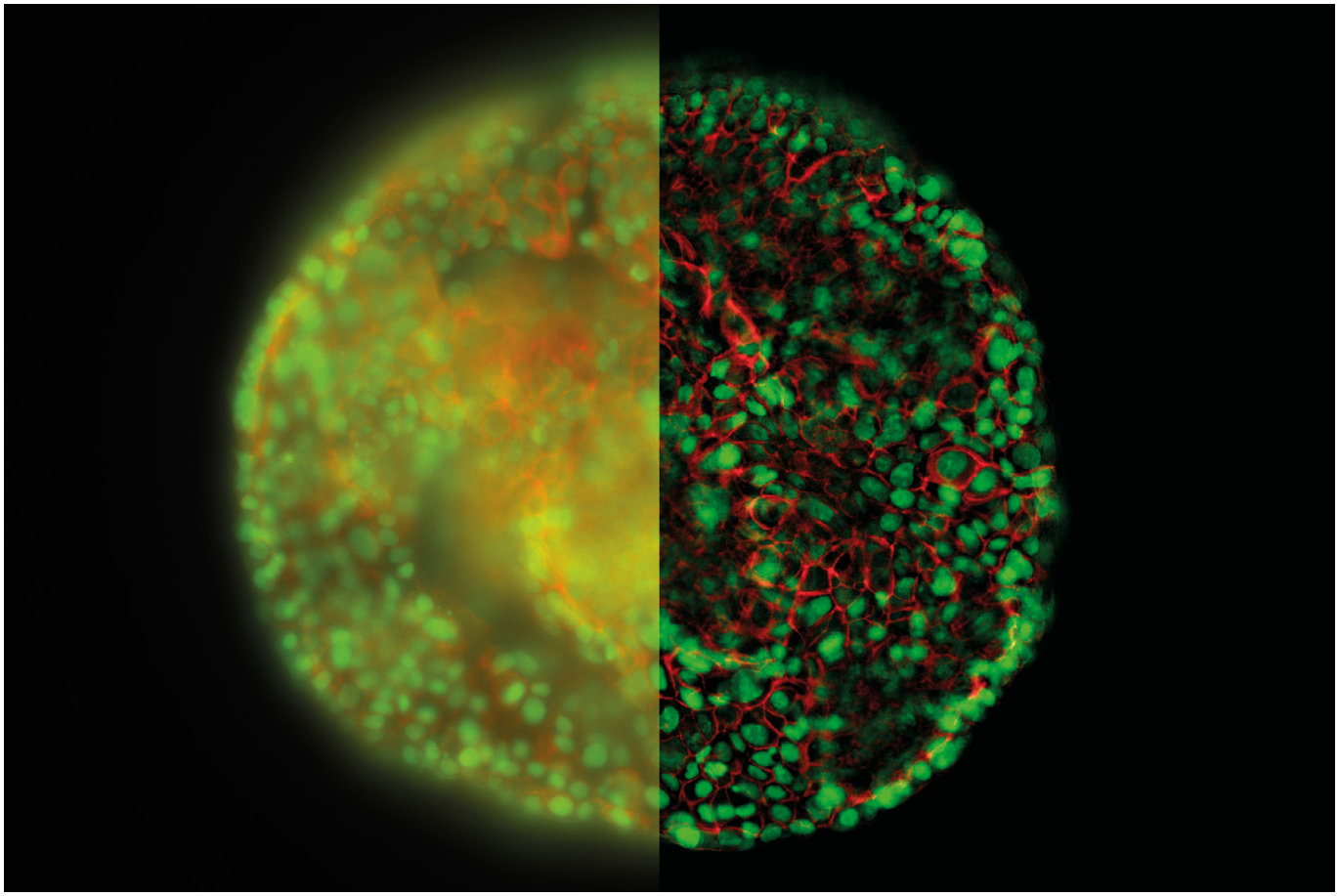

Figure 1: Computational clearing provides greater resolution and contrast. HeLa cell spheroid stained with Alexa Fluor 568 Phalloidin (actin) and YOYO 1 iodide (nucleus). Left side is raw data. Right side shows the image after THUNDER computational clearing. Image taken by Dr. Jan Schumacher, Leica Microsystems, Germany.

Visualizing the Mitotic Spindle in Cancer Cells

In eukaryotic cells, the mitotic spindle, composed of hollow microtubules, is instrumental in building the cytoskeletal structure of a duplicate cell and separating duplicated chromosomes from the original cell during mitosis. In cancerous cells, such as sarcomas, the triggers of mitotic catastrophe are identified by examining dysfunctions of the mitotic spindle (5).

When imaging mitotic spindles, a technique that can quickly image them and achieve sharp, high-contrast 3D imaging, where important details are clearly resolved, is most practical. Conventional widefield microscopy is fast and offers detection sensitivity, but unfortunately, images of thick specimens often show an out-of-focus blur or haze, which reduces the contrast (6). Unraveling the role of mitotic instability in a complex disease like cancer requires the correlation of multiple biological markers in the same sample (multiplexing).

Can Pretreatment Sensitize Cancer Cells to Radiation?

Sarcomas are a family of cancers that develop in connective tissues such as muscle or bone. Ewing's sarcoma and rhabdomyosarcoma, which develop in bone and muscle, respectively, are pediatric cancers with a tendency to occur adjacent to areas of active bone growth. Ionizing radiation is one treatment used for these cancers, but this may permanently damage growing bone. The

severity of bone injury is largely proportional to the dose of radiation the bone receives. So, strategies that selectively sensitize tumor tissue to radiation could lower the dose of radiation needed to achieve local control and minimize collateral injury of adjacent healthy tissue.

Pre-treatment with mRNA synthesis inhibitor Mithramycin A was tested to see if it could selectively radiosensitize EWS:Fli1+ tumor cells by altering the transcriptional response to radiation injury using *in vitro* testing and in murine xenograft model systems (7).

Ewing sarcoma cells (SK-ES-1) were stained for α-tubulin, γ-tubulin, and DNA to visualize the mitotic spindles. After staining, slides were coverslipped using ProLong® Glass Antifade media and imaged with a THUNDER Imager Tissue using a 63×/1.4 NA (numerical aperture) oil immersion objective. LVCC (6) was applied to the image data set, and a maximum intensity projection was generated.

Benefits of Tissue Multiplexing and Computational Clearing for Studying Mitotic Spindles

Figure 2 demonstrates how the THUNDER technology enables visualization of more details of mitotic spindles in sarcoma cells. The α-tubulin (green) forms the mitotic spindles to which chromatids (blue) attach during mitosis, while γ-tubulin (red) localizes to the spindle poles in dividing cells. The sharper images reveal clear structures which can easily be segmented and used for further analysis. The results of this study showed that Mithramycin A can significantly radiosensitize EWS:Fli1+ cells *in vitro* and *in vivo* by inhibiting transcription of genes involved in DNA damage repair, leading to tumor cell death by apoptosis (7). Revealing more details of mitotic spindles in sarcoma cells using the THUNDER Imager and LVCC, compared to conventional widefield imaging, can help cancer researchers gain useful insights.

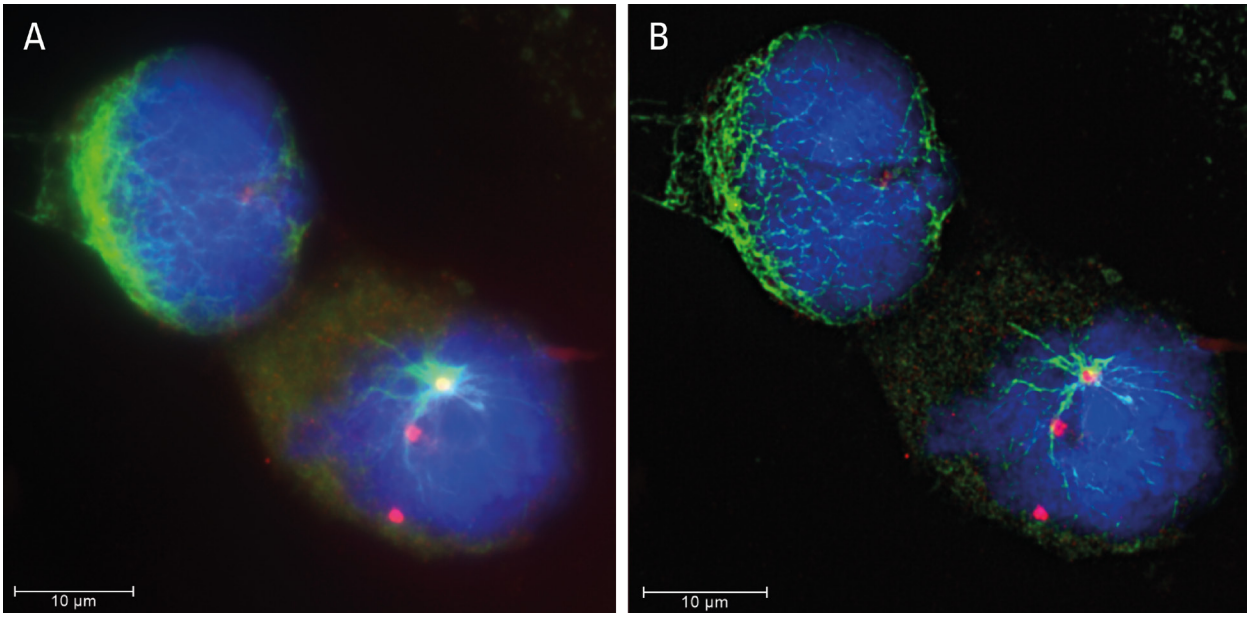

Figure 2: Maximum intensity projection of SK-ES-1 cells stained for α-tubulin (green), γ-tubulin (red), and DNA (blue). (A) raw image data; and (B) THUNDER image with LVCC.

Investigating Epithelial Morphogenesis and the Growth of Epithelia

The follicles or egg chambers of Drosophila fruit flies are often used as a model system for cancer studies to understand how epithelial organization prevents tumor formation and how tumors kill their hosts (6,7). To study the effect of various genetic mutations on the apicobasal polarity of the follicle, specimens of whole Drosophila follicles expressing fluorescently-tagged aPKC, an apical marker, and Dlg, a basolateral marker, were imaged with a THUNDER Imager using a 63x/1.4 NA oil-immersion objective (8,9).

Two main challenges arise when imaging thick follicles or egg chambers of Drosophila model organisms with conventional widefield microscopy:

1. The ability to quickly identify follicles in the stage of interest, especially those with small structures

2. The haze or out-of-focus blur produced by light scattering (7,8), which can obscure interesting structures inside the follicle

Benefits of Rapid Switching Between Low and High Magnification

A critical and time-consuming part of the workflow is identifying the correct stage and genotype of Drosophila follicles to image when searching over the whole coverslip area of a specimen of dissected ovaries. The THUNDER Imager 3D Assay with LAS X Navigator made screening more rapid (10). Clusters of dissected follicles were quickly located at low magnification, and then the THUNDER Imager objective was switched to the 63x oil-immersion objective to find the desired stages of follicle development. A parfocal and parcentric imaging solution allowed for a seamless transition between the lower and higher magnification scans. After imaging a follicle, LVCC was applied (11,12).

The images in Figure 3 show two follicles from a single ovariole. The larger follicle seen on the left is at a later stage of development than the one on the right. The follicle epithelial cells exhibit apicobasal polarity, which is revealed by the juxtaposition of the apical marker aPKC (green) and the basolateral marker Dlg (magenta). The follicle on the left is a mutant for the polarity gene, *Scrib* (8). It has lost polarity, as evidenced by the disorganized architecture and loss of segregation between aPKC and Dlg. The follicle on the right is wild type. The difference in the distribution of apicobasal proteins between wild-type and mutant egg chambers is more clearly distinguished with THUNDER images.

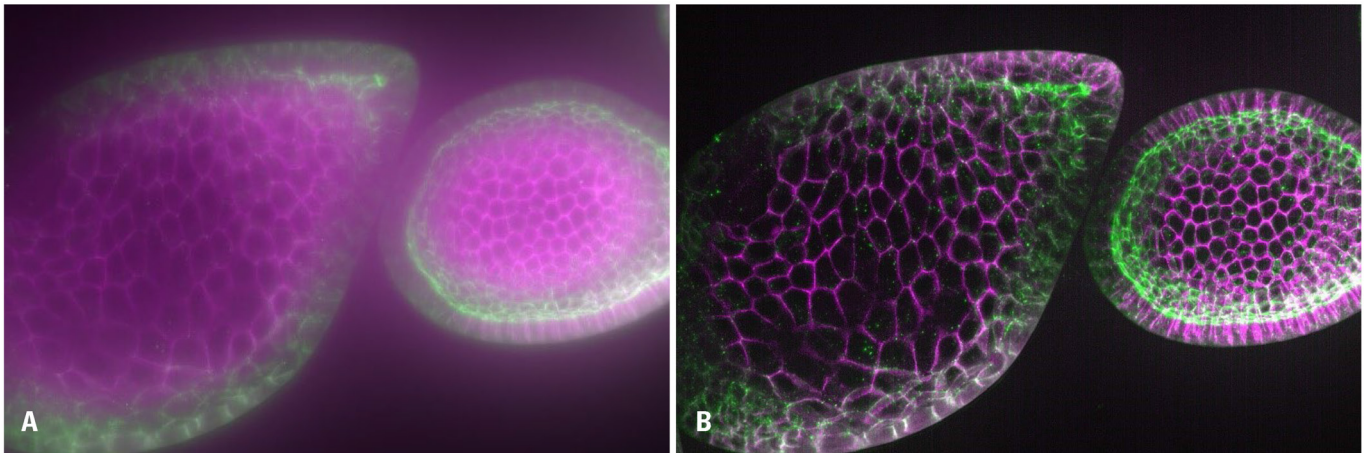

Figure 3: Imaging two follicles from a single ovariole. (A) Raw widefield and (B) THUNDER image representing a 3D maximum intensity projection of a 27.5 µm thick z-stack of Drosophila follicles. The THUNDER image was processed with LVCC. Green indicates aPKC and magenta Dlg. Images courtesy of Mark Khoury and Dr. David Bilder, University of California, Berkeley, USA.

Better Multiplexed Imaging with Computational Clearing

The increased resolution of THUNDER images with LVCC applied means that distributions of apicobasal proteins can be more clearly distinguished between mutant and wild-type Drosophila follicles or egg chambers. Such haze-free, rapidly acquired THUNDER images are therefore useful for studying epithelial morphogenesis in cancer research.

The benefit of studying 3D tissues over 2D cultures is that they can offer greater insight as they are more representative of cancer *in vivo*. However, there is less manipulation possible when working on tissues, meaning *in vitro* work is still necessary. 3D cultures, such as organoids and spheroids, are an intermediary, offering the flexibility of *in vitro* work while being more representative of the *in vivo* environment.

The next chapter discusses multiplexing methods to image multiple biomarkers simultaneously.

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Chapter 4: Imaging Over 60 Biomarkers with Cell DIVE™

As discussed in Chapter 3, multiplexed immunofluorescence imaging, or multiplexing, allows researchers to visualize multiple biomarkers in a single tissue sample. New technologies and techniques have allowed researchers to visualize over 60 targets in a single tissue.

Multiplexing Considerations

Not all multiplex imaging methods are equal. Imaging greater numbers of targets (20-60+) in a single tissue requires multiple rounds of staining, destaining, and imaging steps. Destaining steps can damage tissues because of the chemical destaining process or the physical damage of repeatedly adding and removing coverslips. Some multiplexing technologies use quenching of the fluorescence signal to limit the damage of repetitive destaining.

Choosing and validating antibodies or probes against tens of targets can be time consuming. Several multiplex imaging methods simplify the study design and target selection process by using kitted reagent panels, although this limits the flexibility of biomarkers that you can image (1).

Researchers should consider throughput capabilities carefully for studies involving tens or hundreds of slides, as some multiplexing methods are limited to processing slides one at a time or in small batches (less than 3).

Multiplexed Immunofluorescence Suggests a T-Cell Subtype Survival Marker In Colorectal Cancer Patients

Colorectal cancer (CRC) is one of the most common cancers worldwide, with high incidence and mortality rates. In stage III CRC, postoperative chemotherapy only benefits a minority of patients, and many will still develop metastases. There is a lack of biomarkers for identifying patients at high risk of recurrence following adjuvant chemotherapy.

A recent study shows how multiplexed immunofluorescence imaging can identify markers that may enable clinicians to predict the possibility of tumor recurrence in CRC patients (2).

Identifying New Biomarkers for CRC

The study was conducted on 117 stage III CRC patients who had received fluorouracil-based adjuvant chemotherapy. Tissue microarrays were prepared with up to three cores per patient.

Single sections underwent multiplexed immunofluorescence staining for immune cell markers, CD45, CD3, CD4, CD8, FOXP3, PD1, and tumor/cell segmentation markers.

The images were analyzed using a machine learning probabilistic classification algorithm that identified different types of immune cells and quantified their presence within the tumor and tumor microenvironment.

One of the significant advantages of multiplexed imaging is the ability to identify multiple markers simultaneously, providing a more comprehensive understanding of the immune microenvironment within the tumor and allowing for the identification of specific immune cell subtypes.

This study found that a multi-marker classification of regulatory T cells (Tregs) was significantly associated with disease-free survival and overall survival of folinic acid, fluorouracil-, and oxaliplatin (FOLFOX)-treated patients. Specifically, $CD3^{+}/CD4^{+}FOXP3^{+}/PD1^{-}$ Tregs were associated with improved survival, while $PD1^{+}$ Tregs were not.

Results from an independent FOLFOX-treated cohort of 191 stage III CRC patients support this finding, where higher $PD1^{-}$ Tregs were associated with increased overall survival.

Quantification of Immune Cell Types

Another significant advantage of multiplexed imaging is that it can accurately quantify immune cell types. Traditional methods of quantifying immune cell populations, such as manual counting, are subjective and prone to variability between observers.

In contrast, the probabilistic classification algorithm used in this study allowed for objective and accurate quantification of immune cell populations. Results were validated by comparing the automated scores from the machine learning algorithm to manual assessments made by an expert pathologist using the Cell DIVE™ multiplexing method. The two sets of data showed excellent agreement.

Spatial Information from Multiplexed Imaging

The study also demonstrated how multiplexed imaging can provide information on the spatial distribution of immune cells within the tumor and tumor microenvironment.

This information can provide insights into the functional roles of different immune cell populations and their interactions with tumor cells. For example, the study found that higher stromal and total immune cell content was associated with better outcomes, suggesting the presence of immune cells within the tumor microenvironment is critical for an effective antitumor immune response.

Overall, the study highlights the potential of multiplexed immunofluorescence imaging to provide valuable insights into the immune microenvironment within the tumor and its relationship with patient outcomes. Multiplexed imaging offers a potentially powerful tool for biomarker development, predicting patient response to treatment, and identifying patients at high risk of cancer recurrence.

Spatial Landscape of the Tumor Microenvironment Using Multiplexed Imaging

Pancreatic tumors are highly heterogenous, often with more aggressive regions responsible for invasion and metastasis. These aggressive pancreatic ductal adenocarcinoma (PDAC) cells undergo epithelial-to-mesenchymal transition and create subregions that evade treatments and provide a critical support niche for continued tumor growth and metastasis. Despite advancements in chemotherapy and targeted therapies, the 5-year survival rate remains at around 40%, showing the need for improved treatments for all pancreatic cancer patients.

A PDAC formalin-fixed paraffin-embedded section was iteratively stained with 30 biomarkers and imaged using Cell DIVE™ multiplexed imaging solution to define aggressive tumor cells spatially (3). The markers used in this study define metabolic and hypoxic, apoptotic, epithelial–mesenchymal transition, and immune cell status spatially within the tumor (Figure 1A). Specifically, the hypoxia marker Glut1, and two PanCK biomarkers separate cancer cells with increased glucose uptake, which is a cancer hallmark (Figure 1B).

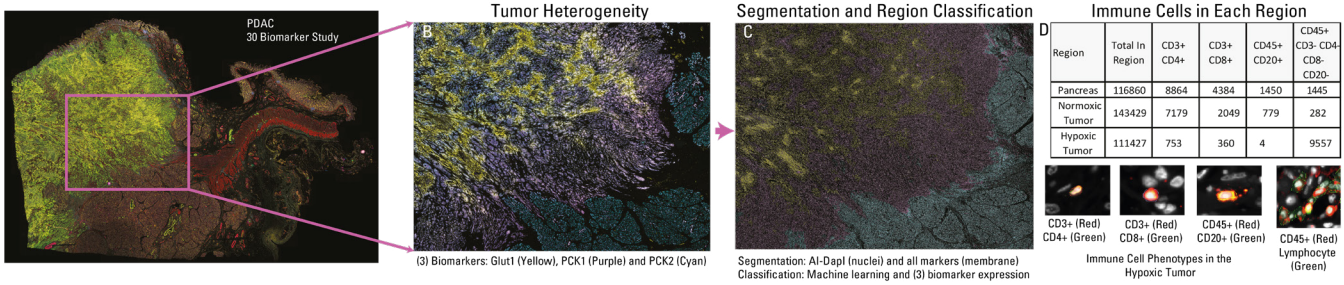

Figure 1: Spatial definition of aggressive tumor cells using iterative staining of 30 Biomarkers. (A) View of tissue following iterative staining. (B) Biomarkers Glut1, PCK1, and PCK2 separate cancer cells with increased glucose uptake. (C) Classification of heterogenous regions of the tumor following segmentation. (D) Quantification of immune cells in each region shows an overall reduction in lymphocytes and increase in $CD45^{+}$ populations in hypoxic tumor regions (3).

Following segmentation using the complete set of 30 biomarkers, a small subset of biomarkers was used to classify and spatially characterize heterogeneous regions of the tumor (Figure 1C). Such analysis reduces heterogeneity by analyzing cell types in more aggressive tumor regions separately from cells in less aggressive regions or normal tissue.

Reduction of Lymphocytes in Hypoxic Tumor Region

When quantifying immune contribution, there is an overall reduction in lymphocytes and increased CD45$^+$ populations in hypoxic tumor regions compared with normoxic tumor and normal pancreas, and increased CD45$^+$ populations, even though the total cells in all regions are similar (Figure 1D). Representative phenotype images are shown (Figure 1D, bottom panel). Clustering further defines the cell populations without bias. Cluster analysis and dimension reduction revealed fewer populations in the hypoxic tumor region than in other regions (Figure 2). Similar to immune cell counts based on phenotyping, very few lymphocytes are present in the hypoxic tumor regions (Figure 2C cluster and UMAP).

Interestingly, several clusters reveal populations of cancer-associated fibroblasts (CAFs) with apparent collagen deposition (Figure 2C, orange box). Further analysis of spatial cluster relationships clearly defines cell types commonly found in the same spatial neighborhood as the CAFs (Figure 2C, neighborhood charts). For example, Cluster 3 (SMA$^+$ VIM$^+$ collagen$^+$ CAFs cluster) and Cluster 7 (immune cell; CD45$^+$, collagen$^+$) are common neighbors, indicative of inflammation and tumor desmoplasia commonly associated with poor survival outcomes in PDAC patients (4).

The Power of Multiplexed Imaging and Single-cell Analysis Combined

The study demonstrated iterative staining and imaging of a single PDAC section with 30 biomarkers using the Cell DIVE™ multiplexed imaging solution. This combination of multiplexed imaging and single-cell analysis within the tumor microenvironment is powerful and enables a detailed examination of single-cell biomarker expression in the context of the aggressive region of a tumor. Here, the contribution of the extracellular matrix, inflammatory cells, and CAFs have been spatially defined within the tumor microenvironment.

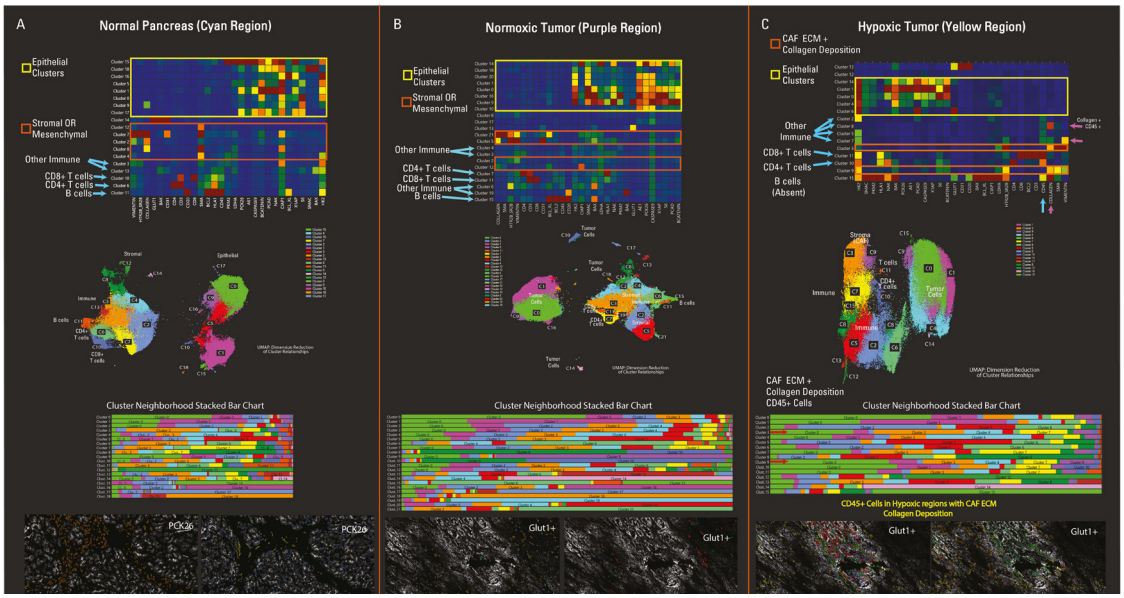

Figure 2: Cluster analysis and dimension reduction reveal fewer populations in the hypoxic tumor region. Analysis of (A) normal pancreas, (B) normoxic tumor, (C) hypoxic tumor (3).

Characterizing the tumor microenvironment in aggressive tumor regions can help elucidate the mechanisms that contribute to poor patient outcomes. Understanding these mechanisms may lead to the discovery of novel interventions to improve future therapeutic success in treating PDAC.

Advantages and Limitations of Cell DIVE™ Multiplexing

Cell DIVE™ multiplexing solution is tissue preserving, and biomarker expression can continue to be explored on the same tissue section and overlayed with all previously stained biomarkers from the study. Multiplexing also allows access to information on large numbers of targets in the same tissue.

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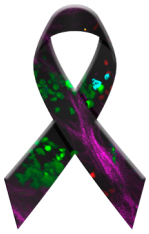

Chapter 5: Fluorescence Lifetime Imaging and Stimulated Emission Depletion Microscopy

Fluorescence Lifetime Imaging Microscopy (FLIM)

While the vast majority of imaging experiments measure the intensity of fluorescence, FLIM uses another key property of fluorescence to add a rich source of information to research – its lifetime. A fluorescent dye has a characteristic emission spectrum and also a fluorescence lifetime which reflects how long the dye stays in the excited state. Fluorescence lifetimes are exquisitely suited to report on biomolecular functional states, because the time a molecule stays in the excited state is highly dependent on its environment and interactions with other nearby species (1,2). As lifetime information is independent of fluorophore concentration, it is a method of choice for functional imaging (3).

An added benefit of FLIM is it is extremely sensitive to the microenvironment surrounding the fluorophore, such as the pH, temperature, viscosity, and presence of specific biomolecules. Because these conditions are dynamic and vary depending on the physiological state of a given cell, FLIM provides quantitative information about the molecular environment of cells that intensity-based methods cannot and is the gold standard to measure Förster resonance energy transfer (FRET). FRET is the gold standard to study the interaction of two fluorescently tagged proteins.

FLIM can provide deep insights into cancer pathophysiology by revealing the interactions between cancer cells and their surrounding microenvironment. It can also inform the development of new diagnostic and therapeutic strategies by validating the delivery of candidate therapeutics. Futhermore, it can use both exogenous fluorophores and endogenous ones, like the metabolic enzyme cofactor NADH which exhibits autofluorescence. In the latter case, FLIM is label-free.

FLIM in Action: Example Applications in Cancer Imaging

During cell metabolism, NADH is oxidized to NAD^+ via oxidative phosphorylation. Glycosylation reduces NAD^+ back to NADH. Thus, cells exhibiting more glycosylation have more NADH. Furthermore, the oxidation:reduction ratio is related to the free:protein-bound NADH ratio (Figure 1).

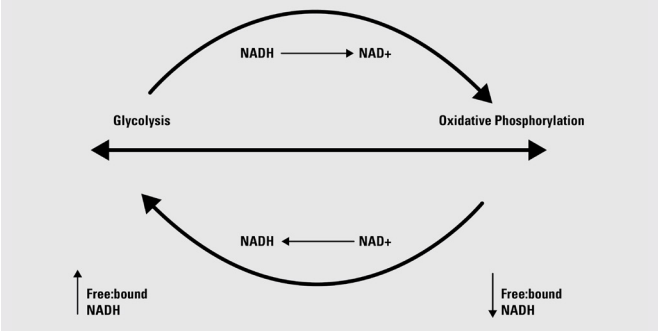

Figure 1: The configurational difference between free NADH and bound NADH impacts the fluorescence lifetime of NADH. Correlated with the redox status of the enzyme cofactor, the relative amount of free and bound NADH is a measure of cellular metabolic status along a spectrum from glycolysis to oxidative phosphorylation.

Since the fluorescence lifetime of protein-bound NADH is longer than free NADH, FLIM can discriminate between the two species, providing information about cell metabolism and any oxidative stress it is experiencing (4).

Stimulated Emission Depletion (STED) Microscopy

The resolution of conventional microscopes is restricted by the diffraction limit of visible light (~ 250 nm). Advances in microscopy techniques such, as stimulated emission depletion microscopy overcome this limitation, allowing higher resolution imaging.

Stimulated emission depletion (STED) nanoscopy has revolutionized the life sciences bringing resolution well below the diffraction limit of confocal microscopy, allowing a better understanding of the spatial distribution and relationships of cellular components and processes (5). Conventional STED is built on a confocal system, scanning the sample with an excitation beam together with a donut–shaped STED beam. In this way, fluorophores return to the ground state via stimulated emission and the effective observation volume is smaller than the diffraction-limited confocal volume.

The new TauSTED technology from Leica Microsystems sets the new standard for straightforward, gentle STED using lifetime-based information (6).

In cancer biology, STED microscopy offers many benefits. For example, it can help visualize the distribution and interaction of molecules within cancer cells in unprecedented detail, providing new insights into the mechanisms underlying cancer growth and metastasis. It has also allowed the study of the nanoscale structure of cancer cells and tissues, providing valuable information about their morphology and organization.

STED in Action: Towards a Better Understanding of DNA Replication In Cancer Cells

Collisions between the DNA replication machinery and co-transcriptional R-loops can impede DNA synthesis and are a source of genomic instability in cancer cells.

R-loops are three-stranded structures that form when a single-stranded RNA molecule hybridizes with one strand of a DNA double helix during transcription. These structures play fundamental roles in gene expression regulation and DNA repair but are also associated with genomic instability and disease when not correctly regulated. Plus, their accumulation has been linked to DNA damage and genomic instability (7).

INO80 is a chromatin remodeling complex involved in DNA repair and gene expression regulation that is recruited to chromatin by R-loops. Dysregulation of the INO80 complex has also been associated with breast cancer (8), prostate cancer (9), and melanoma (10), making it an important target for new cancer therapies.

A study in *Nature Communications* used fluorescently labeled structures called INO80 foci and R-loop foci to help enhance our understanding of DNA replication in cancer cells (11).

STED Distinguishes Between True and False Molecular Collision Events

Using STED microscopy, the researchers visualized the interaction between the INO80 complex and R-loop foci. STED microscopy, which has a higher resolution than traditional confocal microscopy, allowed the researchers to distinguish between 'true' and 'false' colocalization events, and they found that INO80 foci colocalized with R-loop foci in the nucleus (Figure 2).

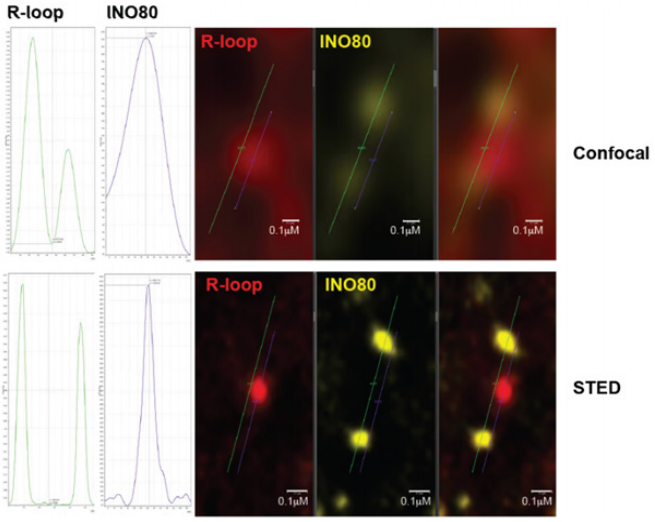

Figure 2: STED resolves the relative position of INO80 and R-loop.

In particular, the R-loops that colocalized with INO80 were significantly more intense and had greater volume and length than their non-colocalizing counterparts, suggesting that the INO80 complex preferentially associates with the largest and most enriched R-loop domains in the nucleus and may play a role in resolving these structures.

They also demonstrated that INO80 prevents genotoxic conflicts between transcription and replication machinery and promotes efficient, healthy DNA synthesis by preventing R-loop accumulation.

Given the links between R-loops, INO80, and cancer, this study demonstrates how STED can enhance our understanding of cancer development and progression by elucidating how INO80 dysregulation contributes to the disease. It may also benefit cancer treatment research by helping identify new therapeutic targets.

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Chapter 6: Electron Microscopy and Correlative Methods

Ultrastructure Imaging with Electron Microscopy (EM)

Electron microscopy (EM) uses electrons instead of light to image the ultrastructure of biological samples at extremely high resolution. There are two types of EM:

1. Transmission EM (TEM), where a beam of electrons is transmitted through a thin sample section, producing an extremely high-resolution image, sometimes reaching atomic resolution
2. Scanning EM (SEM) scans a focused beam of electrons across the surface of a sample to produce a 3D image of the sample's surface morphology

EM has several advantages over light microscopy. It offers much higher resolution, with the ability to visualize individual subcellular structures such as organelles and protein complexes at angstrom resolution. Also, visualization of thick or opaque samples, such as bone or metal implants, is easier.

In cancer research, EM is used to study the ultrastructure of cancer cells and tissues, including their morphology, intracellular organization, and even the atomic structure of protein complexes. This helps understand the mechanisms of cancer growth and metastasis at a level of detail that would not be possible with fluorescence microscopy.

Advantages of Combining Light and Electron Sources

In correlative microscopy, samples are imaged using light (usually fluorescence) and EM. The main advantage of correlative microscopy is integrating complementary information obtained from different imaging modalities, resulting in a more comprehensive understanding of biological processes.

For example, correlative microscopy enables the visualization of both the overall structure and the ultrastructure of cells or tissues, revealing details such as the location and distribution of proteins or subcellular structures in the context of the wider cellular environment. Correlative microscopy is particularly useful for studying the morphology and structural biology of cancer cells and the molecular interactions between tumor cells and their microenvironment.

Cryo-correlative Light and EM (CLEM)

Cryo-correlative light and EM (cryo-CLEM) is a correlative microscopy technique combining the benefits of fluorescence microscopy and EM with the added advantage of studying samples in their near-native state using cryogenics.

The fluorescence microscopy component enables researchers to image live cells, affording physiological relevance to the data that EM alone cannot provide because of the harmful nature of the illumination source.

It also enables researchers to judiciously select which samples and sample regions they wish to study downstream using EM, helping to reduce time and cost.

Cryo-CLEM in Action: Combining Live Cell Imaging and Transmission Electron Microscopy (TEM) Using Leica Microsystems' Coral Life Workflow

Live cell imaging benefits cancer research as cancer is dynamic and complex. Imaging live cells is crucial for investigating time-dependent processes such as disease initiation, progression, and treatment response, which may differ cell-to-cell.

A significant drawback of EM is the lethality of the electron

beam and vacuum within the microscope. The cells of interest must be preserved in a specific static state or studied with light microscopy instead. However, certain physiologically relevant cellular stages and structures are transient and too small to resolve using light microscopy alone. A recent application note demonstrates how to combine live cell imaging with cryo-EM using the streamlined Coral Life workflow and is summarized below (1).

Preparation of Sapphire Disks and Live Cell Imaging

The physical division of a cell into two daughter cells is known as cytokinesis. The final stage of cytokinesis is the cutting of the intercellular bridge between the two cells, which is called abscission. To successfully image abscission, it is first necessary to identify cells currently undergoing cell division.

Light microscopy allowed the selection of an appropriate subset of HeLa cells before downstream ultrastructural analysis using TEM (Figure 1).

6 mm sapphire disks were cleaned with hydrochloric acid (HCl 30%) for 2 hours and washed three times for 5 minutes with doubly distilled water. A finder grid pattern was evaporated onto the clean sapphire disks to ensure easy sample position retrieval. Then, a 10 nm carbon coating was applied with the Leica EM ACE600 carbon thread using 'pulse mode', and the sapphire disks were ready.

HeLa cells were cultivated, and 66,000 cells were plated per sterile SampleLink chamber and incubated for 24 hours to achieve a cell confluency of approximately 70%.

SampleLink chambers were placed in a Leica THUNDER imager. The observation was started with cytokinesis onset, and the sample was fixed after 60 minutes.

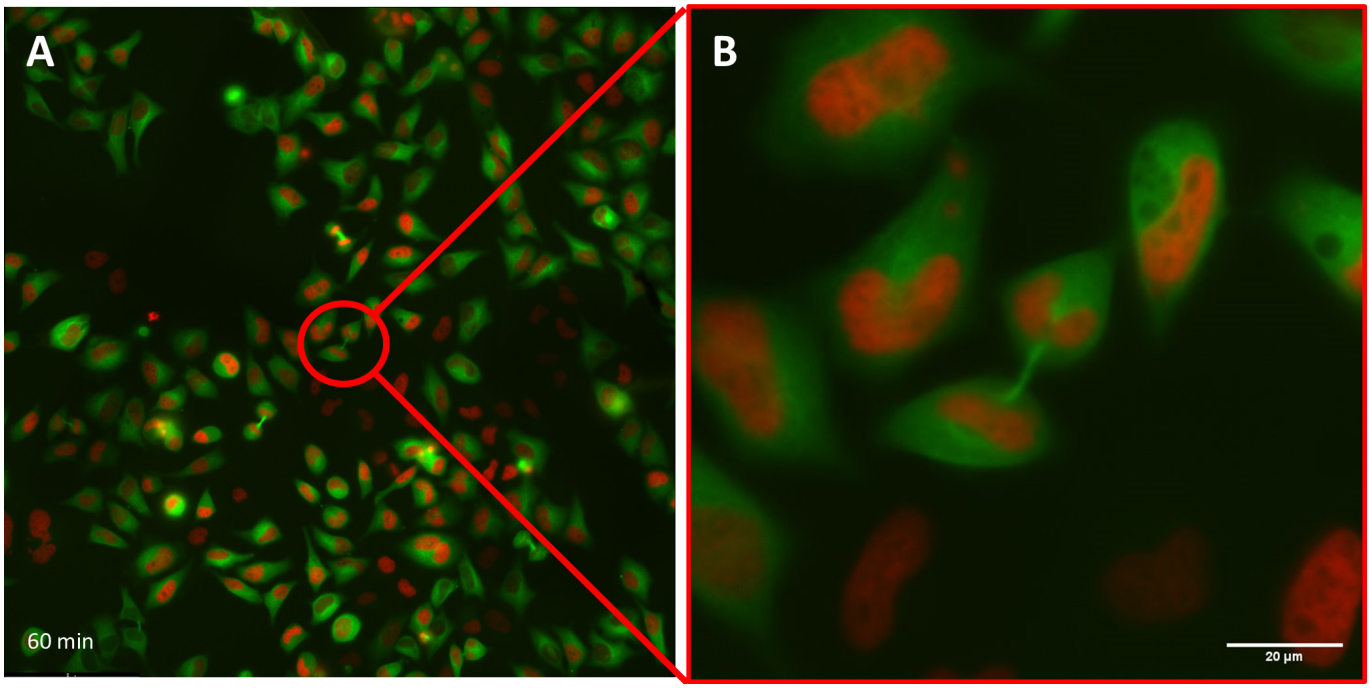

Figure 1: Identification of the region of interest using fluorescence light microscopy. HeLa Kyoto HKF1 cells expressing H2B-mCherry (nuclei in red) and alpha tubuline-mGFP (cytosol in green). (A) Spiralscan creating an overview acquired with the 40x/1.1 NA sapphire-corrected objective. (B) Region depicted by the circle in *A*, magnified. The dividing cell of interest is in the center.

Freeze Substitution and Embedding

An en bloc freeze substitution protocol was used, with a freeze substitution cocktail containing 1% OsO_4 + 0.2% uranyl acetate + 2.5% water in dry acetone. While strong metal staining inside the sample provides good contrast during SEM imaging, it also can be used for TEM imaging; however, it slightly reduces the resolution due to the metal accumulation on lipids. Samples were placed under liquid nitrogen in the freeze substitution cocktail and transferred into the pre-cooled Leica EM AFS2 system at -90°C.

Sample Retrieval, Sectioning, and TEM

Retrieval of the region of interest is critical for correlative methods. In this study, it was located using the finder grid pattern and trimmed using the Leica EM TRIM2.

Serial sectioning was performed because only 5-7 sections contained the target feature, and the sections were mounted on slot grids. As an en bloc freeze substitution and infiltration protocol were used, no further staining was required. Raw data were post-processed using THUNDER technology, and an overlay of the light microscopy and EM data was created using landmarks placed under both the light and electron microscopes.

Data Correlation

The Coral Life workflow enables the overlay of live cell and EM images to capture transient, ultrastructural cellular details that are impossible to resolve with either light or EM alone (Figure 2).

Click here to access the full, step-by-step Coral Life workflow.

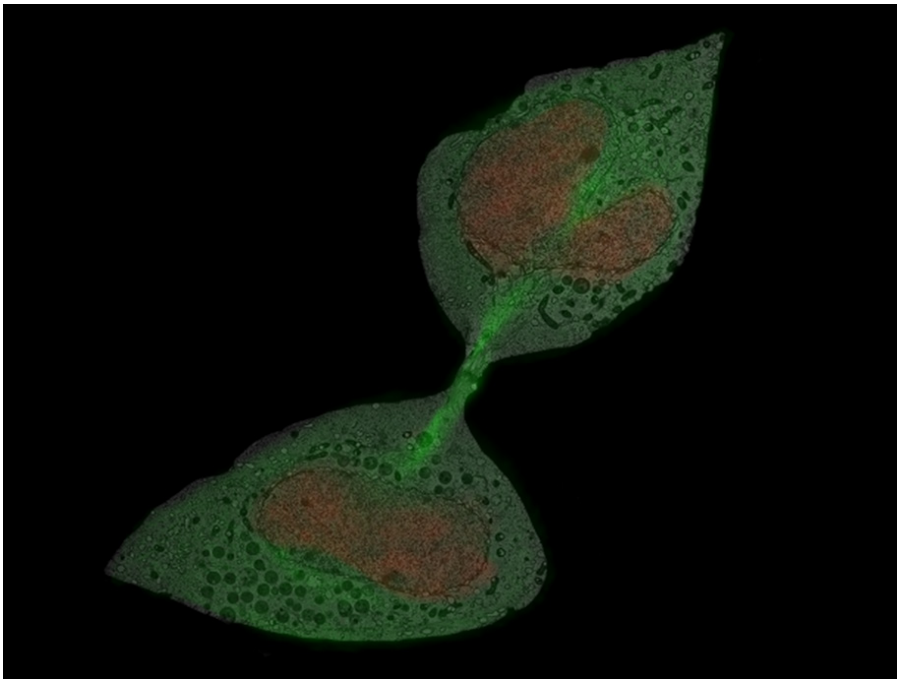

Figure 2: Image overlay of the light microscope and EM image.

How to Successfully Perform Live Cell CLEM: The Coral Life Workflow

Improving Live Cell Imaging and Cryo-CLEM of Thick Samples with Leica THUNDER Technology

Model organisms, including *Drosophila melanogaster* and *Caenorhabditis elegans*, allow researchers to understand the fundamental cellular functions that become dysregulated in cancer.

In live cell fluorescence micrographs of *C. elegans* embryos expressing SPD5-GFP, a green marker for centrioles, the centrioles appear dim and are hard to distinguish from the background.

Removal of the inherent haze using the Leica THUNDER Imagers' Large Volume Computational Clearing (LVCC)

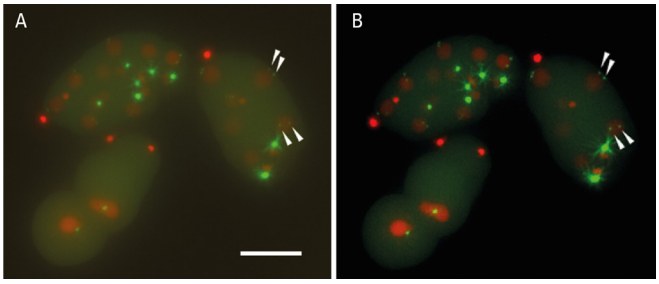

Figure 3: *C. elegans* cells expressing SPD5-GFP, a centriole marker (green), and Histon 2B- Cherry visualizing nuclei (red). (A) Maximum projection of a z-stack before Instant Computational Clearing (ICC) and Small Volume Computational Clearing (SVCC). There are two pairs of centrioles in interphase cells marked by arrows. (B) Maximum projection of a z-stack after ICC and SVCC. Scale bar is 20 µm.

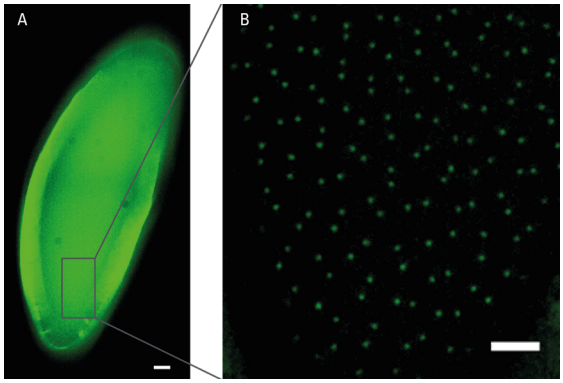

Figure 4: Drosophila embryos labelled with the centriole marker Ana2-GFP. (A) Maximum projection of a mosaic z-stack of the complete embryo prior to ICC and LVCC. Scale bar is 50 µm. (B) Maximum projection of a z-stack after ICC and LVCC. Scale bar is 10 µm. After haze removal, the paired centrioles can be followed easily with further analysis in the EM.

technology allowed the detection of the centrioles (Figure 3). Haze removal is beneficial for screening and identifying the best conditions to image small, dim structures in thick samples (2).

To further emphasize the advantage of LVCC for thicker samples, Drosophila embryos expressing Ana-2-GFP were imaged to visualize the centrioles dividing synchronously. The aim was to create a timeline of the centriole division in the EM. The eggshell has a high fluorescent background making it difficult to detect the central signal. However, once the images were processed with ICC and LVCC to remove the eggshell background fluorescence, the presence and location of the centrioles were clear (Figure 4) (2).

Advantages and Limitations of EM

EM provides incredibly high-resolution images of cellular structures allowing intricate details to be imaged. However, one limitation is that samples must be fixed and sectioned, which can lead to artifacts and distortions. Additionally, EM cannot be used to study living cells, as the electron beam damages biological samples.

Confocal and fluorescence microscopy, on the other hand, allow for live cell imaging and can provide dynamic information about cellular processes in real-time. However, the resolution of these techniques is limited compared to EM. Ultimately, the choice of microscopy method depends on the research question and what information is needed to answer it.

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Chapter 7: What Else Does Leica do for Cancer Research?

Leica Microsystems has shaped the future of microscopy solutions for over 175 years, with a strong tradition of innovation and commitment to nurturing close collaborations with the scientific, medical, and industrial communities. Drawing on users' ideas and needs, and developing solutions tailored to meet their requirements, we continue to set new standards in the markets we serve. We pride ourselves not only on product development but also on the expert technical application and post-sales support we offer across all areas of microscopy.

It is easy to focus on the 'classic' role that microscopes play in pathological diagnosis and biomedical research of cancer. However, Leica microscopes serve many different disciplines, from the research lab performing the most elemental cell biology that forms the basis of cancer research, and the translational research that helps develop new ways to diagnose and treat disease, through to surgical oncology, where microscopes help improve patient outcomes.

As our innovation evolves, the sophistication of the microscopy systems relevant to this important field has likewise evolved into advanced integrated systems that combine our technology with the know-how of our scientific and clinical collaborators.

In previous chapters, we have showcased how Leica's imaging systems, STELLARIS, Thunder, and Mica, are helping researchers better understand the molecular interactions and regulatory mechanisms behind tumor initiation, progression, and response to treatment.

We have also shown how our Cell DIVE™ platform, developed for scientists by scientists, enables researchers to build accurate maps of the cellular architecture of healthy and diseased tissues.

In translational cancer research, Leica microscopes equipped with laser microdissection help extract purely cancerous tissue without contaminating the sample with healthy cells. These Leica technologies are at the heart of visualizing, identifying, and quantifying significant cancer biomarkers, enabling researchers to predict better therapeutic outcomes.

For example, during surgical resection of a malignant glioma in the brain, maximum removal of tumour cells with minimal impact on brain tissue is critical to the outcome of the patient.

Leica's latest surgical microscope is equipped with GLOW400 3D Augmented Reality fluorescence for brain tumour surgery. This enables surgeons to visualize suspected Grade III and IV gliomas and see anatomical details more clearly thanks to multispectral imaging technology, as well as previously hidden details such as vessels and bleeding.

We are also committed to sharing our knowledge with all branches of science. Our customers can choose from a wide range of open educational resources, such as the Leica Science Lab knowledge hub, and free live scientific events, such as webinars and workshops, to learn more on the topic of choice, including cancer research.

Our novel technologies and our commitment to supporting scientists with both innovation and information are helping to create a better and healthier world.

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