Tips and Tricks for Cell-Based Imaging Assays: Part 3, Time-Lapse Imaging

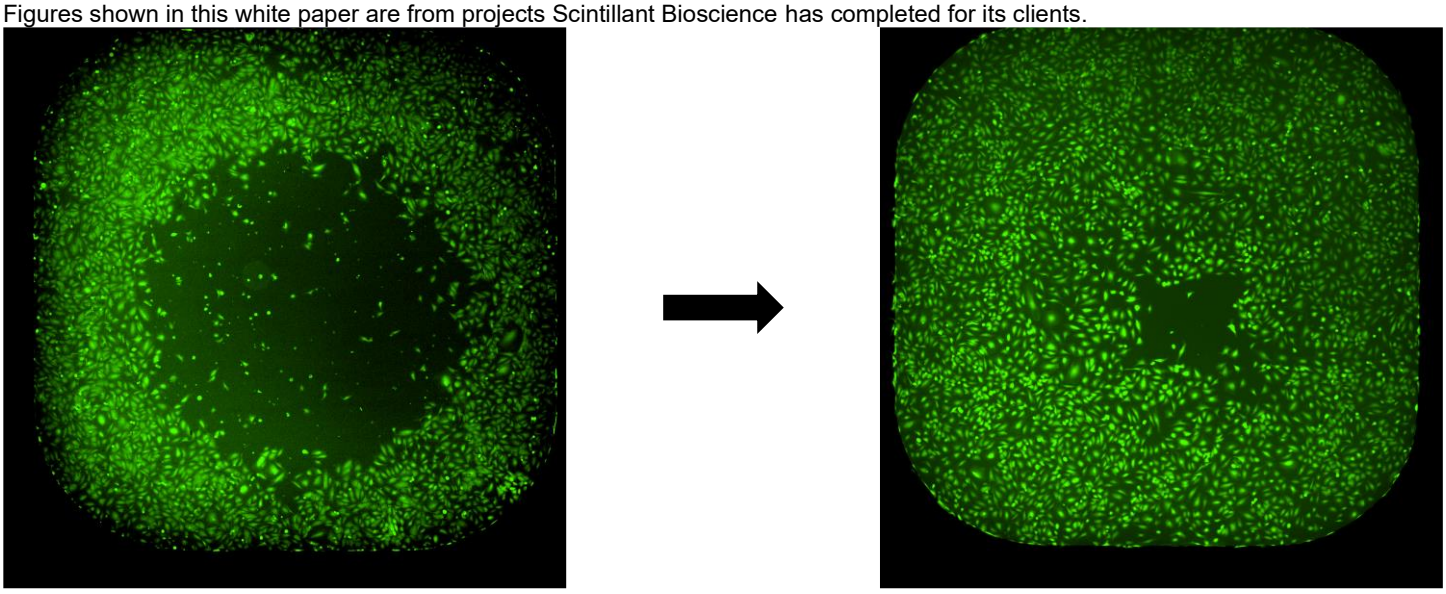
Time-lapse imaging has emerged as an indispensable tool for drug discovery, revolutionizing the way researchers study and understand dynamic cellular processes. Unlike traditional endpoint assays, time-lapse imaging empowers researchers with the ability to capture continuous data streams, allowing the real-time observation of cellular and subcellular changes in response to genetic, pharmacological, or cellular perturbations. Similar to endpoint assays, time-lapse imaging offers the advantage of dose-response testing achieved through automated intermittent imaging of multiple wells across microwell plates, while additionally enabling time-response analysis. This distinctive feature opens up new avenues for high-content analysis, enabling the identification and examination of rare cellular events that may have previously eluded detection. The invaluable insights gleaned from these assays are instrumental in identifying promising drug candidates, refining lead compounds, and unraveling drug mechanisms, underscoring the importance of integrating time-lapse imaging into drug discovery workflows.

In this white paper, we present an overview of the applications of time-lapse imaging in drug discovery and practical tips and tricks for developing, optimizing and implementing time-lapse imaging drug-discovery assays.

Applications of Time-Lapse Imaging for Drug Discovery

Time-lapse imaging has numerous current and potential applications in drug discovery. For example, it plays a pivotal role in studying cell migration during chemotaxis and wound healing assays, enabling the tracking and quantification of cellular movement in response to chemical gradients or wound closure. This information is crucial for assessing the effects of drug candidates on cell motility and tissue repair, with implications for wound healing therapies and cancer metastasis interventions.

Figures shown in this white paper are from projects Scintillant Bioscience has completed for its clients.



Time = 0 hours

Time = 24 hours

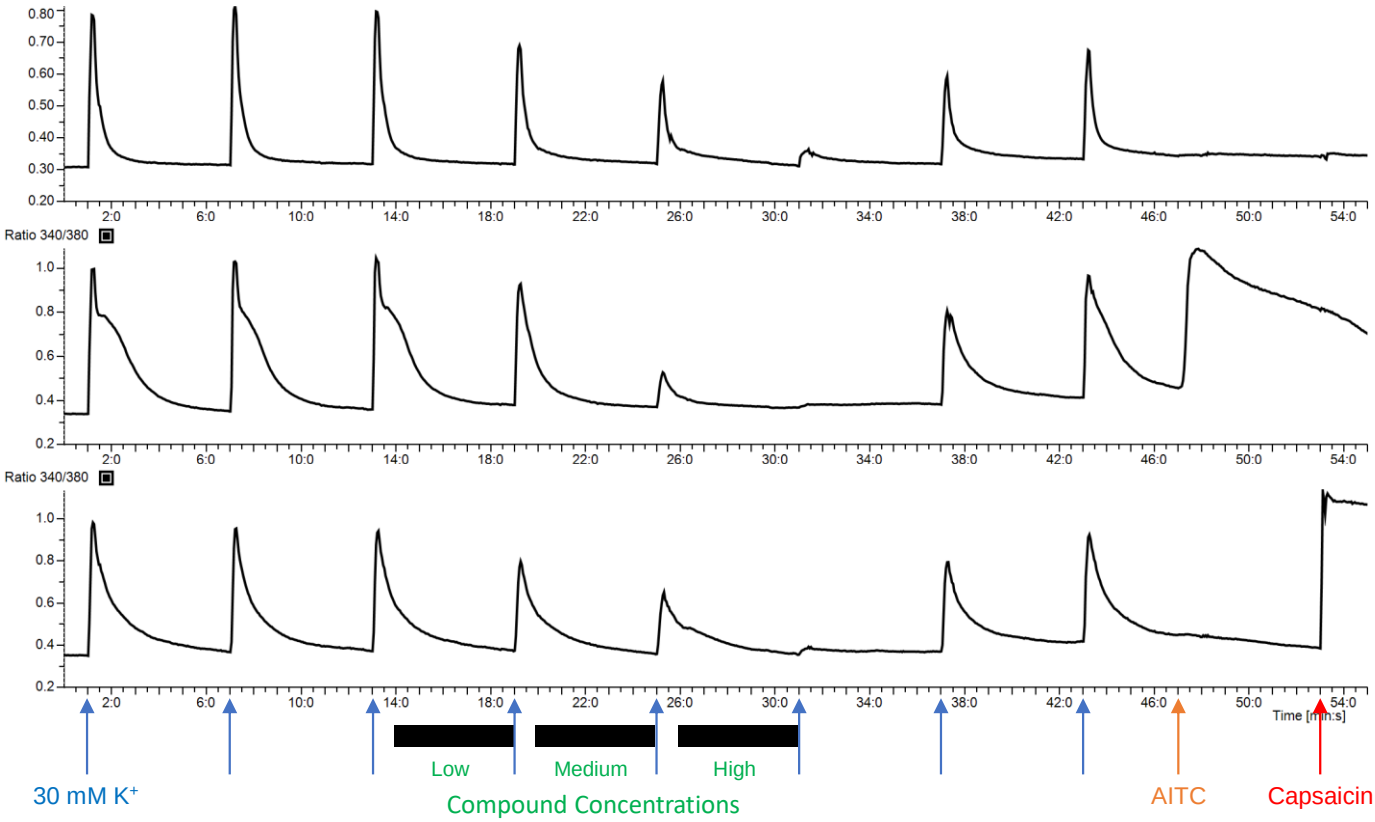
Example of a time-lapse imaging assay used to evaluate cell migration. In this case, the cells were plated in 384-well format, using the Oris™ Pro 384 Cell Migration Assay kit from Platypus Technologies. Each well of the 384-well plate contains a biocompatible gel in the center that dissolves shortly after seeding cells, creating a detection zone in which cells migrate over time. Cell migration can be monitored by time-lapse imaging or at an assay endpoint. Cell migration may be monitored by brightfield or phase contrast imaging, or cells may be loaded with a non-toxic vital fluorescent dye for monitoring cell migration through fluorescence microscopy.

Additionally, time-lapse imaging offers a unique perspective on stem cell and embryo development, providing real-time insights into differentiation and morphological changes. Understanding stem cell behavior is vital for regenerative medicine and tissue engineering, while observing embryo development helps identify potential teratogenic effects of drugs. Time-lapse imaging facilitates the identification of compounds that can positively

influence stem cell behavior or mitigate developmental abnormalities, advancing regenerative medicine and safe drug development.

Moreover, time-lapse imaging serves as an essential tool for monitoring cell proliferation over time, tracking division rates, assessing viability, and identifying cytotoxic effects of drug compounds. It is also employed in examining angiogenesis through tube formation assays, offering valuable insights into blood vessel development and potential therapeutic interventions for angiogenesis-related diseases like cancer and retinopathies.

Calcium imaging is a widely used technique to assess the impact of drugs on ion channels expressed in specific neuronal cell types and other excitable cells. It can also be applied to non-excitable cells expressing calcium-permeable ion channels and/or G-protein coupled receptors (GPCRs) that modulate cytosolic calcium levels. The figure below illustrates the extensive information obtained from these assays, where multiple pharmacological challenges can be applied during a one-hour experiment. This approach allows researchers to analyze various drug potencies in hundreds of individual neurons, encompassing diverse cell types, all in parallel.



Example traces from a calcium-imaging assay using primary mouse dorsal root ganglion (DRG) neurons. Neurons were cultured overnight and loaded with Fura-2-AM for calcium imaging. Each trace represents the response of a single neuron over time (X-axis in minutes, Y-axis indicates increases in cytosolic calcium concentration). Monitoring changes in Fura-2 fluorescence allows simultaneous observation of hundreds of individual neurons, with each region of interest defined as the cross-sectional area of a cell, reflecting changes in cytosolic calcium concentration. In the assay, neurons were depolarized with 30 mM extracellular potassium (K^+) at 6-minute intervals (indicated by blue arrows), eliciting responses showing rapidly rising increases in cytosolic calcium, followed by a slower return to baseline. After the first three control applications of K^+, three different concentrations of a compound were then applied between the 30 mM K^+ applications, leading to dose-dependent inhibition of the responses to 30 mM K^+. After compound washout, responses gradually recovered over time. Neuronal subclasses were distinguished based on responses to Allyl Isothiocyanate (AITC) and capsaicin.

While the current and potential applications of time-lapse imaging in drug discovery are vast and diverse, the few examples provided above illustrate its significance in advancing our understanding of cellular behaviors and drug effects, ultimately contributing to the development of safer and more effective therapeutic interventions. Time-lapse imaging assays are not only used to predict the *in vivo* efficacy of small-molecule drugs, but are also used to predict the efficacy of gene and cell therapies, among other therapeutics.

Practical Tips and Tricks for Developing Time-Lapse Imaging Assays for Drug Discovery

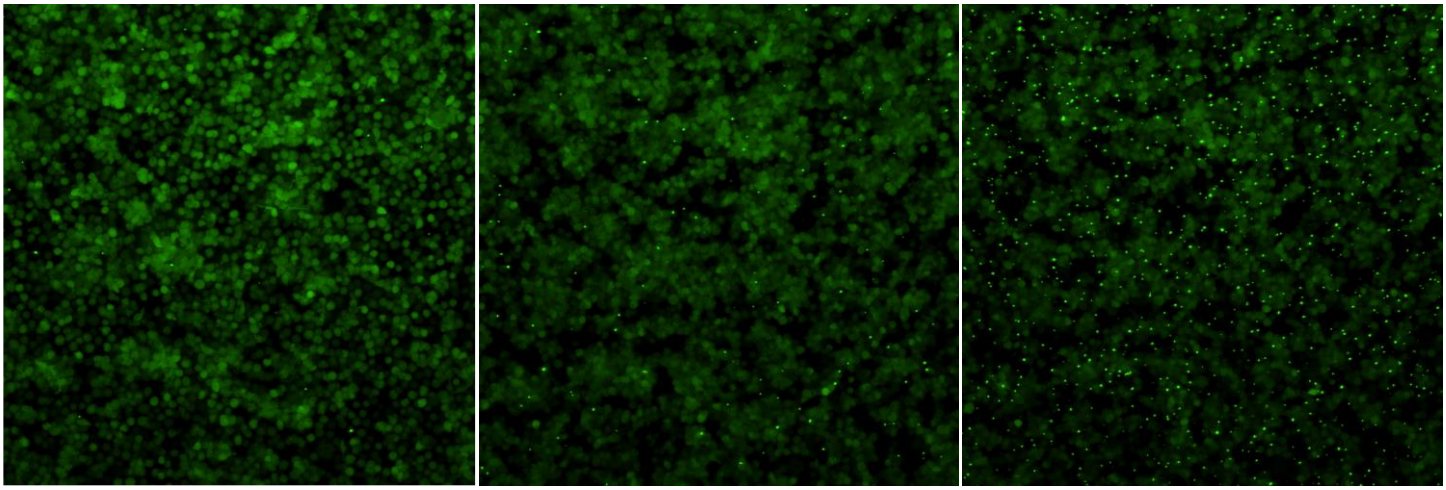
Choosing the Right Cell Model

One critical factor in selecting a cell model for time-lapse imaging is its biological and disease relevance, which is also true of endpoint assays. Immortalized cell lines and cancer cells are widely available and cost effective. However, in some cases they do not represent the relevant physiological complexity to model a disease *in vitro*. Primary cells, on the other hand, better mimic *in vivo* conditions but they are directly isolated from tissues which impacts their cost, availability, and scalability. Human induced pluripotent stem cells (iPSCs) or embryonic stem cells can be programmed to differentiate into specific cell lineages, providing greater relevance in disease modeling and drug screening. They can also be derived from patients with specific diseases, often expressing gene mutations associated with a disease of interest, which makes them highly relevant, but obtaining iPSCs and differentiating them into cell types of interest is very time consuming and expensive.

Beyond traditional 2D cell culture, 3D organoids offer a higher level of complexity and tissue architecture, which in many cases has the potential to model a disease more accurately than 2D cell culture. As an example for time-lapse imaging, 3D cell-culture models can be used to study angiogenesis in a more physiologically relevant environment, employing spheroids or organoids containing endothelial cells and other relevant cell types to observe the formation of complex vascular structures.

Selecting the Best Fluorescent Probes

Time-lapse imaging assays often necessitate fluorescent labeling of subcellular structures to monitor specific cellular events, though some assays can be performed label-free using brightfield or phase contrast imaging. The selection of exogenous fluorescent labels is limited to cell-permeant probes that exhibit minimal toxicity and photobleaching, which limits the scope of potential live-cell time-lapse imaging assays. Genetically encoded fluorescent reporters play a crucial role in addressing these limitations. One common approach involves creating a cell line that stably expresses a fluorescent protein fused to an endogenous protein to enable the tracking of protein abundance and movement. As shown in the figure below, the THP-1 human monocyte cell line has been genetically engineered to express green fluorescent protein (GFP) fused to ASC, a component of the NLRP3 inflammasome. This inflammasome is a protein complex that assembles and initiates a signaling cascade leading to the secretion of the inflammatory cytokine, IL-1beta. By utilizing such genetically encoded fluorescent reporters, we gain valuable insights into the dynamics of cellular processes from time-lapse imaging.



Time = 0 minutes | Time = 20 minutes | Time = 60 minutes

THP1-ASC-GFP cells (from InvivoGen) are shown at three time points in a time-lapse imaging experiment. Prior to Time = 0, the cells were primed to induce expression of NLRP3 inflammasome components by treating cells with the bacterial endotoxin, lipopolysaccharide (LPS). Priming is evident from ASC-GFP expression throughout the cytosol. Cells were then treated with the bacterial ionophore, nigericin, which triggers assembly of the NLRP3 inflammasome and subsequent activation. The consolidation of ASC-GFP into a single fluorescent speck per cell indicates that the NLRP3 inflammasome has assembled and initiated a signaling cascade to release IL-1beta.

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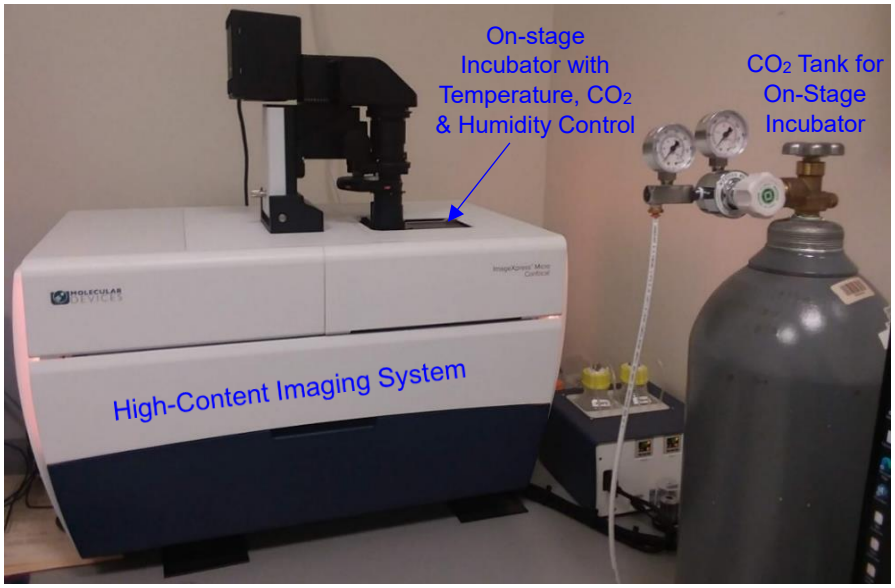
Implementing Drug Perturbations

Drug penetration and metabolism can differ among cell models, potentially affecting the interpretation of drug screening data. 3D organoids better recapitulate the diffusion gradients found in tissues and may provide more accurate drug response predictions. Primary cells and iPSC-derived cell types can express drug-metabolizing enzymes that are more similar to the target tissue compared to cell lines, impacting drug metabolism and response.

An advantage of time-lapse imaging assays is that the effects of drugs may be monitored continuously to evaluate toxic and/or therapeutic effects, rather than guessing at an appropriate drug incubation time and assay endpoint. For time-lapse imaging assays that require capturing cell images before and after drug application, it may be desirable or necessary to implement an automated drug-delivery system. Many high-content imaging systems integrate with liquid-handling automation, although these systems are usually limited to one liquid addition per well. The software that controls automated liquid addition must be integrated with the software that controls the microscope stage movements and the camera for precise and synchronized drug perturbation across multiple wells.

Optimizing Cell Culture Conditions for Prolonged Time-Lapse Imaging

For prolonged time-lapse imaging assays, it is important to replicate physiological conditions as much as possible, which includes maintaining nutrients, optimal pH, temperature, humidity and gases required for cell health in culture. For time-lapse imaging assays that impose high metabolic demands on cells, such as the calcium-imaging experiment shown on page 2, cells need nutrients they can quickly metabolize, including glucose. Some time-lapse imaging assays are conducted in a physiological salt solution supplemented with glucose and other nutrients. Other time-lapse imaging is done in normal growth media. Most cell-culture media contains a bicarbonate buffer requiring a 5% CO_2 environment to bring the media to neutral pH. Many high-content imaging systems have microscope stage-top incubators to replicate the conditions of a cell-culture incubator, including 5% CO_2, temperature at 37 °C, and high humidity, as shown in the figure to the right.



Minimizing Photobleaching of Fluorophores and Phototoxicity of Cells

Achieving high-quality time-lapse imaging while minimizing photobleaching of fluorescent probes is crucial. Careful selection of appropriate fluorescent probes or genetically encoded reporters that can enter live cells and maintain fluorescence after repeated exposure to high-intensity light is essential. To avoid photobleaching, it is beneficial to reduce light intensity to the minimum level necessary for detecting a sufficient signal above background. Increasing the time interval between image captures also helps to minimize photobleaching. However, there is a trade-off between capturing more frames for higher temporal resolution of subcellular events (which may lead to more photobleaching) and capturing fewer frames to reduce photobleaching.

Phototoxicity, or photodamage, to cells is another consideration in time-lapse imaging. High-intensity light, especially in the ultraviolet (UV) range, can be damaging to cells. To mitigate phototoxicity, it is advisable to use

low light intensity, even if this requires longer exposures, instead of relatively shorter exposure times with high light intensity. For experiments involving UV light, such as Fura-2 calcium imaging, the use of a neutral density filter in the light path can reduce light intensity. Although this requires longer exposure times to achieve the necessary signal from the fluorophore above background, cells tend to tolerate this approach better than shorter exposures with higher-intensity UV light. In laser-based systems, it is crucial to reduce laser intensity to the lowest setting possible for time-lapse imaging and increase time intervals between exposures to minimize phototoxic effects on cells.

Phototoxicity during time-lapse imaging may be reduced by adding anti-oxidants to the cell culture media and/or other extracellular solutions used during time-lapse imaging. Some anti-oxidants that have been used include the following: Vitamin C (ascorbic acid), a water-soluble antioxidant that can neutralize free radicals; Vitamin E (α-tocopherol), a lipid-soluble antioxidant that protects cell membranes from lipid peroxidation by reactive oxygen species (ROS); Trolox, a water-soluble analog of vitamin E with potent antioxidant properties; N-acetylcysteine, a precursor of the antioxidant glutathione, which can directly neutralize ROS; Glutathione, a major cellular antioxidant that plays a key role in protecting cells from oxidative stress; Superoxide dismutase, an enzyme that catalyzes the dismutation of superoxide radicals into oxygen and hydrogen peroxide. Use of such antioxidants requires optimization of concentration and duration of application for different cell types and applications.

Optimizing Microscope Setup and Other Integrated Instrumentation

When conducting time-lapse imaging across multiple wells in parallel in a microwell plate format, it is crucial to use a microscope equipped with a precision motorized stage capable of repeatedly returning to precise X, Y, and Z coordinates in each well throughout the experiment. The microscope should also support well-by-well automated focusing and in some cases drift correction may also be necessary to ensure accurate imaging throughout the entire duration of the experiment. To achieve high-quality results, it is essential to employ a suitable microscope with advanced imaging capabilities, such as high numerical aperture (NA) objectives and high-quality, high-sensitivity CCD or sCMOS cameras. Additionally, the use of microwell plates optimized for cell growth and imaging quality is important.

As mentioned earlier, for prolonged time-lapse imaging, it is advisable to conduct the experiments within an on-stage incubator to replicate the physiological cell culture environment, providing optimal conditions for cell viability and growth. Depending on the experimental requirements, automated liquid additions may be necessary or desirable. In such cases, it is crucial to ensure that the automation system seamlessly interfaces with the microscope's software, which controls various aspects of the instrumentation, including the motorized stage and the camera. This integration allows for efficient and precise handling of liquid additions, contributing to the success and reliability of time-lapse imaging experiments.

Acquiring and Analyzing Images

Determining an appropriate imaging frequency is critical to capture relevant cellular events without overwhelming data storage or causing excessive photobleaching or phototoxicity, as discussed earlier. Before initiating time-lapse imaging experiments, it is essential to ensure that sufficient data storage capacity is available for image acquisition and data backups. It's worth noting that writing data to a storage device during time-lapse image acquisition can become a bottleneck, potentially leading to delays in the intended intervals between image capture and, in some cases, causing software crashes.

Accurate image-segmentation algorithms are indispensable for the quantification of cellular features and phenotypes, a fundamental aspect of all microscopy image analysis. In the context of time-lapse imaging, object tracking may also be necessary for precise analysis and interpretation of the time-lapse video. Additionally, it is crucial to thoroughly inspect acquired data for artifacts or irregularities that may impact accurate image analysis. These artifacts can include the presence of large fluorescent particles within the field of view or sudden changes in background fluorescence, among other potential issues. Regularly checking for such artifacts ensures data integrity and the reliability of subsequent analyses.

Conclusion

In conclusion, time-lapse imaging has transformed drug discovery and development by enabling the real-time observation of dynamic cellular processes. Its continuous data streams provide valuable insights into cellular behaviors, aiding in drug candidate identification, lead compound optimization, and drug mechanism elucidation. The versatility of time-lapse imaging extends to various applications, such as cell migration, stem cell differentiation, embryo development, angiogenesis studies, calcium imaging, and more, all enhancing our understanding drug effects in living cells and our ability to predict drug effects in human patients.

To develop successful time-lapse imaging assays, several factors need careful consideration. These include selecting appropriate cell models, fluorescent probes, and optimal imaging conditions. It is crucial to prevent photobleaching and phototoxicity, which can be achieved by making well-informed choices regarding fluorescent probes, light intensities, exposure times, and image-acquisition frequencies.

For prolonged time-lapse imaging, it is essential to maintain physiological conditions using on-stage incubators. Moreover, when conducting time-lapse imaging experiments that involve multiplexing different wells simultaneously, employing microscope setups with precision motorized stages and advanced imaging capabilities becomes crucial for accurate imaging. It is equally important to consider all instrumentation requirements, such as microscope objective lenses, cameras, and data storage capacity, as they play a vital role in achieving successful time-lapse imaging. Another aspect that should not be overlooked is planning ahead for robust image analysis and proper quality control of images. By doing so, you can maximize the value of your projects and ensure the reliability and accuracy of the data obtained.

In this white paper, we have presented an overview of the applications and practical tips for time-lapse imaging in drug discovery. By integrating these insights into drug discovery workflows, researchers can leverage the power of time-lapse imaging to accelerate drug development, predict therapeutic efficacy, and advance precision medicine approaches. With continuous advancements in imaging technologies and analysis tools, the potential of time-lapse imaging in drug discovery is boundless, promising to reshape the landscape of pharmaceutical research and patient care.

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