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Review

The role of 3D cell cultures in understanding mitosis and tissue architecture

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Abbreviations:
2D – two-dimensional
3D – three-dimensional
ASCs – adult stem cells
CPC – chromosomal passenger complex
ECM – extracellular matrix
ESCs – embryonic stem cells
iPSCs – induced pluripotent stem cells
Lgr5+ – Leucine-rich repeat-containing G protein-coupled receptor 5 positive
LSCM – Laser Scanning Confocal Microscopy
LSFM – Light Sheet Fluorescence Microscopy
OCT – Optical Coherence Tomography
PDMS – polydimethylsiloxane
PSCs – pluripotent stem cells
SAC – spindle assembly checkpoint
SDCM – Spinning Disc Confocal Microscopy

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Abstract

Traditional cell biology research has long relied on two-dimensional (2D) cell cultures, where cells are cultivated on flat, rigid surfaces. Although these 2D systems have contributed to significant discoveries, they often fall short in replicating the complex three-dimensional (3D) environments found in living organisms. The development of 3D cell cultures, such as spheroids and organoids, provides a more physiologically relevant model by better simulating natural tissue architecture, spatial orientation, and cellular interactions. This advancement addresses limitations of 2D cultures, including discrepancies between preclinical drug efficacy and clinical outcomes. 3D cultures exhibit greater cellular heterogeneity and altered proliferation rates, which also affects drug sensitivity and gene expression profiles. One key area where 3D cultures have shown considerable impact is in the study of mitosis—a vital cellular process for growth, development, and tissue repair—often inadequately captured by 2D models. By maintaining natural cell-environment interactions, 3D cultures facilitate a more profound understanding of mitosis and its regulation, thereby enhancing our comprehension of human biology and related diseases, including cancer.

INTRODUCTION

The majority of research in cell biology has traditionally relied on two-dimensional (2D) cell cultures, where cells are grown on flat, rigid surfaces. Although these systems have led to significant discoveries, they often fail to replicate the complex, three-dimensional (3D) environment found within living organisms (Figure 1). 3D cell cultures offer a more physiologically relevant model by allowing cells to grow within a surrounding that simulates natural tissue architecture *(1)*.

The limitation of research in 2D cultures, where anti-tumor drugs that showed promising results, often proved less effective in clinical trials. Consequently, the need for 3D cell cultures, which more closely mimic the architecture and microenvironment of tissues *in vivo*, has become increasingly clear *(1, 2)*. As a result, 3D cultures have revolutionized fields such as cancer research, tissue engineering, and developmental biology, providing more accurate insights into cellular processes, disease mechanisms, and drug responses.

One key area where 3D cultures have made a significant impact is in the study of mitosis, the process by which a cell divides its chromosomes into two daughter cells. Mitosis is critical for growth, development, and tissue repair, and any errors in this process can lead to conditions like aneuploidy, which is associated with various diseases, including cancer

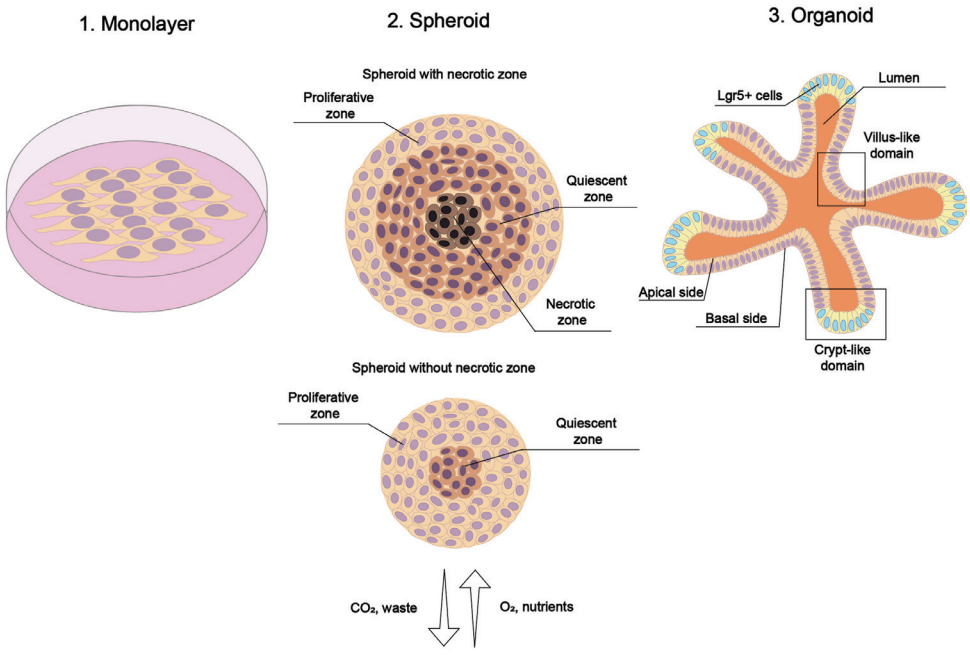

Figure 1. *Comparison between 2D and 3D cell culture models. The first diagram illustrates a 2D monolayer culture where cells grow attached to the surface of a culture flask or Petri dish in a homogeneous manner. The second diagram depicts a 3D spheroid structure with a heterogeneous organization, comprising a proliferative zone (dividing cells), a quiescent zone (non-dividing cells), and a necrotic zone (dead or dying cells). The third diagram represents the architecture of a small intestine organoid, featuring a rigid tissue structure with well-defined cell polarity. It exhibits key features of the native intestine, including a villus-like domain (differentiated cells), crypt-like domain (dividing stem cells), and a lumen (containing mucus and cellular debris).*

(3). Traditional 2D cell cultures, while valuable, often do not capture the full complexity of mitosis as it occurs in the 3D environment of living tissues *(2)*. By preserving the natural interactions between cells and their surroundings, 3D cultures offer deeper insights into the regulation of mitosis and other cellular processes, enhancing our understanding of human biology and disease.

3D CELL CULTURES AS ADVANCED RESEARCH MODELS

3D cell cultures are *in vitro* systems that grow in three dimensions, aiming to closely replicate tissue morphology and functionality by simulating the tissue microenvironment *(4, 5)*. The first 3D cultures were developed by Boiron in 1968, marking the beginning of their important role in cell culture research *(6)*. These cell cultures are categorized into scaffold-based and scaffold-free methods. Scaffold-based techniques use solid or gel scaffolds, while culturing methods without scaffold include hanging drop, low adhesion plates, rotating bioreactors, and magnetic levitation method *(7, 8)*. Scaffold-based methods produce more complex cultures, whereas scaffold-free methods allow cells to spontaneously form aggregates that vary in size, density, and cell number *(8)*. Depending on the morphology of the 3D cultures, which includes the complexity of their architecture and the type of cells forming them, in this study we will classify them into multicellular spheroids and organoids, with a focus on small intestine organoids, as mitosis was studied into these systems.

Multicellular Spheroids

Multicellular spheroids are widely used as a model for studying solid tumors *(9–11)*. The cellular arrangement within spheroids is heterogeneous, and as in tumors, it can be divided into several layers: proliferative cells on the periphery, quiescent cells in the middle, and necrotic cells at the core (Figure 1) *(2, 7, 9, 12, 13)*. The thickness of these layers depends on the spheroid's size *(14)*, treatments, and exposure to stress *(15)*. Smaller spheroids may lack a necrotic core and proliferative cells can be evenly distributed *(13)*. Spheroids also exhibit gradients of metabolic gasses and nutrients, with outer layers having better access to oxygen, glucose, amino acids, and other medium components, while in the inner layers the availability is lower with the accumulation of lactate and other metabolites, leading to lower pH *(12, 16, 17)*. Considering that spheroids exhibit cellular gradients, and they have hypoxic regions that are deprived of nutrients and accumulate metabolites, they are an excellent model for studying micro metastases and non-vascularized tumor regions (Figure 1) *(12)*. It is important to note that spheroids can

exhibit various shapes and complexities, however, we will focus on the type of multicellular spheroids described in this paragraph as mitosis studies were conducted on them.

Organoids

Organoids are 3D miniaturized and simplified versions of organs created *in vitro*, designed to mimic certain aspects of an organ's structure and function. These structures originate from stem cells, including pluripotent stem cells (PSCs) like embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), as well as adult stem cells (ASCs). PSCs have the remarkable ability to differentiate into any cell type in the body, while ASCs can self-renew and differentiate into cell types specific to their tissue of origin. This capability allows stem cells to self-organize into the diverse cell types that constitute an organ *(18–20)*.

Since the establishment of PSC lines, researchers have leveraged insights from developmental biology to generate organoids representing various organs, including the brain, retina, lungs, liver, and kidneys *(20–22)*. This process typically involves guiding PSCs through specific developmental stages to form complex tissue structures. Organoids derived from ASCs have also become a significant area of research due to their potential to model specific tissues and organs *in vitro*. For example, intestinal organoids can be generated from Leucine-rich repeat-containing G protein-coupled receptor 5 positive (Lgr5+) stem cells, exhibiting key features of the native intestine, such as villus-like structures, functional crypts and lumen (Figure 1) *(23)*. Similarly, liver organoids created from adult liver stem cells replicate various aspects of liver function, making them valuable for studying liver disease and drug testing *(24)*. However, challenges exist, including the limited proliferative capacity of ASCs compared to PSCs, which can impact the scalability of organoid production. Additionally, ASCs often require specific growth factors and conditions to maintain their stemness and promote differentiation, making them more challenging to work with *(20, 25)*.

Despite these challenges, ongoing advancements in stem cell biology and tissue engineering continue to enhance the robustness and applicability of organoid technology. As research progresses, both PSC- and ASC-derived organoids are expected to play an increasingly significant role in personalized medicine, disease modelling, and regenerative therapies. For example, organoids derived from patient-specific induced pluripotent stem cells can serve as personalized models for investigating individual treatment responses and developing tailored therapeutic strategies *(19)*. Organoids represent a groundbreaking advancement in biological research, offering more accurate and physiologically relevant models of human organs than traditional 2D cultures, with significant promise for studying development, diseases, and therapeutic responses *(19, 20)*.

OVERVIEW OF MICROSCOPY AND MITOSIS

Microscopy

Microscopy is an essential tool in the field of biological science, allowing scientists to observe and investigate structures at magnifications beyond the capability of the human eye. Advances in microscopy have significantly enhanced our capacity to examine the morphology, composition, and dynamic processes within cells, tissues, and materials *(26)*. Modern microscopy includes a diverse array of techniques, each tailored to obtain specific types of information from a sample.

Bright-field microscopy is a widely used technique to observe and measure spheroids and organoids shape and size in real time without requiring special preparation or staining *(27–29)*. Its non-invasive nature makes it ideal for tracking changes in 3D cell culture morphology over time, though finer structural details can be missed due to the resolution limitations *(28)*.

Fluorescence Microscopy is a technique that uses specific wavelengths to excite the fluorophores in the sample. Excitation is followed by the emission of photons that have lower energy, and higher wavelength than the absorbed light. Fluorescence microscopy allows detailed imaging of biological structures at the nanometer scale *(30)*. This technique is highly valuable for visualizing organoid structure, cell types, biomolecules and protein expression levels *(28)*, although it has limitations, such as sample damage from fluorescent dyes and phototoxicity from reactive oxygen species *(31–33)*. Balancing image quality with minimizing photobleaching and phototoxicity remains a challenge in live imaging *(34–36)*. Laser Scanning Confocal Microscopy (LSCM) is a widely used imaging technique for organoid research, offering high-resolution, depth-specific imaging and 3D reconstruction with a penetration depth of about 100 micrometers, making it ideal for studying subcellular structures and dynamic processes like chromosomal segregation *(28, 37, 38)*. Spinning Disc Confocal Microscopy (SDCM) improves upon traditional LSCM by using a spinning Nipkow disk, enabling rapid, high-frame-rate imaging that is particularly useful for live and high-throughput imaging tasks, with reduced photobleaching and phototoxicity *(39–41)*. It is ideal for capturing dynamic processes like cell division and migration in organoids in real time *(28, 42, 43)*. However, its fixed pinhole size can limit optical slice thickness and imaging resolution *(28, 44)*. Light Sheet Fluorescence Microscopy (LSFM) is an imaging technique that uses a thin sheet of excitation light to illuminate only the focal plane of a sample, leaving other regions unaffected. The fluorescence signal is captured perpendicular to the illumination axis, which allows for accurate imaging of specific planes. LSFM is especially beneficial for organoid research, as it enables rapid, high-resolution 3D imaging of large specimens, with minimal

photobleaching and phototoxicity *(22, 45–47)*. This technique facilitates detailed reconstructions of organoids *(48, 49)*. LSFM is also effective for high-throughput screening, allowing researchers to monitor organoid responses to treatments and capture their development at cellular level over time *(50, 51)*. Additionally, it serves as a powerful tool for visualizing drug penetration and automatically quantifying multiple tumor parameters in organoid models *(50)*.

Modern microscopy techniques have enabled scientists to visualize molecular interactions and dynamics at the nanoscale within living cells, thereby providing unprecedented insights into cellular processes. The advantage of confocal microscopy is its higher resolution compared to LSFM. However, it has limitations, including longer imaging times, higher phototoxicity, and restricted sample thickness, which can prevent full imaging of larger 3D cultures or spheroids floating in the cell culture medium. LSFM is currently the best option for long-term live imaging of 3D cell cultures, despite generating large amounts of data (terabytes), requiring substantial storage and advanced data analysis tools for processing and interpretation *(28)*.

Mitosis

The eukaryotic cell cycle involves four coordinated stages: G1 (cell growth), S (replication of DNA), G2 (second cell growth), M (mitosis). The cells have two states during the cell cycle, the interphase state and mitosis state. The first state, interphase, is when DNA is replicated, preparing the cell for the second state, mitosis state, the process by which an eukaryotic cell splits into two daughter cells. M phase has two main stages: mitosis, where the duplicated chromosomes are divided, and cytokinesis, where the cytoplasm and organelles are distributed to each new cell *(52)*. The cell cycle is regulated by various intracellular and extracellular signals. If conditions are unfavorable or a phase cannot be completed, the regulatory system will halt the cycle *(53)*. Transitions between cell cycle phases are controlled by the cell cycle control system, which activates and monitors processes like DNA replication, mitosis, and cytokinesis based on the cell's conditions and environment *(3)*.

Mitosis is subdivided into prophase, prometaphase, metaphase, anaphase, and telophase. During prophase, chromatin condenses to form chromosomes, each with two sister chromatids joined at the centromere *(54)*. The mitotic spindle, composed of alpha and beta tubulin polymers, starts to form between two centrosomes outside the nucleus. The transition from prophase to prometaphase is marked by the breakdown of the nuclear envelope, allowing chromosomes to attach to microtubules via kinetochores, protein structures on the chromosomes. For successful mitosis, the spindle must be bipolar, with sister chromatids attached to the opposite poles, which has to be accomplished until metaphase, where all chromosomes are aligned at the cell's equatorial plane. Anaphase begins when chromosomes separate due to the shortening of microtubules, which pull the kinetochores toward the poles as the centrosomes move further apart. Telophase follows, with the disassembly of the mitotic spindle, reformation of the nuclear envelope around the separated chromosomes, and chromatin decondensation, marking the end of mitosis. Cytokinesis begins concurrently with anaphase, where a contractile ring of actin and myosin filaments forms and constricts to divide the cytoplasm, resulting in two new daughter cells *(3)*.

In summary, mitosis is a multi-stage process in eukaryotic cell division where a single cell divides into two daughter cells. Mitosis is vital for growth and tissue repair in multicellular organisms. It guarantees the precise distribution of genetic material to daughter cells, ensuring genetic stability. Studying mitosis is crucial for understanding cell function and development, as well as the causes of diseases like cancer, which can result from mitotic errors leading to uncontrolled cell division.

KEY DIFFERENCES AND LIMITATIONS OF 2D AND 3D CELL CULTURE MODELS

Traditionally, cells are grown as monolayers, also known as 2D cell cultures, where cells adhere to the surface of a culture flask or a petri dish, making it a simple and widely used culturing method (Figure 1) *(55)*. Even though this method of cell culturing is convenient and efficient, cells lack proper cell-cell interactions, tissue architecture, distribution of forces, biochemical signals, and extracellular matrix (ECM) *(1, 17, 56, 57)*.

As previously mentioned, cells within spheroids are heterogeneous, comprising both dividing and non-dividing layers and potentially a necrotic core, while monolayers are homogeneous, with most cells being proliferative *(8)*. Cancer cells grown in monolayers have unlimited access to nutrients and oxygen from the medium. Because all cells are equally exposed to the medium, there is no accumulation of metabolic gases and waste in specific areas. In spheroids, due to their architecture resembling tumors, the distribution of nutrients can be uneven, and metabolites can accumulate in certain parts *(8, 17)*. It is important to point out a major difference between organoids and other 3D cultures. Unlike spheroids, organoids have a rigid tissue architecture with well-defined cell polarity, similar to that of a healthy tissue. Organoids are composed of various cell types, including stem cells, which are difficult to maintain in 2D cell culture, so their research is limited. Moreover, a cocktail of growth factors and supplements is required to sustain organoids in cell culture, which can affect the research outcomes *(20, 23, 58)*.

Spheroids Show Lower Proliferation Rates Compared to 2D Cultures

Chignola et al. *(59)* showed that proliferation rate in multicellular spheroids better depicts proliferation rates in tumors than 2D cell cultures. Lower cell proliferation was shown in multiple cell lines when cultured in 3D in comparison to 2D cultures, such as prostate cancer cell lines *(57, 60)*, colorectal cancer cell lines *(61)*, osteosarcoma and breast cancer cell lines *(62)*. Proliferation in spheroids is influenced by the amount and composition of the ECM produced by the cells. It was shown that different glioma cell lines produce varying amounts of hyaluronic acid. Those cell lines that produced more hyaluronic acid also exhibited higher proliferation rates, although this was not correlated with the extent of the ECM *(63)*.

The Role of Extracellular Matrix in 3D Cultures

ECM is a mesh of different matrix proteins (e.g., collagens), glycoproteins (e.g. fibronectin), proteoglycans (e.g. heparan sulphate), cytokines and chemokines *(1, 64–66)*. It is a scaffold that provides a physical support to tissues, controls morphogenesis and differentiation, as well as cell proliferation. ECM affects cell adhesion and migration, and it is crucial for cell communication. Although involved in many cellular processes, it is not produced by cells growing in monolayers, highlighting a key difference between 2D and 3D cultures *(17, 63, 67)*.

It has also been shown that ECM can influence tumor progression. Non-tumorigenic HMT-3522 mammary cells grow in organized acini with well-defined cell polarity, while a tumorigenic subline grows in irregular colonies. Blocking β1-integrin in tumorigenic cells leads to reversion to a normal phenotype, where cells grow in acini, reassemble the basement membrane, and reorganize the cytoskeleton. Similarly, inhibiting α6/β4 integrin heterodimers in healthy cells results in the loss of tissue structure, causing them to grow in irregular colonies like tumorigenic cells *(68)*.

Numerous studies have shown that cells growing in monolayers are much more sensitive to drugs than cells growing in 3D cultures, which produce ECM components *(1, 2, 10, 57, 69–71)*. One such drug is paclitaxel, a widely used chemotherapeutic that stabilizes microtubules, thereby preventing tumor cell division *(72)*. HN12 cells grown in scaffold-based 3D cultures were resistant to paclitaxel concentrations up to 25 times higher than those lethal for the same cells cultured as monolayers *(69)*.

The increased drug resistance associated with ECM is also due to the smaller fraction of proliferative cells and the unequal distribution of the drug among the cells *(57)*. Research on anti-cancer drugs done on monolayer cultures typically targets proliferative cells, which dominate these cultures. However, there should be a greater emphasis on studying quiescent, necrotic, and hypoxia-adapted cells, as they are an integral part of the tumor's structure *(73, 74)*. When screening anti-cancer drugs on spheroids, many of the effective compounds for non-proliferative cells were found to be microtubule inhibitors *(70)*. Drug resistance can even be heterogeneous, with outer layers of spheroids being resistant and inner, ECM-deprived layers, being sensitive *(75)*.

The ECM significantly affects cell shape, interactions, proliferation, and various cellular processes. Its role in influencing drug sensitivity is particularly important in cancer research, as demonstrated in multiple studies. For future research on anti-cancer drugs and tumor cell division, careful consideration should be given to the ECM produced in the chosen experimental model. Research on anti-tumor drugs targeting mitotic cells should be conducted using 3D cultures to enhance their effectiveness, as these cultures respond differently to microtubule inhibitors commonly used in anti-tumor therapies.

Differences in Gene Expression Between 2D and 3D Cell Cultures

2D and 3D cultures also differ in gene expression. Interestingly, different results were observed in three different prostate cancer cell lines. Some genes were upregulated in spheroids compared to 2D cultures, while others were downregulated. For example, in PC-3 and LNCaP spheroids, ANXA1 and CD44 were upregulated, while in DU145 spheroids, they were downregulated *(57)*. This is significant because ANXA1 plays a role in apoptosis and proliferation *(76)*, and CD44 is involved in cell-cell interactions and adhesion *(77)*. Proteomic analysis of 2D and 3D neuroblastoma cultures showed overexpression of proteins related to metabolism, stress response, as well as tubulin β-2 chain and actin, crucial components in cell-cell interactions *(78)*. These studies have revealed differences in the expression of genes critical for mitosis, along with distinctions at the proteome level. This further confirms that findings from mitosis research conducted on 2D cultures cannot be directly translated to tissues.

CELL DIVISION IN MULTICELLULAR SPHEROIDS AND ORGANOIDS

Challenges Mitotic Cells Encounter in 3D Environment

Mitotic cells in multicellular tumor spheroids encounter many challenges. Mechanical stress caused by tumor microenvironment and confinement can have a negative impact on mitosis. Stress caused by the 3D environment can cause mitotic arrest, changes spindle polarity, and division axis alterations, which can lead to cytokinesis failure *(10, 15, 79, 80)*. Molla et al. observed unsuccessful mitoses within multicellular tumor spheroids, finding

that the number of binucleated cells increased by 20% in spheroids formed from three different cell lines: murine p53WT TSA/pc, HEK293, and HeLa *(10)*. Binucleated cells can form during cytokinesis failure, when a cell fails to divide into two daughter cells *(81)*. To investigate the cause, they examined various signals important for mitosis progression. The chromosomal passenger complex (CPC), which controls the spindle assembly checkpoint (SAC), was activated, allowing cells to proceed into anaphase. Furthermore, all cells showed phosphorylated histone H3 at Ser10, essential for chromosome condensation *(82)*. These findings confirmed that spindle assembly checkpoint was satisfied, and cells could proceed to anaphase. However, time-lapse imaging revealed rotation of the metaphase plate in all cells, leading to incomplete chromosome segregation. An increase in lagging chromosomes was also noted compared to 2D cultures. As a control, spheroids were placed on glass and allowed to spread overnight, where the cells divided normally into two daughter cells. They concluded that cytokinesis failure might result from destabilization of the division axis due to contact with neighboring cells or an unstretched cytoskeleton in interphase, but further investigation will be needed *(10)*.

The tumor microenvironment can apply compressive stress on a tumor, potentially limiting its growth *(5)*. It is important to investigate how this stress affects mitotic cells within tumors, and multicellular tumor spheroids serve as an excellent model for such research. Mechanical stress has been found to affect mitosis in multicellular tumor spheroids composed of HCT116 cells. Stress conditions were generated using a polydimethylsiloxane (PDMS) microdevice, which restricted spheroid growth, causing them to adopt a rod-like shape *(79)*. In spheroids with confined growth, proliferative cells were distributed throughout the whole spheroid, whereas in freely growing spheroids, they are primarily located in the outer layer, a finding noted by others *(10, 13, 79)*. Mitotic cells accumulate in the central region of confined spheroids because they are in mitotic arrest. Additionally, issues with bipolar spindle assembly were observed in confined spheroids, which had only 63% bipolar spindles compared to 89% in the control group. Notably, 22% of the spindles were monopolar *(79)*, suggesting possible problems with centrosome separation or duplication *(83)*. Because there were no problems in cell rounding, Desmaison et al. *(79)* concluded that mechanical stress causes mitotic arrest in multicellular tumor spheroids.

Contrary to this study, it has been demonstrated that the stronger the force applied to CT26 cell line spheroids, the number of proliferative cells decreases, and is localized in an increasingly thinner outer layer *(15)*. It is important to note that different methods for generating mechanical stress and varying exposure times were used in these studies, which might explain the observed differences. It is possible that CT26 cells became quiescent under the conditions of mechanical stress, because these are not optimal conditions for cells to go into mitosis. It was also shown that although the force applied to the spheroids was isotropic, it spread anisotropically. Pressure on the spheroid's surface decreased while increasing toward the center, as cells on the surface rearranged under stress *(15)*.

Additional evidence that confinement affects mitosis in spheroids was provided. In freely growing spheroids of HCT116 cells, nuclei were elongated and oriented parallel to the spheroid surface as well as the division axis in the outer layers of the spheroid *(80)*. When spheroid growth was confined with 1% agarose gel nuclei elongation decreased, and cells were less parallel to the spheroid surface. Division axis also became less parallel *(80)*. These changes were observed only in outer layers of the spheroid *(80)*, indicating that cells in the center of the spheroid are already in confined conditions because of the adjacent cells, and additional external forces don't impact them. Confinement caused prometaphase prolongation which is in the agreement with previous findings that mechanical stress in spheroids causes mitotic arrest *(79, 80)*.

Spindle positioning in cells within spheroids

Tissue architecture, polarity, and cell-cell interactions have a major role in spindle positioning and the orientation of cell division. Most studies in this field have been conducted on monolayers, which lack the complexity of the tissue and proper cell communication. In monolayers, cell polarity is artificial. Cells adhere to the substrate through focal adhesions, while their upper side is exposed to the medium *(84)*. The orientation of cell division is vital for maintaining tissue structure and is especially important in morphogenesis *(85)*.

Research on cell division orientation was mostly focused on intrinsic factors, but there is now growing interest in the influence of extrinsic factors, especially with the advancement of 3D cultures and *in vivo* studies *(86)*. Intrinsic cues that regulate spindle positioning consist of polarized cortical signals, which act as a bait for microtubule plus-end binding proteins, thereby aligning the spindle along a specific axis *(87)*. Extrinsic cues are mechanical forces that act on the cell, generated by the substrate in monolayers and by adjacent cells and components of the ECM in 3D cultures and tissues *(86)*. Direct evidence of spindle orientation being influenced by external cues was demonstrated by applying unidirectional stretch to HeLa cells and keratinocytes, causing the spindle to rotate toward the stretch direction. This occurs because external forces polarize subcortical actin, affecting spindle orientation *(88, 89)*.

In monolayers, the spindle typically aligns with the long axis of the interphase cell, which is also known as the Hertwig's rule *(90)*. It was demonstrated that when HeLa cells are cultured on different micropatterns, they

adjust their shape accordingly. During the subsequent mitosis, the spindle aligns with the long axis of the newly acquired shape *(91)*. To ensure proper bipolar spindle assembly and correct orientation during cell division, proper cell rounding is essential. Although not essential in isolated cells, in confined environments, the actin cortex generates forces that support the pressure increase within cells at the onset of mitosis, leading to cell rounding *(92)*.

It has been shown that mechanical confinement can impair mitotic cell rounding and induce aberrant mitosis in HeLa cells cultured as monolayers (92, 93). In contrast, Desmaison et al. *(79)* demonstrated that mechanical stress does not cause issues with mitotic rounding in HCT116 cells cultured as spheroids. While the use of different cell lines might explain the opposing results, another explanation could be that cells in a 3D system have a mechanism to distribute forces among themselves, reducing the negative impact on mitotic cells. Further research is needed to clarify this issue. This example highlights the importance of conducting research in 3D cell cultures, as they can produce results that differ significantly from those obtained in monolayers.

Another example why spindle positioning is important are symmetrical and asymmetrical divisions in epithelium, which are essential for proper development and barrier function. Epithelial cells are polarized, with distinct apical and basal sides. During symmetric divisions, the spindle is oriented perpendicular to the apical-basal axis, resulting in the formation of two identical basal cells. This process helps to expand and sustain the basal layer. In the asymmetrical division, the spindle rotates for 90° becoming parallel to the apical-basal axis, generating one basal cell that continues to proliferate and one suprabasal cell that begins to differentiate, contributing to the formation of the skin's protective barrier *(86, 87)*. Cortical complex consisting of LGN, NuMA and dynein/dynactin has a key role in orienting spindles in asymmetric divisions *(89)*.

Organoids as a Model for Understanding Mitosis in Tissues

Organoids have become a valuable tool in biological research, especially for studying mitosis, as they provide a more accurate representation of the natural 3D environment of tissues compared to traditional 2D cultures. This makes them an appropriate model for understanding how cells divide in conditions that closely resemble those in living organisms. For example, brain organoids mimic the organization and diversity of cells in the developing human brain, allowing researchers to observe how the orientation of the mitotic spindle affects cell fate during neurogenesis *(19)*. This 3D environment also helps in studying how physical constraints and cell interactions influence mitosis, something that is difficult to achieve with 2D cultures.

Sato and Clevers *(23)* developed methods to cultivate and manipulate genetically engineered intestinal organoids, enabling in-depth study of mitosis. These organoids form structures similar to those in the intestine, with a similar distribution of cell types. Proliferating cells are found at the base of the crypts, in the stem cell niche, where a Wnt3 gradient helps regulate cell division and behavior. To maintain tissue integrity, the organoids are composed of an epithelial layer with densely packed, columnar-shaped cells. Inside the organoids, there is a lumen. The cells are polarized, each having an apical side facing the lumen, a basal side facing the basement membrane, and lateral sides in contact with neighboring cells (Figure 2) *(23)*.

Cells have adapted their mitosis to maintain the integrity of the epithelial layer. In interphase, the nucleus is located on the basal side, while centrosome is on the apical side. At the onset of mitosis, the nucleus moves apically in a process called interkinetic nuclear migration. The cell rounds up on the apical side and stays connected to the basement membrane via actin cables, ensuring it returns to its correct position after mitosis *(94)*. In some cells it is also possible that the mitotic rounding is only partial, at the apical side, and cells remain elongated in the apical-basal direction *(95)*. Neighboring cells expand to fill the basal gap during mitosis. The metaphase plate aligns perpendicular to the apical side, and cells divide symmetrically. After cytokinesis, they begin moving towards the basal side. There are two ways in which daughter cells can reintegrate into the epithelial layer. They can either remain next to each other and become neighboring cells, or, alternatively, the daughter cells can be separated by neighboring interphase cells, adopting their characteristic columnar shape (Figure 2) *(42, 94, 95)*.

In research on mitosis in intestinal organoids, it has been observed that the localization patterns of NuMA and LGN proteins, which are known to be involved in spindle orientation, differ from expectations. While NuMA and LGN are typically found at the lateral cortex, where they connect astral microtubules with the cortex, in intestinal organoids, both proteins are localized at the basal side of the cell. This suggests that in this system, NuMA and LGN might not play a role in spindle orientation *(95)* highlighting the importance of studying mitosis in systems that more closely resemble actual tissue.

Disruptions in the process of spindle orientation can have serious consequences. For instance, the loss of Dlg-1, a protein needed for correct spindle orientation, can cause misoriented divisions in ISCs, leading to problems in cell migration and potentially contributing to cancer development *(96)*. Similarly, Tacc3, a protein that helps to stabilize microtubules and ensures proper spindle function, is essential for mitosis. When Tacc3 is depleted in mouse intestinal crypts, cell proliferation stops *(97)*. In organoids derived from mice with APC mutations, which model

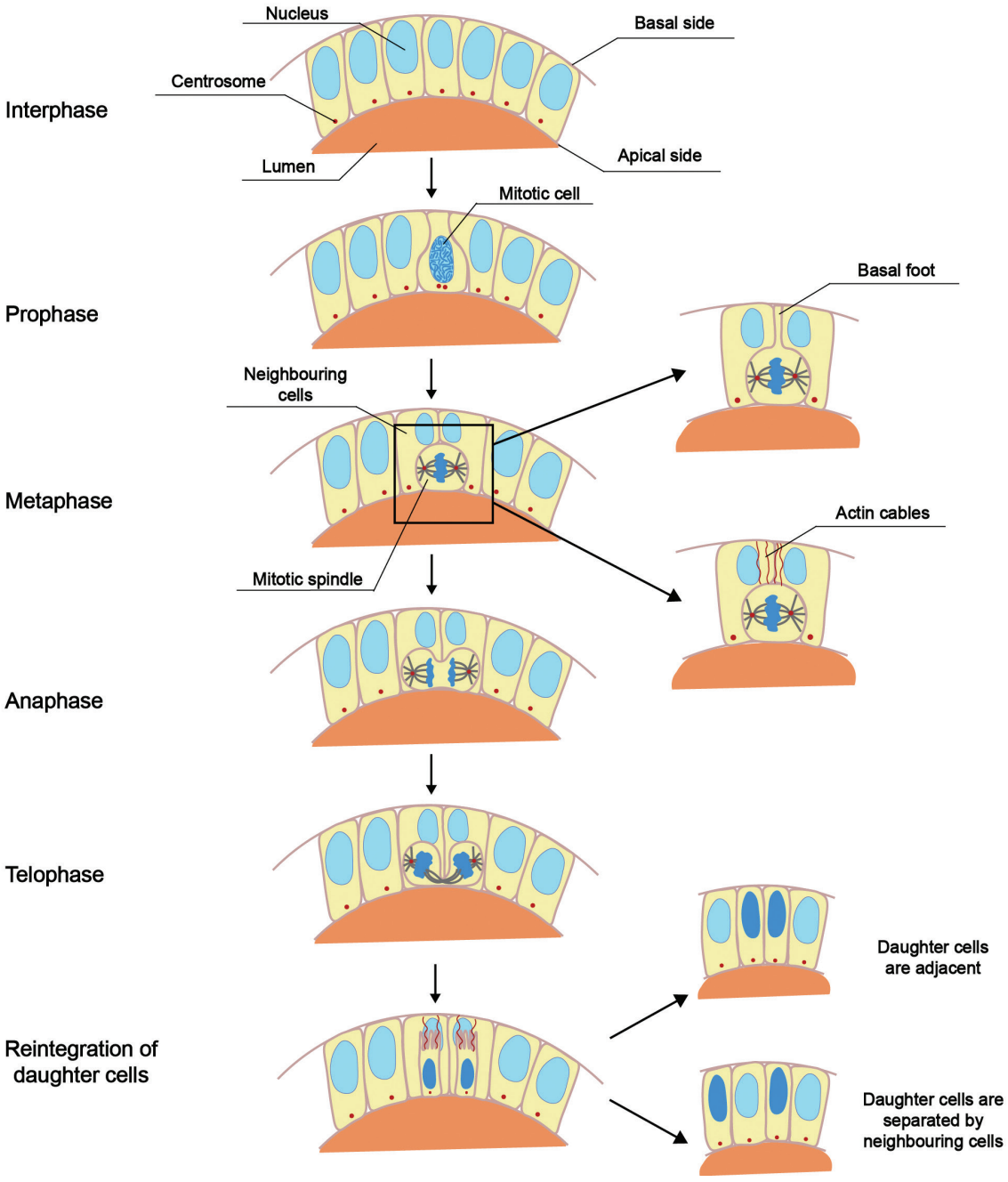

Figure 2. *Mitosis in small intestine organoids. During interphase, the nucleus is positioned on the basal side of the cell, while centrosomes are located on the apical side. In prophase, the nucleus shifts towards the apical side. The cell rounds up on the apical side and remains connected to the basement membrane through actin cables, or the cell elongates as part of the membrane fills the basal gap. During mitosis, neighboring cells expand to fill the basal gap. The metaphase plate aligns perpendicular to the apical surface, resulting in symmetric cell division. After cytokinesis, the cells begin migrating towards the basal side to reintegrate into the epithelial layer. The daughter cells can either reintegrate by becoming adjacent to one another, or they may be separated by neighboring interphase cells.*

colorectal cancer, knocking out Tacc3 leads to chromosome misalignment and defective mitotic spindles, causing either prolonged mitosis or mitotic arrest. These defects closely resemble those seen in living organisms *(97, 98)*, highlighting the relevance of organoids in studying mitotic errors related to cancer.

Knouse et al. *(99)* highlight the critical role of organoids in maintaining accurate chromosome segregation during mitosis by preserving tissue architecture. Their examination of chromosome segregation fidelity across various mammalian cell types in both tissues and cultured cells revealed that epithelial cells maintain high fi-

delity in their native environments. However, this fidelity diminishes when tissue structure is disrupted. The integrity of tissue structure, including the function of integrins, is crucial for correcting errors in chromosome attachment. Disruption of this structure in organoids can lead to chromosome instability *(99)*, a phenomenon often observed in cancer *(100)*. This finding underscores the value of organoids as a more accurate model for studying mitosis compared to traditional cell cultures.

One of the key advantages of using organoids is the ability to observe mitosis in real-time within a structure that closely mimics actual tissue. Studies have shown that intestinal organoids maintain a functioning stem cell niche, enabling researchers to study stem cell division and behavior in conditions that closely resemble those of the living intestine *(23, 101)*. This approach enhances our understanding of tissue maintenance and regeneration. Despite significant advances in mitosis research involving organoids, long-term live imaging remains a major challenge. The Liberali lab made significant progress by developing protocols for long-term live imaging using light-sheet fluorescence microscopy and software for in-depth cellular analysis, which allows for tracking mitotic cells over multiple generations and monitoring organoid growth *(102)*. This advancement enhances our understanding of cell division.

In summary, organoids offer a better model for studying mitosis compared to 2D cultures. Their ability to mimic the complex 3D structure of tissues allows researchers to observe cell division in a way that more closely resembles what happens in living organisms, leading to a better understanding of cell division in development, tissue maintenance, and disease.

DISSCUSION

The transition from traditional 2D cell cultures to more advanced 3D cultures marks a significant development in cellular biology, primarily due to the enhanced physiological relevance of 3D models. Unlike 2D cultures, where cells grow as monolayers attached to a flat surface, 3D cultures, such as spheroids and organoids, provide a more realistic representation of the *in vivo* environment, as they better mimic the complex architecture, cellular interactions, and microenvironment found within tissues and tumors *(17, 20, 55–57)*.

Why 3D Cultures are Necessary?

In 2D cultures, cells lack the spatial orientation and interaction dynamics that are essential for maintaining tissue architecture and function. Cells in monolayers do not experience the same mechanical forces, biochemical gradients, or cell-cell interactions as they would in a 3D structure. Cells in monolayers are homogeneous, primarily consisting of proliferative cells, whereas in 3D cultures, they are heterogeneous, containing proliferative cells, quiescent cells, and in some spheroids, necrotic cells as well *(8, 17)*. Proliferation rates in 3D cultures are generally lower than in 2D cultures, which is also related to the fact that not all cells in spheroids have equal access to nutrients and oxygen, which are necessary for cells to enter mitosis *(59)*.

ECM plays a critical role in providing structural support, regulating cell signaling, and maintaining tissue integrity. In 3D cell cultures, cells actively produce components of the ECM, closely mimicking the natural environment found in tissues. In contrast, in 2D cell cultures, only certain cell lines are capable of producing ECM components, and the ECM formed in these conditions often has a different, less complex structure, highlighting a significant limitation of traditional cell culture methods *(1, 64–66, 84)*. Moreover, 3D cultures are less sensitive to chemotherapy drugs compared to 2D cultures. This is likely due to the reduced proliferative capacity and the protective effect of the ECM, which can impede drug penetration and distribution *(1, 2, 57, 69)*. Studies have shown that the gene expression profiles of cells in 2D and 3D cultures can be markedly different, affecting key processes such as apoptosis, cell adhesion, and cellular metabolism *(57, 78)*. This variability underscores the importance of using 3D cultures to more accurately model *in vivo* conditions.

Depending on whether mitosis is being studied in healthy or diseased tissue, a specific type of 3D culture should be selected. Organoids are used as a model for healthy tissue, tumors and for precancerous stages of a tissue *(20)*. In contrast, multicellular tumor spheroids are commonly used in the study of solid tumors *(9–11)*. It is important to note that in this type of research it is better to use tumor cell lines, because non-tumor cells will not divide in this system due to the growth arrest caused by contact inhibition *(103)*.

The Role of 3D Cultures in Advancing Our Knowledge of Mitosis

The study of mitosis in 3D cultures has revealed insights that would be difficult to obtain from 2D cultures. For instance, in spheroids, mitotic cells often experience incomplete cytokinesis, resulting in a higher number of binucleated cells *(10)*. This incomplete cell division might be due to mechanical stress and altered spindle dynamics, which are influenced by the 3D structure and the surrounding microenvironment *(79)*. Additionally, in intestinal organoids, the orientation of cell division is carefully regulated to maintain tissue architecture, ensuring proper cell placement post-mitosis *(42, 94, 95)*. Disruptions in these processes can lead to significant consequences, such as cancer development.

Although most of the key experiments in the field of mitosis have been conducted using 2D cell cultures, these

examples highlight how mitosis in 3D models of both healthy and tumor tissues differs significantly from that observed in 2D cultures. It is crucial to continue research in 3D systems to fully understand these differences and their implications.

Future Directions in Microscopy for Studying Mitosis in Complex 3D Models

Among its many applications, microscopy is particularly vital for studying mitosis. Techniques like bright-field microscopy allow real-time observation of cellular structures, however it has a significant resolution limit *(27, 28)*. Fluorescence microscopy is valuable for visualizing chromosomes and the mitotic spindle during cell division, despite challenges like photobleaching and phototoxicity *(28, 33)*. Advanced methods like LSCM and LSFM further enhance our ability to study mitosis by providing high-resolution, 3D imaging with minimal phototoxicity *(28)*.

Many findings in the field of mitosis have been discovered with the help of microscopy, especially fluorescence microscopy. Monolayer samples are thin and fixed to a substrate, making them easier to image. However, thicker samples like 3D cultures pose a challenge since their thickness exceeds the objective's working distance. Imaging can also be challenging due to the strong background signal from surrounding cells.

The selection of a microscopy technique should depend on the specific research goals. For studying mitosis in individual cells and capturing detailed intracellular images, higher resolution is required, making confocal microscopy a recommended choice. For studies requiring prolonged imaging, such as observing organoid growth and cell division across multiple generations, LSFM is the best option due to its reduced phototoxicity compared to confocal microscopy. For imaging thick 3D cultures, Optical Coherence Tomography (OCT) is also recommended as it provides deep penetration into samples, up to 3 mm *(28)*.

Although in recent years 3D cell cultures have proven to be a more suitable model for studying mitosis compared to traditional monolayers, mitosis in these systems remains relatively unexplored. Future research could focus on cell cycle regulation to determine the conditions under which cells undergo mitotic arrest as opposed to those where mitosis does not occur, and cells enter a quiescent state. Additionally, findings about the effects of the ECM, neighboring cells, and confinement, largely based on research on 2D cultures, should be validated in 3D systems to provide a more accurate representation of tissue events. Finally, investigations into the occurrence and repair of mitotic errors should be conducted using 3D models of both healthy and tumor tissues to gain a clearer understanding of the aneuploidy mechanisms that contribute to tumor development and progression.

CONCLUSION

The transition from 2D to 3D cell cultures has significantly advanced our understanding of cellular behavior and drug responses. Unlike 2D cultures, 3D models such as spheroids and organoids better replicate the complexity of tissue environments, including ECM interactions and spatial constraints. These models reveal critical differences in cell proliferation, drug resistance, and gene expression, offering more realistic insights into tumor biology and therapeutic efficacy. The enhanced physiological relevance of 3D cultures underscores their importance in cancer research and drug development. Future studies utilizing these advanced systems will likely lead to more accurate predictions of treatment outcomes and a deeper understanding of cellular dynamics. Although most of the pivotal experiments in the field of mitosis have been conducted using 2D cell cultures, these examples illustrate the significant differences in mitosis between 3D models of healthy and tumor tissues compared to 2D cultures, highlighting the importance of further research in 3D systems.

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REFERENCES

1. LANGHANS S A 2018 Three-Dimensional in Vitro Cell Culture Models in Drug Discovery and Drug Repositioning. Front Pharmacol 9: https://doi.org/10.3389/fphar.2018.00006
2. DESOIZE B and JARDILLIER J-C 2000 Multicellular resistance: A paradigm for clinical resistance? Critical reviews in oncology/hematology 36: 193–207 https://doi.org/10.1016/S1040-8428(00)00086-X
3. ALBERTS B, JOHNSON A, LEWIS J, RAFF M, ROBERTS K, and WALTER P 2002 Molecular Biology of the Cell, Garland Science https://doi.org/10.1093/aob/mcg023
4. OVSIANIKOV A, MIRONOV V, STAMPFL J, and LISKA R 2012 Engineering 3D cell-culture matrices: multiphoton processing technologies for biological and tissue engineering applications. Expert Review of Medical Devices 9(6): 613–633 https://doi.org/10.1586/erd.12.48
5. TAUBENBERGER A V, GIRARDO S, TRÄBER N, FISCHER-FRIEDRICH E, KRÄTER M, WAGNER K, KURTH T, RICHTER I, HALLER B, BINNER M, HAHN D, FREUDENBERG U, WERNER C, and GUCK J 2019 3D Microenvironment Stiffness Regulates Tumor Spheroid Growth and Mechanics via p21 and ROCK. Advanced Biosystems 3(9): 1900128 https://doi.org/10.1002/adbi.201900128
6. BOIRON M, GUILLEMAIN B, BERNARD C, PERIES J, and CHUAT J C 1968 Presence in Murine Sarcoma Virus Stocks of a Third Component which alone initiates Cellular Conversion. Nature 219(5155): 748–749 https://doi.org/10.1038/219748a0

7. LIN R-Z and CHANG H-Y 2008 Recent advances in three-dimensional multicellular spheroid culture for biomedical research. Biotechnol J 3(9–10): 1172–1184 https://doi.org/10.1002/biot.200700228
8. CHEN Q and WANG Y 2020 The application of three-dimensional cell culture in clinical medicine. Biotechnol Lett 42(11): 2071–2082 https://doi.org/10.1007/s10529-020-03003-y
9. HIRSCHHAEUSER F, MENNE H, DITTFELD C, WEST J, MUELLER-KLIESER W, and KUNZ-SCHUGHART L A 2010 Multicellular tumor spheroids: An underestimated tool is catching up again. Journal of Biotechnology 148(1): 3–15 https://doi.org/10.1016/j.jbiotec.2010.01.012
10. MOLLA A, COUVET M, and COLL J-L 2017 Unsuccessful mitosis in multicellular tumour spheroids. Oncotarget 8(17): 28769–28784 https://doi.org/10.18632/oncotarget.15673
11. NUNES A S, BARROS A S, COSTA E C, MOREIRA A F, and CORREIA I J 2019 3D tumor spheroids as in vitro models to mimic in vivo human solid tumors resistance to therapeutic drugs. Biotechnology and Bioengineering 116(1): 206–226 https://doi.org/10.1002/bit.26845
12. SUTHERLAND R M 1988 Cell and environment interactions in tumor microregions: the multicell spheroid model. Science 240(4849): 177–184 https://doi.org/10.1126/science.2451290
13. LAURENT J, FRONGIA C, CAZALES M, MONDESERT O, DUCOMMUN B, and LOBJOIS V 2013 Multicellular tumor spheroid models to explore cell cycle checkpoints in 3D. BMC Cancer 13(1): 73 https://doi.org/10.1186/1471-2407-13-73
14. SOUZA G R, MOLINA J R, RAPHAEL R M, OZAWA M G, STARK D J, LEVIN C S, BRONK L F, ANANTA J S, MANDELIN J, GEORGESCU M-M, BANKSON J A, GELOVANI J G, KILLIAN T C, ARAP W, and PASQUALINI R 2010 Three-dimensional tissue culture based on magnetic cell levitation. Nat Nanotechnol 5(4): 291–296 https://doi.org/10.1038/nnano.2010.23
15. DOLEGA M E, DELARUE M, INGREMEAU F, PROST J, DELON A, and CAPPELLO G 2017 Cell-like pressure sensors reveal increase of mechanical stress towards the core of multicellular spheroids under compression. Nat Commun 8(1): 14056 https://doi.org/10.1038/ncomms14056
16. TRÉDAN O, GALMARINI C M, PATEL K, and TANNOCK I F 2007 Drug Resistance and the Solid Tumor Microenvironment. JNCI: Journal of the National Cancer Institute 99(19): 1441–1454 https://doi.org/10.1093/jnci/djm135
17. PAMPALONI F, REYNAUD E G, and STELZER E H K 2007 The third dimension bridges the gap between cell culture and live tissue. Nat Rev Mol Cell Biol 8(10): 839–845 https://doi.org/10.1038/nrm2236
18. EIRAKU M and SASAI Y 2012 Self-formation of layered neural structures in three-dimensional culture of ES cells. Curr Opin Neurobiol 22(5): 768–777 https://doi.org/10.1016/j.conb.2012.02.005
19. LANCASTER M A and KNOBLICH J A 2014 Organogenesis in a dish: modeling development and disease using organoid technologies. Science 345(6194): 1247125 https://doi.org/10.1126/science.1247125
20. CLEVERS H 2016 Modeling Development and Disease with Organoids. Cell 165(7): 1586–1597 https://doi.org/10.1016/j.cell.2016.05.082
21. CHERRY A B C and DALEY G Q 2012 Reprogramming cellular identity for regenerative medicine. Cell 148(6): 1110–1122 https://doi.org/10.1016/j.cell.2012.02.031
22. CHEN K G, MALLON B S, MCKAY R D G, and ROBEY P G 2014 Human pluripotent stem cell culture: considerations for maintenance, expansion, and therapeutics. Cell Stem Cell 14(1): 13–26 https://doi.org/10.1016/j.stem.2013.12.005
23. SATO T, VRIES R G, SNIPPERT H J, WETERING M VAN DE, BARKER N, STANGE D E, ES J H VAN, ABO A, KUJALA P, PETERS P J, and CLEVERS H 2009 Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. Nature 459(7244): 262–265 https://doi.org/10.1038/nature07935
24. HUCH M, GEHART H, VAN BOXTEL R, HAMER K, BLOKZIJL F, VERSTEGEN M M A, ELLIS E, VAN WENUM M, FUCHS S A, DE LIGT J, VAN DE WETERING M, SASAKI N, BOERS S J, KEMPERMAN H, DE JONGE J, IJZERMANS J N M, NIEUWENHUIS E E S, HOEKSTRA R, STROM S, VRIES R R G, VAN DER LAAN L J W, CUPPEN E, and CLEVERS H 2015 Long-Term Culture of Genome-Stable Bipotent Stem Cells from Adult Human Liver. Cell 160(1–2): 299–312 https://doi.org/10.1016/j.cell.2014.11.050
25. SATO T, ES J H VAN, SNIPPERT H J, STANGE D E, VRIES R G, BORN M VAN DEN, BARKER N, SHROYER N F, WETERING M VAN DE, and CLEVERS H 2011 Paneth cells constitute the niche for Lgr5 stem cells in intestinal crypts. Nature 469(7330): 415–418 https://doi.org/10.1038/nature09637
26. MURPHY D B and DAVIDSON M W 2012 Fundamentals of Light Microscopy and Electronic Imaging, John Wiley & Sons https://doi.org/10.1002/9781118382905
27. MONZEL A S, SMITS L M, HEMMER K, HACHI S, MORENO E L, WUELLEN T VAN, JARAZO J, WALTER J, BRÜGGEMANN I, BOUSSAAD I, BERGER E, FLEMING R M T, BOLOGNIN S, and SCHWAMBORN J C 2017 Derivation of Human Midbrain-Specific Organoids from Neuroepithelial Stem Cells. Stem Cell Reports 8(5): 1144–1154 https://doi.org/10.1016/j.stemcr.2017.03.010
28. FEI K, ZHANG J, YUAN J, and XIAO P 2022 Present Application and Perspectives of Organoid Imaging Technology. Bioengineering (Basel) 9(3): 121 https://doi.org/10.3390/bioengineering9030121
29. MAHMOUD L, COUGNOUX A, BEKIARI C, ARACELI RUIZ DE CASTROVIEJO TEBA P, EL MARRAHI A, PANNEAU G, GSELL L, and HAUSSER J 2023 Microscopy-based phenotypic monitoring of MDA-MB-231 spheroids allows the evaluation of phenotype-directed therapy. Experimental Cell Research 425(2): 113527 https://doi.org/10.1016/j.yexcr.2023.113527
30. SANDERSON M J, SMITH I, PARKER I, and BOOTMAN M D 2014 Fluorescence microscopy. Cold Spring Harb Protoc 2014(10): pdb.top071795 https://doi.org/10.1101/pdb.top071795
31. DIXIT R and CYR R 2003 Cell damage and reactive oxygen species production induced by fluorescence microscopy: effect on mitosis and guidelines for non-invasive fluorescence microscopy. Plant J 36(2): 280–290 https://doi.org/10.1046/j.1365-313x.2003.01868.x
32. VROUENRAETS M B, VISSER G W M, SNOW G B, and DONGEN G A M S VAN 2003 Basic principles, applications in oncology and improved selectivity of photodynamic therapy. Anticancer Res 23(1B): 505–522
33. BERNAS T, ZAREBSKI M, DOBRUCKI J W, and COOK P R 2004 Minimizing photobleaching during confocal microscopy of fluorescent probes bound to chromatin: role of anoxia and photon flux. J Microsc 215(Pt 3): 281–296 https://doi.org/10.1111/j.0022-2720.2004.01377.x
34. ETTINGER A and WITTMANN T 2014 Fluorescence live cell imaging. Methods Cell Biol 123: 77–94 https://doi.org/10.1016/B978-0-12-420138-5.00005-7
35. ICHA J, WEBER M, WATERS J C, and NORDEN C 2017 Phototoxicity in live fluorescence microscopy, and how to avoid it. Bioessays 39(8): https://doi.org/10.1002/bies.201700003
36. KIEPAS A, VOORAND E, MUBAID F, SIEGEL P M, and BROWN C M 2020 Optimizing live-cell fluorescence imaging conditions to minimize phototoxicity. J Cell Sci 133(4): jcs242834 https://doi.org/10.1242/jcs.242834

37. DROST J, JAARSVELD R H VAN, PONSIOEN B, ZIMBERLIN C, BOXTEL R VAN, BUIJS A, SACHS N, OVERMEER R M, OFFERHAUS G J, BEGTHEL H, KORVING J, WETERING M VAN DE, SCHWANK G, LOGTENBERG M, CUPPEN E, SNIPPERT H J, MEDEMA J P, KOPS G J P L, and CLEVERS H 2015 Sequential cancer mutations in cultured human intestinal stem cells. Nature 521(7550): 43–47 https://doi.org/10.1038/nature14415

38. BRÉMOND MARTIN C, SIMON CHANE C, CLOUCHOUX C, and HISTACE A 2021 Recent Trends and Perspectives in Cerebral Organoids Imaging and Analysis. Front Neurosci 15: https://doi.org/10.3389/fnins.2021.629067

39. GRÄF R, RIETDORF J, and ZIMMERMANN T 2005 Live cell spinning disk microscopy. Adv Biochem Eng Biotechnol 95: 57–75 https://doi.org/10.1007/b102210

40. STEHBENS S, PEMBLE H, MURROW L, and WITTMANN T 2012 Imaging intracellular protein dynamics by spinning disk confocal microscopy. Methods Enzymol 504: 293–313 https://doi.org/10.1016/B978-0-12-391857-4.00015-X

41. OREOPOULOS J, BERMAN R, and BROWNE M 2014 Spinning-disk confocal microscopy: present technology and future trends. Methods Cell Biol 123: 153–175 https://doi.org/10.1016/B978-0-12-420138-5.00009-4

42. MCKINLEY K L, STUURMAN N, ROYER L A, SCHARTNER C, CASTILLO-AZOFEIFA D, DELLING M, KLEIN O D, and VALE R D 2018 Cellular aspect ratio and cell division mechanics underlie the patterning of cell progeny in diverse mammalian epithelia. Elife 7: e36739 https://doi.org/10.7554/eLife.36739

43. BOCK N and RÖHL J 2019 Real-Time and 3D Quantification of Cancer Cell Dynamics: Exploiting a Bioengineered Human Bone Metastatic Microtissue. Methods Mol Biol 2054: 59–77 https://doi.org/10.1007/978-1-4939-9769-5_3

44. CONCHELLO J A and LICHTMAN J W 1994 Theoretical analysis of a rotating-disk partially confocal scanning microscope. Appl Opt 33(4): 585–596 https://doi.org/10.1364/AO.33.000585

45. MERTZ J 2011 Optical sectioning microscopy with planar or structured illumination. Nat Methods 8(10): 811–819 https://doi.org/10.1038/nmeth.1709

46. REYNAUD E G, PEYCHL J, HUISKEN J, and TOMANCAK P 2015 Guide to light-sheet microscopy for adventurous biologists. Nat Methods 12(1): 30–34 https://doi.org/10.1038/nmeth.3222

47. CHATTERJEE K, PRATIWI F W, WU F C M, CHEN P, and CHEN B-C 2018 Recent Progress in Light Sheet Microscopy for Biological Applications. Appl Spectrosc 72(8): 1137–1169 https://doi.org/10.1177/0003702818778851

48. LI Y, MUFFAT J, OMER A, BOSCH I, LANCASTER M A, SUR M, GEHRKE L, KNOBLICH J A, and JAENISCH R 2017 Induction of Expansion and Folding in Human Cerebral Organoids. Cell Stem Cell 20(3): 385-396.e3 https://doi.org/10.1016/j.stem.2016.11.017

49. WANG Q, DONG X, LU J, HU T, and PEI G 2020 Constitutive activity of a G protein-coupled receptor, DRD1, contributes to human cerebral organoid formation. Stem Cells 38(5): 653–665 https://doi.org/10.1002/stem.3156

50. DOBOSZ M, NTZIACHRISTOS V, SCHEUER W, and STROBEL S 2014 Multispectral fluorescence ultramicroscopy: three-dimensional visualization and automatic quantification of tumor morphology, drug penetration, and antiangiogenic treatment response. Neoplasia 16(1): 1–13 https://doi.org/10.1593/neo.131848

51. HOF L, MORETH T, KOCH M, LIEBISCH T, KURTZ M, TARNICK J, LISSEK S M, VERSTEGEN M M A, LAAN L J W VAN DER, HUCH M, MATTHÄUS F, STELZER E H K, and PAMPALONI F 2021 Long-term live imaging and multiscale analysis identify heterogeneity and core principles of epithelial organoid morphogenesis. BMC Biology 19(1): 37 https://doi.org/10.1186/s12915-021-00958-w

52. COOPER G M and COOPER G M 2000 The Cell, Sinauer Associates

53. GALDERISI U, JORI F P, and GIORDANO A 2003 Cell cycle regulation and neural differentiation. Oncogene 22(33): 5208–5219 https://doi.org/10.1038/sj.onc.1206558

54. MCINTOSH J R, MOLODTSOV M I, and ATAULLAKHANOV F I 2012 Biophysics of mitosis. Q Rev Biophys 45(2): 147–207 https://doi.org/10.1017/S0033583512000017

55. BRESLIN S and O'DRISCOLL L 2013 Three-dimensional cell culture: the missing link in drug discovery. Drug Discovery Today 18(5): 240–249 https://doi.org/10.1016/j.drudis.2012.10.003

56. KAPAŁCZYŃSKA M, KOLENDA T, PRZYBYŁA W, ZAJĄCZKOWSKA M, TERESIAK A, FILAS V, IBBS M, BLIŹNIAK R, ŁUCZEWSKI Ł, and LAMPERSKA K 2018 2D and 3D cell cultures – a comparison of different types of cancer cell cultures. Arch Med Sci 14(4): 910–919 https://doi.org/10.5114/aoms.2016.63743

57. SOUZA A G, SILVA I B B, CAMPOS-FERNANDEZ E, BARCELOS L S, SOUZA J B, MARANGONI K, GOULART L R, and ALONSO-GOULART V Comparative Assay of 2D and 3D Cell Culture Models: Proliferation, Gene Expression and Anticancer Drug Response. Current Pharmaceutical Design 24(15): 1689–1694 https://doi.org/10.2174/1381612824666180404152304

58. CLEVERS H 2013 The intestinal crypt, a prototype stem cell compartment. Cell 154(2): 274–284 https://doi.org/10.1016/j.cell.2013.07.004

59. CHIGNOLA R, SCHENETTI A, ANDRIGHETTO G, CHIESA E, FORONI R, SARTORIS S, TRIDENTE G, and LIBERATI D 2000 Forecasting the growth of multicell tumour spheroids: implications for the dynamic growth of solid tumours. Cell Prolif 33(4): 219–229 https://doi.org/10.1046/j.1365-2184.2000.00174.x

60. ADCOCK A F, TRIVEDI G, EDMONDSON R, SPEARMAN C, and YANG L 2015 Three-Dimensional (3D) Cell Cultures in Cell-based Assays for in-vitro Evaluation of Anticancer Drugs. Journal of Analytical & Bioanalytical Techniques 6(3): 1 https://doi.org/10.4172/2155-9872.1000249

61. LUCA A C, MERSCH S, DEENEN R, SCHMIDT S, MESSNER I, SCHÄFER K-L, BALDUS S E, HUCKENBECK W, PIEKORZ R P, KNOEFEL W T, KRIEG A, and STOECKLEIN N H 2013 Impact of the 3D Microenvironment on Phenotype, Gene Expression, and EGFR Inhibition of Colorectal Cancer Cell Lines. PLoS One 8(3): e59689 https://doi.org/10.1371/journal.pone.0059689

62. FALLICA B, MAFFEI J S, VILLA S, MAKIN G, and ZAMAN M 2012 Alteration of Cellular Behavior and Response to PI3K Pathway Inhibition by Culture in 3D Collagen Gels. PLOS ONE 7(10): e48024 https://doi.org/10.1371/journal.pone.0048024

63. GLIMELIUS B, NORLING B, NEDERMAN T, and CARLSSON J 1988 Extracellular matrices in multicellular spheroids of human glioma origin: Increased incorporation of proteoglycans and fibronectin as compared to monolayer cultures. APMIS 96(1–6): 433–444 https://doi.org/10.1111/j.1699-0463.1988.tb05327.x

64. AUMAILLEY M and GAYRAUD B 1998 Structure and biological activity of the extracellular matrix. J Mol Med 76(3): 253–265 https://doi.org/10.1007/s001090050215

65. STREULI C 1999 Extracellular matrix remodelling and cellular differentiation. Current Opinion in Cell Biology 11(5): 634–640 https://doi.org/10.1016/S0955-0674(99)00026-5

66. HOHENESTER E and ENGEL J 2002 Domain structure and organisation in extracellular matrix proteins. Matrix Biology 21(2): 115–128 https://doi.org/10.1016/S0945-053X(01)00191-3

67. KLEINMAN H K, PHILP D, and HOFFMAN M P 2003 Role of the extracellular matrix in morphogenesis. Current Opinion in Biotechnology 14(5): 526–532 https://doi.org/10.1016/j.copbio.2003.08.002

68. WEAVER V M, PETERSEN O W, WANG F, LARABELL C A, BRIAND P, DAMSKY C, and BISSELL M J 1997 Reversion of the Malignant Phenotype of Human Breast Cells in Three-Dimensional Culture and In Vivo by Integrin Blocking Antibodies. Journal of Cell Biology 137(1): 231–245 https://doi.org/10.1083/jcb.137.1.231

69. BULYSHEVA A A, BOWLIN G L, PETROVA S P, and YEUDALL W A 2013 Enhanced chemoresistance of squamous carcinoma cells grown in 3D cryogenic electrospun scaffolds. Biomed Mater 8(5): 055009 https://doi.org/10.1088/1748-6041/8/5/055009

70. FAYAD W, RICKARDSON L, HAGLUND C, OLOFSSON M H, D'ARCY P, LARSSON R, LINDER S, and FRYKNÄS M 2011 Identification of Agents that Induce Apoptosis of Multicellular Tumour Spheroids: Enrichment for Mitotic Inhibitors with Hydrophobic Properties. Chemical Biology & Drug Design 78(4): 547–557 https://doi.org/10.1111/j.1747-0285.2011.01170.x

71. DHIMAN H K, RAY A R, and PANDA A K 2005 Three-dimensional chitosan scaffold-based MCF-7 cell culture for the determination of the cytotoxicity of tamoxifen. Biomaterials 26(9): 979–986 https://doi.org/10.1016/j.biomaterials.2004.04.012

72. AWOSIKA A O, FARRAR M C, and JACOBS T F 2024 Paclitaxel, In: StatPearls, StatPearls Publishing, Treasure Island (FL),

73. HUTCHINSON L and KIRK R 2011 High drug attrition rates—where are we going wrong? Nat Rev Clin Oncol 8(4): 189–190 https://doi.org/10.1038/nrclinonc.2011.34

74. ANTONI D, BURCKEL H, JOSSET E, and NOEL G 2015 Three-dimensional cell culture: a breakthrough in vivo. Int J Mol Sci 16(3): 5517–5527 https://doi.org/10.3390/ijms16035517

75. MURANEN T, SELFORS L M, WORSTER D T, IWANICKI M P, SONG L, MORALES F C, GAO S, MILLS G B, and BRUGGE J S 2012 Inhibition of PI3K/mTOR Leads to Adaptive Resistance in Matrix-Attached Cancer Cells. Cancer Cell 21(2): 227–239 https://doi.org/10.1016/j.ccr.2011.12.024

76. SHAO G, ZHOU H, ZHANG Q, JIN Y, and FU C 2019 Advancements of Annexin A1 in inflammation and tumorigenesis. Onco Targets Ther 12: 3245–3254 https://doi.org/10.2147/OTT.S202271

77. SENBANJO L T and CHELLAIAH M A 2017 CD44: A Multifunctional Cell Surface Adhesion Receptor Is a Regulator of Progression and Metastasis of Cancer Cells. Front Cell Dev Biol 5: https://doi.org/10.3389/fcell.2017.00018

78. KUMAR H R, ZHONG X, HOELZ D J, RESCORLA F J, HICKEY R J, MALKAS L H, and SANDOVAL J A 2008 Three-dimensional neuroblastoma cell culture: proteomic analysis between monolayer and multicellular tumor spheroids. Pediatr Surg Int 24(11): 1229–1234 https://doi.org/10.1007/s00383-008-2245-2

79. DESMAISON A, FRONGIA C, GRENIER K, DUCOMMUN B, and LOBJOIS V 2013 Mechanical Stress Impairs Mitosis Progression in Multi-Cellular Tumor Spheroids. PLOS ONE 8(12): e80447 https://doi.org/10.1371/journal.pone.0080447

80. DESMAISON A, GUILLAUME L, TRICLIN S, WEISS P, DUCOMMUN B, and LOBJOIS V 2018 Impact of physical confinement on nuclei geometry and cell division dynamics in 3D spheroids. Sci Rep 8(1): 8785 https://doi.org/10.1038/s41598-018-27060-6

81. STORCHOVA Z and PELLMAN D 2004 From polyploidy to aneuploidy, genome instability and cancer. Nat Rev Mol Cell Biol 5(1): 45–54 https://doi.org/10.1038/nrm1276

82. LI D W, YANG Q, CHEN J T, ZHOU H, LIU R M, and HUANG X T 2005 Dynamic distribution of Ser-10 phosphorylated histone H3 in cytoplasm of MCF-7 and CHO cells during mitosis. Cell Res 15(2): 120–126 https://doi.org/10.1038/sj.cr.7290276

83. TILLEMENT V, REMY M-H, RAYNAUD-MESSINA B, MAZZOLINI L, HAREN L, and MERDES A 2009 Spindle assembly defects leading to the formation of a monopolar mitotic apparatus. Biology of the Cell 101(1): 1–11 https://doi.org/10.1042/BC20070162

84. CUKIERMAN E, PANKOV R, STEVENS D R, and YAMADA K M 2001 Taking Cell-Matrix Adhesions to the Third Dimension. Science 294(5547): 1708–1712 https://doi.org/10.1126/science.1064829

85. BAENA-LÓPEZ L A, BAONZA A, and GARCÍA-BELLIDO A 2005 The orientation of cell divisions determines the shape of Drosophila organs. Curr Biol 15(18): 1640–1644 https://doi.org/10.1016/j.cub.2005.07.062

86. NESTOR-BERGMANN A, GODDARD G, and WOOLNER S 2014 Force and the spindle: Mechanical cues in mitotic spindle orientation. Semin Cell Dev Biol 34: 133–139 https://doi.org/10.1016/j.semcdb.2014.07.008

87. LU M S and JOHNSTON C A 2013 Molecular pathways regulating mitotic spindle orientation in animal cells. Development 140(9): 1843–1856 https://doi.org/10.1242/dev.087627

88. FINK J, CARPI N, BETZ T, BÉTARD A, CHEBAH M, AZIOUNE A, BORNENS M, SYKES C, FETLER L, CUVELIER D, and PIEL M 2011 External forces control mitotic spindle positioning. Nat Cell Biol 13(7): 771–778 https://doi.org/10.1038/ncb2269

89. SELDIN L, POULSON N D, FOOTE H P, and LECHLER T 2013 NuMA localization, stability, and function in spindle orientation involve 4.1 and Cdk1 interactions. Mol Biol Cell 24(23): 3651–3662 https://doi.org/10.1091/mbc.E13-05-0277

90. HERTWIG O and HERTWIG R 1884 Untersuchungen zur Morphologie und Physiologie der Zelle, Fischer (Studies of the Morphology and Physiology of the Cell, original language German)

91. THÉRY M, RACINE V, PÉPIN A, PIEL M, CHEN Y, SIBARITA J-B, and BORNENS M 2005 The extracellular matrix guides the orientation of the cell division axis. Nat Cell Biol 7(10): 947–953 https://doi.org/10.1038/ncb1307

92. LANCASTER O M, LE BERRE M, DIMITRACOPOULOS A, BONAZZI D, ZLOTEK-ZLOTKIEWICZ E, PICONE R, DUKE T, PIEL M, and BAUM B 2013 Mitotic Rounding Alters Cell Geometry to Ensure Efficient Bipolar Spindle Formation. Developmental Cell 25(3): 270–283 https://doi.org/10.1016/j.devcel.2013.03.014

93. TSE H T K, WEAVER W M, and CARLO D D 2012 Increased Asymmetric and Multi-Daughter Cell Division in Mechanically Confined Microenvironments. PLOS ONE 7(6): e38986 https://doi.org/10.1371/journal.pone.0038986

94. CARROLL T D, LANGLANDS A J, OSBORNE J M, NEWTON I P, APPLETON P L, and NÄTHKE I 2017 Interkinetic nuclear migration and basal tethering facilitates post-mitotic daughter separation in intestinal organoids. Journal of Cell Science 130(22): 3862–3877 https://doi.org/10.1242/jcs.211656

95. SALEH J, FARDIN M-A, BARAI A, SOLEILHAC M, FRENOY O, GASTON C, CUI H, DANG T, GAUDIN N, VINCENT A, MINC N, and DELACOUR D 2023 Length limitation of astral microtubules orients cell divisions in murine intestinal crypts. Developmental Cell 58(17): 1519-1533.e6 https://doi.org/10.1016/j.devcel.2023.06.004

96. YOUNG M A, MAY S, DAMO A, YOON Y S, HUR M-W, SWAT W, and PARRY L 2019 Epigenetic Regulation of Dlg1, via

Kaiso, Alters Mitotic Spindle Polarity and Promotes Intestinal Tumorigenesis. Molecular Cancer Research 17(3): 686–696 https://doi.org/10.1158/1541-7786.MCR-18-0280

97. YAO R, OYANAGI J, NATSUME Y, KUSAMA D, KATO Y, NAGAYAMA S, and NODA T 2016 Suppression of intestinal tumors by targeting the mitotic spindle of intestinal stem cells. Oncogene 35(47): 6109–6119 https://doi.org/10.1038/onc.2016.148

98. MERENDA A, FENDERICO N, and MAURICE M M 2020 Wnt Signaling in 3D: Recent Advances in the Applications of Intestinal Organoids. Trends Cell Biol 30(1): 60–73 https://doi.org/10.1016/j.tcb.2019.10.003

99. KNOUSE K A, LOPEZ K E, BACHOFNER M, and AMON A 2018 Chromosome Segregation Fidelity in Epithelia Requires Tissue Architecture. Cell 175(1): 200-211.e13 https://doi.org/10.1016/j.cell.2018.07.042

100. BAKHOUM S F and CANTLEY L C 2018 The multifaceted role of chromosomal instability in cancer and its microenvironment. Cell 174(6): 1347–1360 https://doi.org/10.1016/j.cell.2018.08.027

101. SACHS N, TSUKAMOTO Y, KUJALA P, PETERS P J, and CLEVERS H 2017 Intestinal epithelial organoids fuse to form self-organizing tubes in floating collagen gels. Development 144(6): 1107–1112 https://doi.org/10.1242/dev.143933

102. MEDEIROS G DE, ORTIZ R, STRNAD P, BONI A, MOOS F, REPINA N, CHALLET MEYLAN L, MAURER F, and LIBERALI P 2022 Multiscale light-sheet organoid imaging framework. Nat Commun 13(1): 4864 https://doi.org/10.1038/s41467-022-32465-z

103. MEERSON A, MILYAVSKY M, and ROTTER V 2004 p53 mediates density-dependent growth arrest. FEBS Letters 559(1): 152–158 https://doi.org/10.1016/S0014-5793(04)00027-4