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Walk-Away Automation for Live Cell Assays: From Setup to Long-Term Kinetic Assays

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Paul Held: Hello, everyone. Thank you for joining us today for BioTek's webinar event. My name is Paul Held. I'm the applications lab manager here at BioTek Instruments.

There's a couple of administrative things we're going to talk about. First, the audio for today's webinar will be coming from your computer speakers. So, please be sure that they're turned on and the volume is off.

To ask a question, please use the chat feature. It's located at the bottom left hand corner of your screen. And you can send any questions at any time. And I will address those questions at the question and answer session at the conclusion of the event. If we do not get your questions, we'll try to do it via e-mail.

So, I'll start with the presentations today. Again, my name is Paul Held. I'm the lab manager here at BioTek. And what I want to talk about today is some Walk-Away Automation for Live Cell Assays. And, in regards to that, a new product of ours called the BioSpa.

So, I'm really going to take it from the point of setup all the way through some examples of kinetic assays.

Drug discovery over the last 20 or 30 years has really moved from biochemical assays, basically tube-based assays, where either purified drug target was assayed or there were some sort of synthesized peptide-based substrates. And basically, these were all in kind of manmade conventional buffer such as PBS or Tris.

In a drive towards creating more relevancy in terms of the biology, these types of tube-based assays have moved more toward cell-based assays, where now the cells have been modified to some extent and these cells express the drug target in high concentrations, or there's some sort of membrane permeabilization, typically these were end-point assays.

Most recently, new cell-based assays have been developed that basically look at indigenous drug targets. Flourite substrates in cell lines have – that were traditionally immortal cell lines, the move has been towards primary cells. And then, in response to all of these, these assays have all become really live cell-based assays, rather than tube-based assays.

So, the drive for this – the experiments has really been towards utilizing live cells that mimic biological responses much better than the tube-based assays.

As a consequent of that, live cell assays have a number of demands that the typical tube-based assays don't have. For the most part, they tend to take a longer duration because you're looking, you're working with live entities really require bacterial sterilities to ensure that the responses that you get are from the cells that you're working with and not some sort of contamination.

These cells also require a number of environmental controls that tube-based assays (would) certainly have.

Such as temperature control, you know, typical assay would take place at 37 degrees Celsius. The media in many live cell-based assays uses a bicarbonate buffer so that CO2 control, typically five percent or 10 percent is required. And if the assays are looking at hypoxic conditions, the control of oxygen vapor pressure is more less required.

In addition to that, in order to prevent drying, humidity usually has to be preserved. So, all these are kind of environmental things that are necessary for live cell assays.

In addition to that, many of these assays require different reagent concentrations, different cell numbers in different wells, or different drug concentrations. And they also utilize multiple time points for the addition of these drug reagents. And they often span, as I said, longer durations, typically 24 to 48 hours.

So in order to address these live cell assay needs, BioTek has developed the BioSpa Automated Incubator, and as you can see here, in this picture, depicting the BioSpa in the center.

One might ask, "Well, what is it?" The BioSpa 8, as I said, is an automated microplate incubator. Each drawer holds two microplates or other lab ware. So, if we have – you have lab ware adapters, the whole Petri dishes, we can hold Petri dishes for a total of eight microplates with or without lids.

So, how does it work? Basically, it's a robotic arm or the gripper, as you can see, that is depicted as number one. This arm moves side to side, left to right. And the gripper arm, gripper itself moves up and down. It handles microplates with or without lids. So the left would be some sort of liquid handling device from BioTek. These devices include the EL406 full plate washer dispenser, a number of four or five washers.

And, last but not least, the MultiFlo FX which is a reagent dispenser strip washer.

On the right hand side would be a detection device, a BioTek detection device. In this image, they're not shown but it could be an Epoch 2, which is the UV-Vis absorbance meter, Synergy H1 Multi-Mode Reader, Synergy Neo, or the imaging Readers Cytation 3, and 5.

So, as I said, in connect to liquid handler or a dispenser, in fact, can actually connect to both and actually fully automate processing for up to two weeks.

As I mentioned before, it provides temperature control of the 45 degrees C. It has built-in CO2 controllers with open and close valves to control both CO2 as well as oxygen levels. In both cases, the CO2 level is controlled – basically

is required is 100 percent carbon dioxide. There's no need for a mixed gas. Likewise on the oxygen control, all is required is a nitrogen tank with 100 percent nitrogen. And the valves will open and close to maintain the desired conservation of gases. And it also provides humidity, much like a tissue culture incubator, this water pan at the bottom of the incubator.

The top door which isn't open in these pictures easily comes off for cleaning and decontamination. There's a hyper filter inside to filter the circulated gases. This hyper filter is easily removed and can be replaced. And in the front bay, there's actually a leading and deleading station for the BioSpa to remove lids for use on the liquid handlers.

In regards to sterility, most people use some sort of biosafety cabinet to provide a sterile environment for live cell-based assays. In this picture, you can see the BioSpa, with our Cytation imager as well as a portion of the MultiFlo. And these all fit in the conventional six-foot biosafety cabinet. Although the picture we have here is actually a custom cabinet, it's actually deeper than a standard cabinet. Most are just for a little more room. But they will fit in a standard six-foot cabinet.

The software, BioSpa, it has its own software that works in conjunction with the LHC Software which is a BioTek's Liquid Handling Control Software to manage the liquid handling portion of any assay, as well as with Gen5 which is the software that controls reader function as well as data reduction.

So the BioSpa interfaces with both of these pieces of software to control timing and calls up programs from each of these to accomplish the necessary test in a program.

It also can act as a standalone unit. In that it can control environmental parameters of the BioSpa, independent of the reader or the liquid handler. So, this is – and essentially could allow the BioSpa to be used as a standard microplate incubator if no automation is required. So, as you can see in the graphic temperature, can be controlled as well as carbon dioxide levels or O2 levels.

In regards to programming session, it's a multi-step process. First one define what the environmental parameters would be in terms of temperature and gas control. And you do so at both the start and the finish. There's so many example where, if you have CO2 control during the assay but you didn't care to use any CO2 or didn't care that the temperature was alleviated at the finish of the assay, you could have the BioSpa turn off the control at the subsequent end of the assay run.

The next step is to define your steps in terms of timing, whether it's a wash dispense step, a (read) step or an incubation step in kind of a linear fashion.

From there, you would assign a location for your assay plates. The BioSpa software does this in two ways. One, you can auto assign that we're – will – the BioSpa will assign plates as it – so it deserts typically from top to bottom. But alternatively, you can assign them yourself. So, if you have some plates that you've already put in the BioSpa as part of just a standalone incubation and there's no need to move those plates, you can define those plates as being a starting location.

Next, you would generate a timeline where basically by (previewing) the timeline in the – the – in conjunction with the assay process you previously defined as well as a number of plates, the BioSpa program will generate a timeline. And then, subsequent to that, you would just start the session.

As the session begins, it generates a Gantt chart. And as you can see from here, really what we're looking at here is five different plates that are started in a staggered fashion versus an incubation, and then there's a series of repeated steps. And as you can see, that the software not only schedules things, but also writes information as to when the assay is going to be completed and approximate time on when each step is planned to be started and finished.

During a run, the BioSpa monitors gas levels as well as humidity, as well as provides a true Gantt chart as to what actually transpired of someone past the assay mid run, that would be recorded as well in the session.

And in addition to that, the BioSpa software can send out an e-mail or a text message indicating whether or not any parameter is out of range during the run as well as give you a message that the run was completed successfully at the end.

One of the other interesting features of the BioSpa software and the BioSpa is that with live cell assays, often times, many assays are a very long duration and really the plate is more or less in the incubator really not being processed to any extent. But it just best of being, you know, stable or, you know, static, and using a vast amounts of time.

So, in this example, we've got two assays, one is the 6-well microplate dish wash once a day. Likewise, there's another assay where there's four different – 96-well microplates and they're imaged every two hours. Because one assay is at a lot much longer duration with very little process steps, these two assays actually can be combined and run concurrently as long as there is enough incubator slots in the BioSpa.

So, in regards to applications and really what we're going to talk about today are some of the automated live cell assays. And I'll provide some examples next. And I mentioned that the multiple assays can be run concurrently.

But, a number of other different types of assays or processes can be performed such as ELISA automation. Typically, ELISAs are wash dispenser (read) steps, not necessarily with live cell assays but these types of assays can be performed with the BioSpa and the appropriate liquid handler as well as reader.

The BioSpa is really good at automating over nine or over the weekend processes. And I'll give you examples of that in a minute. And then we can also automate kinetic measurements where repeated (reads) or repeated image steps are maintained overtime.

So, to switch gears a little bit and talk about applications, I'll have three examples, the first which is really a – simply a proliferation assay. In this

example, it's a combination of the imager as well as the MultiFlo in conjunction with the BioSpa.

In this example, what we've done is we have used the MultiFlo FX which is a reagent dispenser that has a peri pump and we utilize this – in this example, the seeder cells. And then, after seeding the cells, these plates were incubated overnight in the BioSpa maintaining temperatures, humidity and CO2.

And the following day, these cells were fixed and stained using a 4 percent paraformaldehyde followed by DAPI and fluid stain, along with a wash – in these – the fixed and stained cells were then imaged using a Cytation imager. And, you know, the kind of the premise of this type of assay is that you can utilize these types of experiments to look at proliferative drugs or (inaudible) drugs. And basically do analysis on the images after the effect.

In this case, really what we did is we use the MultiFlo with four plates and we had a linear cell titration where a different number of cells were titrated into different wells. The subsequent wells were fixed and stained the next day and then imaged using the Cytation objective.

In terms of analysis, we have DAPI staining, so what we really did is we counted nuclei. And as you can see in the graph to the right, the linear titrations for all four plates is quite repeatable, even through these four plates were incubated in different locations in the BioSpa, the titrations were relatively close to one another and repeatable.

Cytotoxicity, that's an assay that's used very quite often in drug discovery, particularly in cancerous chemotherapies.

So, in this example, we use the (cAMP on) called oridonin. Oridonin is a diterpenoid (cAMP on) but it was originally isolated from Chinese medicinal herbs. And it's been shown to be a very potent agent for certain leukemias. It's been known to show in low doses into cell cycle arrest at higher doses induced apoptosis and it really inhibits cell migration in migration assays. And in some cases, has really known to have remarkable effects in tumor invasion studies.

So, we're going to use this (cAMP on) as an example (cAMP on) in regards to cytotoxicity assays with the BioSpa.

The crux of the assay is based on staining cells nuclei with either a DAPI or a Hoechst stain, and this will identify total cell numbers. And then, we're going to do a subpopulation analysis with CellTox Green, which is a – was a stain from Promega. It identifies nuclei with a green stain that only cytotoxic or injured cells will allow the stain into the cell. So only cytotoxic cells will stain green in – by expressing the number of green cells as compared to the total number of blue cells as a percentage will generate the percentage of cytotoxicity.

So, the gist of this assay is such that we're going to seed cells with the MultiFlo FX and incubate them overnight. The following day, we're going to add oridonin. We're going to do it one of two ways. The first one is what I call an image-driven process. And really, what we're going to do is because the MultiFlo has a peri pump with one by eight manifold of eight individual tubes, we can use eight different concentrations of oridonin and add them to different rows of microplate.

So, in this case, each row have a different concentration. And then we're going to use the Cytation to image cells that was stained with DAPI and GFP channels every hour and a half.

So the gist of it is, in terms of a plate map on the right, as you can see, the wells in row A will have no oridonin and the wells in row H will have the most. And then, subsequent to that, we're going to image it repeatedly.

This is a Gantt chart of a session, as you can see, we've got a multiple steps going on. Each one of the kind of blue dash is an image. In this experiment, we have three different plates. Each plate was seeded with 10,000 cells per well and we added oridonin at the beginning of the experiment along with Hoechst dye which stains nuclei as well as a CellTox Green.

Images every hour and half, and then we're going to look count as a number of nuclei as well as the number of cytotoxicity nuclei.

Again, this is an image-based process and you can see the – a picture of the BioSpa and the Cytation. So, the first analysis we've looked at was simply cell number. And as you can see, those wells with – or oridonin either 100 or 30 micromolar, the number of cells overtime decreases while those wells that have no drug actually has a slight increase.

So, we know that oridonin is cytotoxic, so this is not a surprising event. What we also looked at was the percentage of cytotoxic cells. And interestingly enough, you see a very big spike with both 30 or 100 micromolar drug concentrations around seven, eight hours post addition of drug.

And then, it falls off quite rapidly. And really, what we're looking at here is the fact that many of the cells actually lies and we actually lose our counts. So, this explains why the previous slide, the cell count went down, but it really shows that the oridonin is cytotoxic, relatively early events.

If one does a drug titration at a specific time point, this – really what we're looking at here is where the peak cytotoxicity events were occurring. As you can see, we've got a nice drug dose response. And we did it on three different plates during the run, as you can see, the three plates are quite repeatable amongst each other.

At the same time, because we were imaging, we actually look at them under bright field as well. And really what we did here is we wanted to look at apoptosis marker which – one of which is cell rounding. It's a very non-invasive test as you can see in the upper left of the untreated cells look markedly different than the three cells six hours later.

And by analyzing a population – by identifying the cells in bright field and then looking at those cells that are round or circulated greater than 0.6, you can see that both 100 micromolar and the 30 micromolar have a substantial

increase in the number of apoptotic cells percentage, whereas the untreated cells remain relatively constant over the 24-hour period.

We think the round cells in the untreated population really are those cells that are undergoing mitosis. One of the cell morphology changes with mitosis is rounding. In this data, it agrees quite well with some work that a colleague of mine, Brad Larson, did with MDA-MB-231 cells, where he looked at similar apoptosis markers using a phase contrast. And this is just some of the data that he generated. And the timing seemed to be very similar for both experiments.

Now, switching gears a little bit, so, the other question is, because the BioSpa works in conjunction with both a liquid handler as well as a reader, we can ask similar questions whereas instead of adding a reagent one time and an imaging multiple times, we generate large amounts of data, we can do similar experiments where instead of imaging things multiple times, we can add reagents at different times. So, in this case, we move the plate to the liquid handler periodically, add reagents at different times.

So again, we're using the same one by manifold. Different rows have different concentrations, but now, what we're going to do is add drug to different strips at different times. So, in the end result, we have a plate that has drug concentration changing in this direction as well as the time in this direction.

In this example, every well really is unique in – on anyone plate. And then really, in terms of statistics, what you really need to do is run multiple plates. Again, what we're going to do is we're going to move the plate and add reagents at different times rather than image it at different times.

The (sketcher) is slightly different, as you can see, the image steps all take place at the very end, where there's a number of different process – liquid handling process events. In terms of setting up the experiment, again, in (apoptotic) cells per well, the oridonin concentrations are the same. We're going to image it 4X objective and look at the DAPI and GFP channels again.

So, the analysis is exactly the same, the process is just – is liquid handled driving rather than image driving.

In this case again, we see different concentrations have different responses. Those with the highest concentration have the greatest response at the earliest time. Likewise, if you do concentration curves, those concentration curves that were taken at longest incubation times result in the greatest amount of cytotoxicity. But these are not unexpected results, very similar to what we found with the image-based analysis as compared to a process-driven analysis.

My last example is actually – is an energy metabolism phenotypic screening. And this is a (biolog) kit that's available for (inaudible). (Biolog) has a number of phenotype-driven assays for bacteria but they also have some for the million cells that we're looking at, that we've looked at.

So the premise of this kind of phenotypic screening is, there's a number of kind of drivers for. You can kind of monitor the stability of your cell lines. Make sure that the cell lines are behaving today as they did a year ago. If there are some sort of mutant phenotypes that you want or looking for, you can detect them.

Gene (faction), you can compare two different cell lines as to how they perform in regards to energy metabolism. You can certainly look at the effect of RNA gene knockdowns, if you're looking at a particular energy metabolism biochemistry and you're looking at specific genes that you want to knock down, you can assess the knockdown with these kinds of strains.

Probably most important is to compare some cell lines, in particularly cancerous versus non-cancerous. One of the kind of critical elements tumor cell lines is – was known as a Warburg effect, where the cancer cells lines tend to metabolize things anaerobically as compared to normal cells. In fact, they maintain a high glycolysis and do it under anaerobic conditions followed by lactate fermentation. So the energy usage of cancer cells tends to be markedly different than normal cells.

And last but not least, you can also use it to test different drug (cAMP ons) in terms of energy metabolisms on cells.

The assay itself is basically very similar to an MTT or an XTT cell proliferation dye in that it's tetrazolium salts that's converted to a formazan dye. And it does so by an NADH-driven production from the mitochondria, and there's some sort of electron shuttle compound that converts the electrons from the NADH to the cell surface which is then used to convert the dyes.

So, in this regards, drug compounds that are mitochondrial inhibitors have a marked effect on dye conversion. And this assay is actually an absorbance-based assay.

So, the assay is quite lengthy. There's the 40-hour incubation post-seeding. Again, we seeded our cells with the MultiFlo FX, incubated them in the BioSpa for two days. This should be an example of an assay where because of its long duration, we could program some sort of short-term assay to run in conjunction with this.

The 48-hour incubation does a number of things. One, is it allows all the glucose present in the normal media, in residual media from seeding the cells to be utilized. And it also provides time for the cells to adapt to whatever new carbon sources is present in the well of that plate. And then following the incubation, there's addition of the dye, and this is converted to a colored product which we then measure.

Again, it's kind of a – this is not so much image driven but rather absorbance-based driven assay.

And this is an example of what the results look like. The basic strain entails four plates. There are a total of 367 metabolic substrates. Some plates have positive controls in similar locations, but it has both carbon energies, both nitrogen substrates and it's a number of different (cap on) including carbohydrates, starches, alcohol, et cetera.

So, as you can see in this example, different substrates work much better than others in regards to conversion of the tetrazolium salt to a colored dye.

We might monitor it kinetically over a period of time. And this is just an example of a Gen5 output – kinetic output. So we had four plates and we measured the absorbance at 500 nanometers every 20 minutes. And really, what we're looking at here is a response to different substrates.

If we zoom it on one well, what we found is that if we use the (vimin) from the first few data points, and that was the first 60 minutes of duct immersion as compared to the whole 12 hours, we got much more repeatable results.

So, what we then did – then utilizes, so we look at analysis of the kinetic curves at the early time points and then generated a heat map. It's little hard to see but we're – really what you're looking at here is the kinetic rate of four different cell lines to a number of these substrates, I think this is roughly 300 of the substrates grouped accordingly to sugars or nucleosides or carbonic acrylic acids. And, the take-home result is that these different cell lines behave differently in regards to what substrates they will utilize or not utilize for growth.

To summarize, I mean, think I've shown that the BioSpa is a robotic microplate incubator, but hopefully, I have provided some information that proves that this – the BioSpa provides a means to automate live cell assays.

As I said earlier, it interfaces with both a BioTek liquid handler or as well as a BioTek reader, controls environmental conditions including gas and humidity, it works with lidded or unlidded plates. And it will also maintain a process log and provides e-mail and text messages at the end, or during the run if there's an error that occurs.

So, at this point, I'd like to address some of the questions submitted during the presentation.

Oh, OK. So, the question entailed were really was mentioned that humidity is important in live cell experiments. And how does the Cytation 5 control humidity. Unfortunately, the Cytation 5 reader itself does not control humidity. It does control CO2 and O2 gases.

In regards to the use of the BioSpa, the idea is that, the reader – the plate will only be in the reader for a short period of time relative to the lengthy – the long expanse of the assay. So, the idea being is, it goes to the reader or imager, it's imaged and then goes back to the BioSpa were – in a humid environment, so its exposure to a dry environment is minimized. And you can also image with the plate lid on to further inhibit evaporative losses.

So, I think that's the questions there are. I'd like to thank everyone for joining us today. We will e-mail a recorded event and transcript to all the participants. If you have further questions or you think of something later, we would certainly welcome you to visit for more information of BioTek products, our Web site, as you can see, the links here on this page.

In addition to that, we have a blog, you can like us on Facebook or follow us on Twitter to hear more about BioTek webinars or to learn more about our products and how they can help you.

Please join us for any upcoming events. And you can – like I said, you can view the complete schedule at biotek.com/events.

Again, thank you for joining us.