3D cellular imaging advances and considerations for high-content screening

Commercially available high-content imaging (HCI) systems, introduced in the 1990s, have provided the scientific community with a platform that offers a unique set of tools ideal for advancing high-throughput biological discoveries and therapeutic development. HCI has made it possible to interrogate specific biological responses on heterotypic populations of cells, sub-populations of cells within a large population, and even within individual single cells. Today, this technology is becoming widespread across many scientific disciplines and has been used in nearly all capacities of biological and biochemical research studies.

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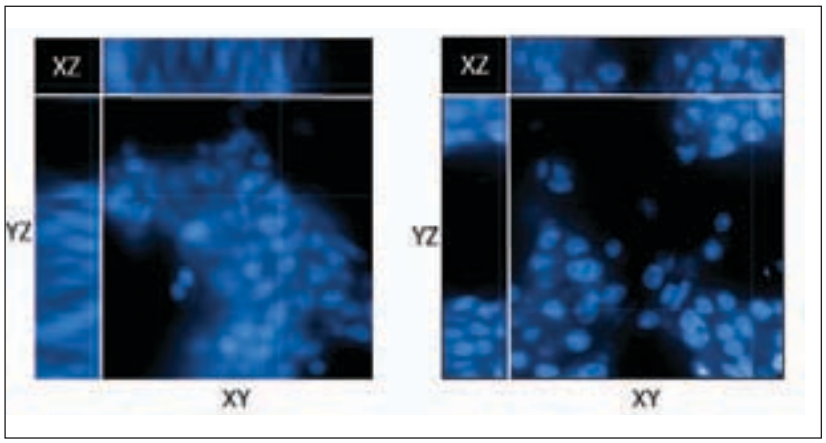
The plethora of experimental assays and cell models designed and used to produce HCI data from these sophisticated imaging systems has now grown from simple two-dimensional (2D) monolayer cell culture models to more complex three-dimensional (3D) organotypic cell models or organoids and whole organisms (examples include, Zebrafish and *C. elegans*) to determine biological outcomes including measuring the phenotypic characteristics in context of the tissue. The superiority of complex 3D cellular models compared to 2D cell models at recapitulating *in vivo* biology is a major driving force innovating the field of HCI in the context of disease pathology, therapeutic development and developmental biology.

As the scientific community continues to migrate towards more relevant 3D cellular models to study the outcome of drug or compound treatment, researchers in the field of high-content imaging are faced with a number of new challenges. The first of these challenges begins with high-content screening (HCS) technology that was originally developed for 2D cellular experiments, which is now being adopted for 3D cell models. Other challenges faced are broad and include, but are not limited to, the type of cell model, the media and growth components and constituents used to grow, support and maintain cellular function, the type of vessel or multiwell plate (microplate), automation and liquid handling approaches, the methods used to label cells with bioprobe markers and, perhaps the most challenging, the type of image acquisition and image analysis tools used to interrogate 3D cellular models. Each of these challenges is crucial to optimise and



3D image XYZ view display of HepG2 liver tumour cells labelled with Hoechst nuclear dye captured using the PerkinElmer Operetta CLS. The XY resolution of the nucleus is clearly observed in both panels, however, the Z position (3D): (A, left) distorted and blurred nucleus is observed in YZ and XZ 3D viewing planes when using a 40x/0.6NA air objective lens; (B, right) clear with sharp details of nucleus observed in YZ and XZ 3D viewing planes with 40x/1.1 NA water immersion objective lens

validate a 3D cellular study. In the subsequent sections we will discuss the challenges HCS practitioners face and potential solutions to overcome these obstacles.

3D organotypic culture models

The ability to measure the outcome of a drug or therapeutic compound in an *in vitro* model system that recapitulates the disease state or organ function from an *in vivo* environment is the 'holy-grail' for scientists to study cellular responses *ex vivo*. In attempts to accomplish this over the past few years, innovative approaches, newer techniques and processes have created methodologies to generate organotypic culture models (OCM) *ex vivo* that are viable in culture for extended periods of time and express both phenotypic and genotypic characteristics found *in vivo*.

OCMs, often referred to as spheroids, have the keen ability to self-assemble in the presence of other cell types (eg fibroblasts) and microenvironment components such as extracellular matrix (ECM)[2-4]. The challenges with many OCM models used with HCI is their placement and location within the well of the microplate. Typically, OCMs are loosely attached to the surface of the microplate or in suspension, and therefore have a tendency to move around in the well before or during image acquisition and analysis. When cultured imbedded within an ECM, OCMs typically form in non-uniform size and shapes from well to well in most microplate formats, creating additional challenges when acquiring and analysing images.

Regardless of the 3D cell model system used, it will be important during assay development and validation to show reproducibility performance of the experiment using data analysis and informatics tools to generate statistical outcomes that demonstrate repeatability and robustness[5].

Microplates

Microplates play a pivotal role in the process of capturing images in three-dimensions. Foremost, creating light penetration deep into the 3D tissue cell models is essential and is directly dependent on the type of physical media used to support the cellular structure, ie, the vessel or microplate. It is absolutely imperative that the materials used to construct these microplates are optically clear and present no interference or generation of unwanted light in the visible spectrum (400-800nm), typical of most bioprobe markers, as this could have a detrimental outcome during 3D image acquisition and/or analysis. The layout or design of these microplates is equally as important in creating an environment that supports and maintains 3D cell formation, and provides placement of tissue or cells in a format that permits ease of imaging, enhances survival and allows detection in all three geometric dimensions, x, y and z. For standardisation and ease of use within an automated workflow, including liquid handlers and imagers, microplates should be conducive to the ANSI/SLAS flat bottom plate standards. Even though a number of standard microplates have been used for many years to support the growth of 3D cell models, typically ex-plant tissues or other co-culture stratifying layers, the initial design was not specifically intended to support 3D spheroid formation. Today, researchers interested in higher throughput technologies have taken advantage of commercial solutions such as those that form a 3D cell suspension model with cells suspended in liquid form to create 3D structures. Another simpler option is the use of ultra-low attachment (ULA) conical round bottom (U-shaped) microplates in 96 and 384-well configurations that typically contain growth-promoting, enhanced substrates such as Hydrogel, Matrigel®, or other similar organic basement membrane matrix materials including agarose, to facilitate the formation of 3D spheroids. Additionally, there are a number of other microplate solutions that are commercially available to facilitate the formation of self-assembled 3D cell models including the use of magnetic beads to rapidly form spheroids, nano-based films and micro-spaced surfaces that promote self-assembly of tumorgenic cell lines into spheroids.

Another confounding challenge in design for 3D spheroid formation and other 3D cell models in general is the need for fabricating microplate bottom thickness equivalent to a #2 microscope coverslip (0.19 to 0.23mm) so they are amenable for higher numerical aperture microscope objective lenses needed to promote deeper light penetration,

thus providing higher pixel resolution for detection of subcellular feature measurements. While this is possible in flat 2D thin-bottom microplates, the ULA microplates are typically more challenging because of the geometry of the U-bottom shape.

Automation and liquid handling

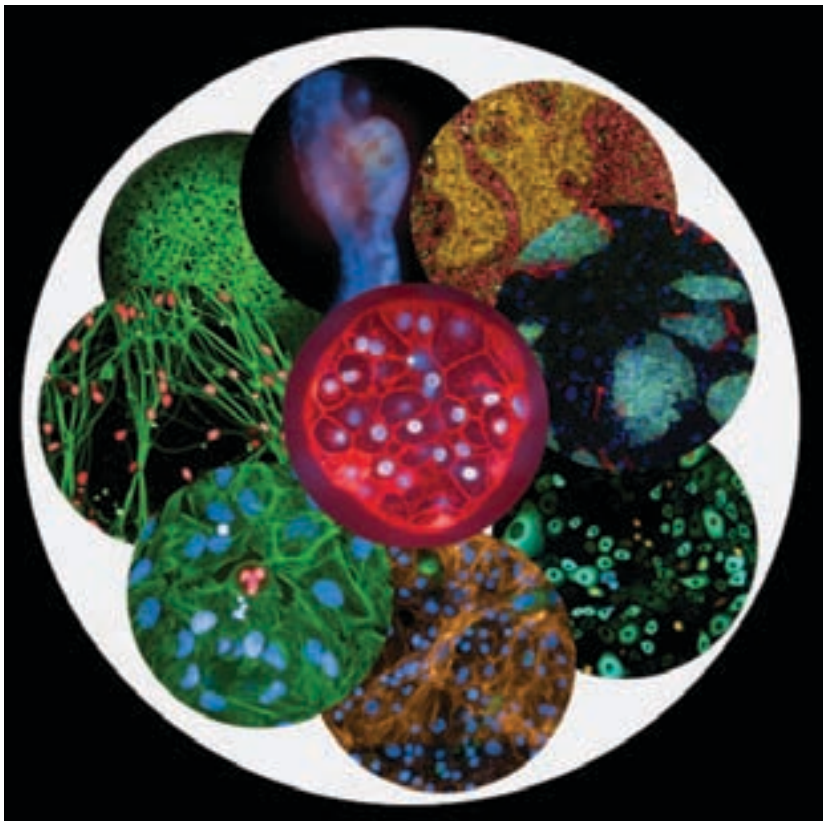
The complexity of today's 3D cellular models requires careful consideration on the approaches to develop methodologies that demonstrate a robust response and that are reproducible. Many of the 3D cell models being used today are created in systems where the cell interface or scaffold structures are easily disrupted during manipulations during medium culture changes, compound treatments, or labelling live cells with bioprobe markers. Each time a microplate is manipulated by movement or introduction of a pipette tip into the well, the chances of dislodging loosely attached spheroid clusters or OCMs increases. A solution most HCS laboratories have instituted is the use of automated liquid handling devices that can minimise this impact by reducing the interaction between the cell and microplate well interface. Furthermore, using these devices provides a means to improve the reproducibility by controlling the physical height of the pipette tips when they are inserted into the wells and the speed of dispensing or aspiration of liquid transfers. Without the use of automated liquid handling devices the likelihood of increased variability in the assay will occur and in some cases complete loss of OCM spheroid clusters will be observed during image acquisition. The automation and liquid handling process solutions need to be designed primarily for ease of use for almost any 3D microplate format. There are, however, some microplate formats that require additional steps in the workflow process to transfer or move cells or tissue from one format to another format that is suitable for HCS imaging.

3D imaging

The acquisition of 3D cell models can be challenging due to the loss of excitable light that can be partially reflected or absorbed in the specimen. Even with the use of highly penetrable two-photon point scanning confocal microscopy systems, which are considered one of the best light engines to move light beyond 200 microns *in situ*, there is still a limitation due to the theory described in the Beer-Lambert law, which describes the attenuation of light as it is passing through the specimen's material[7]. Another concern is the process of how light is focused; in general terms, just shining light on a specimen can generate unnecessary out-of-focus light that is typically scattered and convoluted creating poor quality images. To overcome these obstacles, advanced high-throughput HCS systems today are equipped with continuous laser light engines and in some cases high-powered LED lights that are directed into a spinning-disk confocal systems that streamlines targeted light through a defined pinhole while removing most of the out-of-focus light to achieve high signal to noise ratios. While there are many variables involved in light penetration, in the best case scenario, a light depth penetration limitation of about 200 microns can be achieved pending the type of tissue, microplate, labelling of bioprobe markers inside the tissue and the hardware components equipped on a high-content imager. The evolution of optics from excitation laser light, microscope objective lenses and improved photon detection recorders such as cameras have all played a fundamental role in the advances on high-content imaging devices to penetrate and detect inside 3D cellular tissue. To maximise light throughput, high numerical aperture (NA, amount of light entered through the lens), objective lenses with enhancing liquid materials such as glycerin, oils and water between the objective lens and the specimen's microplate are introduced to reduce the refractive index and increase light transmission for greater homogeneous detection from the specimen. The image resolution of a z-sectioned acquired image in a 3D cell or tissue is directly dependent on the objective lens magnification power and associated NA. Since most microplates are made of polymer composites, the use of water immersion objective lenses provides the best choice for high NA immersion objective lenses. These objective lenses, with magnification power of 20x and higher, typically have a NA of 1.0 or greater to capture individual images at z-position intervals as low as 0.5 microns providing resolution that is superior to any air objective lens with equivalent magnification power. Moreover, because of the higher light penetration in water immersion objective lenses, damage to the cell or bioprobe are reduced with lower energy, shorter exposure times, and faster imaging is achieved.

The generation of a 3D image begins by collecting a series of images, each captured at varying pre-defined z planes at fixed xy geometrical positions. The entire xyz plane series of images can then be reconstructed to represent an image volume or, in some cases, displayed as a collection or series of images through the cell or tissue specimen.

Due to the inherent mobility of 3D culture, HCS practitioners are faced with the challenge of efficiently finding OCMs or spheroids of interest. For



this reason, a subsequent challenge is the need to capture a large scan area within the microplate well using an objective lens with enough magnification and resolution to measure the biological response of interest. This requires capturing many frame fields per well and collecting images that do not necessarily contain cells of interest because the microtissue or spheroids are not always centred. A straightforward solution to overcome this imaging challenging is using the approach of pre-scanning the well or slide with a low magnification objective lens in 2D to locate and identify the global xy coordinate positions of all microtissue or spheroids, and then employ a rescan method only to the areas that contain spheroids, using a higher magnification objective lens in 3D for quantitative analysis.

3D image analysis

Perhaps the most daunting task for HCS practitioners in the 3D cell model workflow is focused on the methodologies to quantitate 3D images. Historically, HCS automated high throughput image analysis software solutions were designed to process 2D images. The introduction of 3D cell models using microplate technology for high throughput HCS can no longer depend on basic 2D image analysis algorithm solutions alone. The challenge for 3D image analysis starts with the processing power required to compute image stacks that can range from just a few images to 50 or more images within a stack, depending on tissue thickness from a single field frame per channel (biomarker probe). While a single field can be processed easily from almost all commercially available software and some open-source imaging software solutions, the sheer volume of high-content screening data from 3D imaging represents an enormous challenge because of the large number of fields per well captured in a plate as well as the number of each bioprobe label used in the experimental assay. As an example, a normal 2D imaging experiment from a four-channel (bioprobe marker) assay that captures four fields per well in a 384-well microplate generates 6,144 images per microplate; while with 3D imaging, by capturing just 21 image stacks per field, the number of images increases to a staggering 129,024 images per microplate. This volume is only amplified when using higher powered magnification objective lenses, as fewer objects are typically detected, therefore to insure a statistical significance measurement more fields are needed. For these reasons, most researchers today typically use a single representative image created from a 3D image stack called 'maximum projection', which is a process of collapsing a defined stack of images to generate a single image. Although this approach offers a solution that is easier for 2D image analysis software to calculate measurements, the method may not be ideal as it could misrepresent or misinterpret the biological response. To explain this point, if a protein target within the 3D cell boundaries crosses into several independent image z-stacks, the maximum projection image would result in an overlay of summed pixel information of a collapsed 2D image rather than maintaining the integrity of the protein in cellular context within the 3D cell volume. The end result is the loss of z-dimensional spatial information and potentially unique phenotypic image structures that may not be properly characterised or identified. Another approach some HCS practitioners are using is measuring each individual z-stack image, but again the loss of z-dimensional spatial information is not in context of the cellular model and this information can be lost. To keep the z-stack of images 'whole' and maintain context of the 3D cell model and the image pixel information within, a volume of images are generated by reconstructing each individual image plane in x, y and z dimensions. Clearly, the process of measuring 3D volume requires additional computational power and time

to reconstruct the entire or partial image stack to measure z-dimensional image volume pixels, called voxels. There is a balance that researchers must consider, do they need 3D volumetric image analysis or will a maximum projection image address the biological question? In target-based discovery projects these questions can be answered during the assay development and validation process. However, in a phenotypic screening campaign, where the target is not necessarily important to the outcome, a collapsed 3D image may not properly represent the cellular phenotypes that otherwise could manifest following drug or compound treatment when the entire cell volume is measured. The experimental design and validation of each approach will provide researchers with the tools to determine which approach is best.

There are commercially available 3D image reconstruction and image analysis software packages, initially designed for low throughput of single images, that may provide a solution for measuring volumetric voxel information, but are not necessarily a long-term solution for higher throughput HCS. As high-content imaging software evolves, it is likely these more advanced 3D imaging tools will become available to improve the overall ease of use to systemically measure 3D volumes from HCS campaigns.

Conclusions and future directions

The recent explosion of today's 3D cell models has rapidly grown in the past five to 10 years with a lot of interest and some speculation in the community with concerns that it may be accelerating faster than scientists can properly determine the validation of the 3D cellular models they are using or understand. It will be critical that scientists using these advanced technological tools, including confocal microscopy and software, to measure 3D images to assist in understanding the mechanisms of how these complex 3D cell models are behaving. As target-based screens are declining, the challenges of information rich phenotypic approaches has only exasperated the interpretation through the advent of *in vitro* heterogeneous co-cultures, *ex vivo* and *in vivo* tissues, and whole organisms, all of which can be presented in a 3D format. The upside of these complex cellular model systems is the promise to provide opportunities for interrogating the phenotypic characteristics while closely resembling the *in vivo* environment.

Early generation HCS imaging systems were specifically designed for simpler 2D high throughput image acquisition and analysis, as many of the imagers did not even have the ability to capture independent z-axis field frames in 3D space. To interrogate 3D cell models, advances in cell imaging instrumentation were designed with technology that could rapidly identify high resolution single cells throughout the 3D cell model sphere or scaffold. Advances of HCI technology required systems to be outfitted with enhanced optical light paths, high power light sources, linear encoded plate stages having submicron resolution and repeatability, improved microscope lenses with high numerical aperture ratings and sensitive cameras to capture photons of light emitted by bioprobe markers deep inside the tissue or 3D cell model. To achieve deeper tissue imaging in 3D models, a solution provided by some of the more advanced instruments on the market today are equipped with laser based confocal units with the necessary high-power in-focus light and high numerical aperture water-immersion objective lenses to promote light penetration deeper into the tissue. Also the recent release of newer high-content imaging software to prescan cells or tissue at low magnification then rescan at higher magnification in 3D confocal mode has provided advances and ease of use for many scientists to rapidly design experiments to generate meaningful data.

Further, the advances in microplate design and fabrication thereof are also required for these newer high-end microscope components on HCS imaging systems to increase the efficiency of image capture, including assisting with centring microtissue or spheroids in microplate wells. The potential of next generation 3D microplates with improved flat bottom architecture and a thin-bottom will enhance 3D imaging resolution, thus providing scientists with the capability to address more complex biological questions. In all cases, these 3D cell model microplates must be amendable for high throughput handling and processing. Any secondary steps required to move cells from their original environment only complicate the workflow and perhaps introduce unwanted variables into the experiment.

Significant barriers for implementing and validating complex 3D cell models for HCI remains and will require new approaches including optimised medium formulations to better understand the biological function or disease characteristics of 3D cell model systems. In addition, many of the tools and consumables such as microplates, chemical and biological probes and reagents to label deep into the tissue or spheroid, and of course the instruments and software required to efficiently provide solutions to rapidly acquire images in 3D space with enough resolution at the single-cell level

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for image analysis. Finally, informatics tools will be a key requirement to assist researchers in the understanding multivariate statistical measurements of the data to make it meaningful.

In conclusion, the power of 3D cellular imaging is here and the promise of newer imaging hardware and software tools to interrogate 3D cell models is an advancement for HCS practitioners in the scientific community. This has generated excitement and the demand to provide a technique that cannot be overlooked as it has the potential to discover new and relevant biology related to human organ function and disease and also help deliver safer, more effective medicines to the world. DDW

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