

The Many Roads to Cell Death: Gaining a Practical Understanding of Apoptosis, Necrosis, and Autophagy

Webinar

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Slide 1

Tianna Hicklin:

Hello and welcome to today's *Science*/AAAS Technology Webinar on The Many Roads to Cell Death, Gaining a Practical Understanding of Apoptosis, Necrosis, and Autophagy. I'm Tianna Hicklin, the Assistant Editor for *Science's* Custom Publishing Office and I'll be your moderator for today's event. In this webinar we'll be discussing the molecular mechanisms underlying cell death including the apoptotic cascade as well as the non-apoptotic pathways, necro-apoptosis, and autophagy.

Cell death is an essential part of life and new discoveries in the field have been enabled by cutting-edge technologies, particularly in the realm of cytometry and cell-death specific-markers. In this webinar, we'll hear some of the latest insights into the cell death pathways, including the molecular markers and cellular changes that characterize these pathways. We will also discuss cytometry-based strategies for dissecting cell death pathways, and how to use the data to better understand the pathophysiology of diseases and to uncover the new targets for drug discovery and development.

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We have with us today an expert panel who will be discussing the different cell death pathways, presenting data from some of their current research, as well as addressing questions from our live audience. It is my pleasure to introduce Dr. John Abrams from the University of Texas Southwestern Medical Center in Dallas, Texas and Dr. Bill Telford from the National Institutes of Health in Bethesda, Maryland. Thank you both for joining us. We're happy you could be here today.

Before we get started I'd like to share some important information for our online viewers. Please note that you can resize or hide any of the windows in your viewing console, and the icons at the bottom of the console allow you to control what you see. Click on these to read speaker

bios, to find additional information about technologies related to today's discussion, or to download a PDF version of the slides.

We'll begin today's webinar with the presentation from each of our speakers and end with a Q&A session, during which our panelist will address the questions that are submitted by our online audience. If you're joining us live now, you can submit your questions at any time by typing them into the box at the bottom left of your viewing console and clicking the Submit button. If you don't see this box, click the red Q&A icon at the bottom of the screen.

Please be aware that concise and broadly applicable questions are the most likely to be put to the panel, and whenever possible, please direct your questions to a specific panelist. You can also login to your Facebook, Twitter or LinkedIn accounts during the webinar to post updates or send tweets about the event. Just click the relevant icons at the bottom of the screen. For tweets you can add the hashtag #sciencewebinar. Finally, thank you to EMD Millipore for sponsoring today's webinar.

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It is now my pleasure to introduce today's first speaker, Dr. John Abrams. Dr. Abrams completed his Ph.D. at Stanford University in California after completing his undergraduate degree at Cornell University. He joined the University of Texas Southwestern Medical Center in 1994 as an assistant professor in the Department of Cell Biology before becoming an associate professor in 2000 and program chair of the genetics and development graduate program in 2004.

Dr. Abrams has been a professor of cell biology since 2006 and his research examines the in vivo molecular networks involved in cell death regulation and explores determinants of chromatic organization, using *Drosophila* as a model system. He is also an Ellison Foundation Scholar and a member of the Faculty of 1,000. Welcome and thank you for being here Dr. Abrams.

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Dr. John Abrams:

Thanks Tianna. Well, to get started I think we all remember probably from high school biology and early that cell death is sort of a natural process that occurs ubiquitously in biological systems. As a refresher it's very prominently sort of seen in developing systems. A classic example is when morphologic patterns are sculpted for example, and in immunity

it's used in a variety of ways to eliminate cells that are infected with either bacteria or viruses.

During viral infection for instance, we now know that there's actually a molecular 'arms race' that exist between the host which encodes genes to attempt to commit suicide when infected, and there are viral genes that are actually inhibitors of the natural suicide process to keep the host cell alive to perpetuate more viruses. Of course there's various situations, tissue damage situations caused by environmental stressors and other context that impose or promote programmed forms of cell death.

Now we also know that in various diseases, the pathways that elicit these forms of cell death can become deranged, and when that happens we see the manifestation of, for example, cancers when programmed cell death fails you get excessive cells, or when some of these pathways are deranged to generate excessive cell death we can see degenerative disorders and it turns out the excessive cell death pathways are activated to eliminate forms of T cells during AIDS.

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So with that introduction let's consider the various classification systems that have been typically used to discuss or build a vocabulary for these forms of cell death. Originally, long ago, there were three types of cell deaths that were described generally in ultrastructural studies, Type I, Type II, and Type III. These essentially were discriminating based on whether or not the neighboring cell was engulfing the dying cell or whether the cell seemed to be self-cannibalizing. That's now known as Type II or autophagic forms of cell death, and then cell death that's associated with no digestion by neighboring cells or professional phagocytes was considered Type III, and now we would refer to that more likely as necrotic cell death.

But the term programmed death has an interesting history. In various context that generally meant naturally occurring forms of cell death that could be predicted, for example in various times and developments, so stereotype situations of cell death were referred to as programmed in the sense that you could predict when and where they'd occur. We now use that term to say that the cell death that we're witnessing is actually an active sailor gene-directed process; perhaps there are dedicated pathways that control those forms of programmed cell death.

So the word programmed is actually taken on a rather different meaning than originally used. But essentially what we're referring to when we talk about programmed forms of cell death is that we're distinguishing those deaths from otherwise passive cytotoxic forms of the death where the cell is not actively committing suicide per se or actively being murdered per se, but it's simply just dying because of passive events that ultimately cause the demise of the cell.

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So now when we consider active forms of cell death there's also various vocabularies and lexicons that are used. Typically and most frequently one will see apoptosis versus necrosis, or apoptosis versus programmed forms of necrosis, and we'll talk more about that later. Often times the lexicon takes on contexts that have to do with the type of specifier that has provoked or elicited the cell death that we're looking at. Is it a natural form of cell death? Is it a natural elicitor? Or is it some kind of non-natural product that is promoting the death?

Also, the lexicon refers to whether or not we're talking about autonomous suicide processes within the cell that's dying, or an active killing process by a neighboring cell for example that is promoting essentially the murder of a neighboring cell, and then ultimately the engulfment of that cell. But I think there's a broad consensus that the best way to describe these forms of cell death is by eliciting and referring to the underlying genetics and biochemistry that promotes and provokes those cell deaths.

Within those kinds of nomenclatures, we can try to dissect or determine whether we're talking about dedicated cell death pathways, or whether we're talking about something called sabotage which is essentially signaling that gets run amok, or you have an active process that somehow becomes deranged that needs to be active that ultimately kills the cells, but that death was not elicited by a dedicated pathway was simply the consequence of another kind of event that went array. For example, generation of reactive oxygen species is a classic example of that.

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One other point to make before we move on is that when cell death community discusses these types of cell death, one of the pivotal features that we're all sort of discussing and attempting to dissect is the so-called point of no return which is demonstrated by the red arrow

there. So there are places in the cell death pathway that are reversible and places that are not, and we're very interested in the field in determining what that transition point is in various contexts.

And the reason is it's important from the standpoint of just basic knowledge, but it's also very important from the standpoint of trying to intervene and prevent cell death in various context because once your path, the point of no return, at least a conceptual framework is such that one cannot intervene and prevent those deaths. So that's another way that the cell death field has historically tried to grapple with those issues.

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And on this slide, the very classic distinction between necrosis toward the left and apoptosis toward the right is illustrated. What you can see here are these forms of cell death are very distinct morphologically. In the apoptotic forms of cell death, as most of us know, the cell shrinks, becomes small little apoptotic corpses or fragments that are rapidly engulfed in vivo situations by professional phagocytes or even neighboring cells.

Necrotic forms of cell death, the pathway to the left is essentially very different morphologically because the cell doesn't shrink, in fact it expands. The mitochondria actually expand and become very distended as well, and ultimately the plasma membrane appears to just disintegrate. So those are very distinct ultrastructural changes that occur, and we now know that the pathway to the left necrosis can occur in passive sabotage situations as well as there are programmed situations that appear to elicit this as well, and we can discuss that later if people are interested.

Slide 10

So on this next slide is a video showing a thousand -- a picture or a video is worth a thousand words or pictures because when you witness apoptotic cell death in culture it becomes very obvious what you're seeing. What you'll see on this video as it plays is cells roundup and appear to boil that is. We prefer to this as blebbing process during apoptosis. And what you'll see is the plasma membrane appears to boil in bubble, and ultimately those so-called blebbing cells will fragment into individual -- many sort of bodies that ultimately inside an animal would be rapidly engulfed.

But in culture we see the remnants of those bodies and that's mostly because in cultured cells frequently engulfment is very inefficient. So probably one of the best ways to visualize or score something as apoptotic is to actually just look at it in a video or in real-time. And what you're looking at there is about a 15-20 minute window of time sped up in this situation so it's not going to look that obvious. But if you do this over time they'll become very -- many of you probably have already seen this in many of your cultures.

Slide 11 and Slide 12

Another way to look at apoptosis is using so-called vital dyes. In this particular slide we're looking at *Drosophila* embryos that have been stained with a vital dye known as Acridine Orange. This is a dye that is specific for apoptotic cells. And what you can see on this next sort of slide is that every one of those little dots is an apoptotic corpse. And if you drill in on a very, very high power, you can see that many of these corpses are bright even after they have been engulfed by phagocytes.

So the nice thing about these dyes is it follows cells not only as they initiate apoptosis, but they stay with the apoptotic corpse for some time, so one can actually follow those corpses even after they've been engulfed.

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Another way obviously to look at apoptotic cells in fixed tissue is, again, just simply by histologically staining, and then when one looks at those, carefully at those sections, one can actually see -- here's a section of thymus, one can actually see, circled here in red, the cells that are essentially caught here in the midst of apoptosis and they're clearly sort of small, pyknotic and overtly blebbing cells. Electron microscopy is another way to look at apoptotic cells.

Slide 15

And here, what I'd like you to appreciate is that the arrow is pointing to the nucleus of an apoptotic cells, and you can witness in these cells very strong chromatin condensation that's classically associated with apoptosis. With the way we really know that this cell is dying is because you may be able to appreciate that it is actually a target for a huge phagocytic macrophage that's only partially visible in this slide, and the arrow points to a process that is emerging from that phagocyte that is

beginning to wrap around the apoptotic cell, and ultimately will engulf it. And that white arrow, the white open arrow that you're seeing is a previously engulfed corpse that is now very, very obviously pyknotic inside the large phagocyte.

Slide 16

But at the molecular level what we know is that a series of enzymes known as Caspases are largely responsible for implement and executing the apoptotic process. These so-called caspases exist in all our cells, in all our bodies as dormant proenzymes, and during apoptosis they are processed and matured into active enzymatic molecules. The classic caspases format for processing is shown in the schematic to the right, and this large proenzyme becomes processed into a large and small subunit. The prodomain does not actually exist together in the final enzymes, but the classic characteristics of caspases are, that they have a cysteine in the active site that's vital for the cleavage process and these cleave at aspartic acetate, aspartate residues.

[0:15:28]

Now when the cleavage of substrates occurs, we know that there are plenty of examples where the substrates for these caspases can be activated or it's very clear that these substrates can also be inactivated. And so, much work going on currently in the apoptosis community is attempting to dissect the consequences of not only processing of caspases, but processing any cleavage of target substrates to understand how these substrates when cleaved bring about the death of the cell. It's very obvious looking at the podium of apoptotic cells and non-apoptotic cells that you don't see a whole cell shredding of the prodium simply because the caspases are activated.

Actually, the caspases targets themselves or rather limited, and so we're truly trying to understand how the cell is converted from a living state to a dying state when the targets themselves are cleaved. And also turns out that many viral genomes encode direct inhibitors of these caspases. These caspases not only function only in program cell death, but they also function in other related processes such as immunity.

Slide 17

So here's an example of how one can actually demonstrate the involvement of caspases in apoptotic cell death. In this form of cell death we're looking at live cultures that are treated with a so-called Smac-

mimetic, we'll see a little bit later what that is, but essentially is a very potent apoptotic inducer of these cells. And the graph down below, one can see that if you sort of follow these cells using a CellTiter-Glo Assay that measures live or living and dead cells, that if you prevent caspase activation by either silencing the initiating caspase in the process, which is the yellow bar on the left end and the right, or by coming in with caspase inhibitors and preventing caspase activation overall, you can prevent the death and keep those cells alive in the culture.

Slide 18

Another way to visualize caspase activation is showed to the right using an antibody that picks up activated caspases in the tissue. You can see that these are reflected as very bright green staining cells, and it's also vital that we appreciate that we can show caspases are not only activated, but they're functionally necessary for the death, and this can be done as I'll show later by genetics and using RNAi; so functional studies are absolutely essential when demonstrating and studying the role of caspases in apoptotic cell death or non-apoptotic cell death.

Slide 19

So here's a general summary of the distinction between apoptotic cell death and necrotic cell death. I won't walk you through it other than to point out that, generally speaking, when we sort of follow necrotic forms of cell death in vivo that is generally a very highly inflammatory event, and generally speaking when we follow apoptotic cell death inflammation is not associated. This is an oversimplification, but that's a reasonable prediction to make.

Apoptotic cell death require caspases and necrotic cell death generally do not, and we can also point out that there are changes in the plasma membrane that are characteristically associated with apoptotic cell death that are not associated with necrotic cell death and this is the phosphatidylserine exposure that many people study in association with apoptosis. This can be detected with various antibodies and markers.

Slide 20

On this next slide we're summarizing the various methods that can be used to distinguish between these forms of cell death and how to actually look at the changes that occur during either necrotic cell death or apoptotic cell death. So for example phosphatidylserine can be detected using Annexin V. Caspases can be detected using various antibodies and

substrate detector systems. DNA cleavage which is characteristically associated with apoptotic cell death, and I'll show that in a minute, uses a so-called TUNEL method to be visualized. Mitochondria are very, very pivotal organelle studied during both necrosis and apoptosis, and various markers and imaging technologies can be used to distinguish changes that are associated with mitochondria during necrosis and apoptosis.

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Just many of you may already know that the TUNEL procedure is classically associated or a classic method for looking at apoptotic cell death. It takes advantage of the fact that there are DNA's enzymes that cleave in the spacer region between nucleosomes in chromatic leaving free ends that it can be labeled as seen here on the next slide.

Slide 22 and Slide 23

Here we're using a so-called TUNEL labeling procedure. TUNEL stands for Terminal Deoxytransferase dUTP Nick End Labeling, and this enzyme adds labeled nucleotides to the free ends that occur during that process. Here we're visualizing it not only in fiberglass but it can also be visualized on the lower right in developing eye disc in drosophila and all those little bright yellow dots are TUNEL positive cells. So it's a very effective way to look at apoptotic cells, but I should point out that it picks up apoptotic cells fairly late in the process.

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Now how do we go about in the field defining the mode of cell death that's actually occurring? And several points have been made here. One is that it's usually not sufficient to simply look at one criteria and make a declaration about what form of cell death you might be visualizing. Generally speaking there are two types of criteria. There are other descriptive criteria or functional criteria. Functional criteria are those that allow us to make statements that define the events that are necessary for the killing process.

Slide 25

Here's an example of once such assay where here we're looking at drosophila strains. In the upper left is a wild-type animal. We're looking at the eye of a wild-type animal, and if you look at the lower left-hand

panel there, we've killed all the cells that will ultimately form the eye using expression of a transgene that elicits apoptotic cell death. And if you look at the lower right panel, at the same time, not only are we promoting cell death using expression of an apoptotic inducer known as grim here, but we're also coming in with a caspase inhibitor known as p35 in transgenic form. And what you can see here is we've rescued virtually the entire eye simply by coming in with a caspase inhibitor.

So this kind of functional assay allows to make a very powerful statement about what the mediators are that are eliciting the death of the cell in this particular context, so those genes when eliminated can prevent apoptotic cell death.

Slide 26

So here's a situation where we've eliminated all the pro-apoptotic genes and you can see that in the embryo in the upper right, there's all those apoptosis dots that are lacking in an animal that is missing the apoptotic genes. And so there's another powerful functional example where when you eliminate programmed cell death genes, you eliminate the cell deaths that otherwise occur allowing you to make a powerful functional statement about the molecules and genes that elicit the death of the cell.

Slide 27

Now, I am actually getting fairly low on time, but I want to show one last slide here in this context and demonstrate that one of the manifestations of failures in cell death is the presentation of extra cells and that's shown here on the lower left here where one can see that an apoptotic mutant on the lower panel, there's extra cells that don't exist in a wild-type animal.

Slide 28

One last example here, what we're looking at here are cells that are either patches of cells and those cells are actually wild-type in green, and other cells that are orange and these orange cells are mutant for a programmed cell death pathways. And what you can see in this particular context is that the wild-type cells will die, whereas the mutant cells will fail to die, and this again is a very powerful example that tells us about genes that are needed to elicit cell death.

So when you eliminate a cell death gene and cell death fails, you have a very powerful argument to be able to make to say that the product

encoded by that gene is necessary for the cell death that you're witnessing. So these kinds of functional assays are vital in order to be able to make definitive statements regarding the kinds of death that one I was referring to.

Slide 29

So in the interest of time, I'm just going to move on to the next slide and just outline that for the cell death community there are generally two types of caspase mediated pathways that are sort of generally referred to both the intrinsic pathway and the extrinsic pathway. Generally speaking the extrinsic pathway is brought about by signaling two caspases through a so-called death ligand interacting with a so-called death receptor that ultimately form apoptotic caspase that mediates caspase-dependent cell death.

[0:25:25]

In the intrinsic pathway often times we're looking at caspase activation platforms that launch from mitochondria and forms so-called apoptosomes that activate initiator caspases that then cleave and activate executioner caspases. And what I'm going to do is, again, I'm going to skip the next two slides which are essentially an experiment that demonstrates how one can screen for new players in the pathway, and I'm just going to skip through that to get to what we refer to as the so-called vocabulary or lexicon of cell death.

Slide 32

And these are various things that you may see in the literature and thought I'd touch on them here very quickly. Generally speaking there's this process known as Anoikis which is essentially an apoptotic response that is seen when adherent cells lose their matrix interactions, and this is believed to be a major tumor suppressive mechanism. You may also encounter a term known as Pyroptosis. This is associated with an inflammation and involves Caspase 1 and it turns out that this particular pathway accounts for the loss of T cells associated with AIDS.

So Pyroptosis in contrast to other forms of classic apoptosis involves Caspase 1, whereas classical apoptosis generally involves Caspase 8 through the death receptors or Caspase 9 through the intrinsic pathway. Necroptosis is a new form of necrotic cell death, generally a new form of necrotic cell death, discovered through studies that loop the TNFR1 signaling, and demonstrated that RIP1 kinase occurs when Caspase 8 is

inhibited and this form of cell death is revealed in the context of inhibition of that abled caspase.

There are basically partial demolition kinds of events that can be referred to as cell death as well. For example, the enucleation of red blood cells, essentially those cells are dying because they lose their nucleus, skin cell keratinization is another example of that. Those are caspase-dependent processes as well. There's a secondary form of necrosis that occurs after a full-blown apoptotic program. This is often times seen in culture when engulfment is compromised.

There are other forms of necrosis listed here, Parthanatos which involves PARP polymerase; Ferroptosis which involves intracellular iron, and autophagic cell death which is associated with high levels of autophagy. And in this particular situation it's important to distinguish between autophagy that a death by autophagy versus death with autophagy, and in one context when we're talking about death by autophagy, we're really suggesting that the autophagy is the lethal event, and frequently though the death at scene with elevated autophagy is more associated with or best described as death with autophagy.

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I'll just leave with this final slide here that outlines the autophagy pathway and the autophagy network, and just to make one last point that we know that there's quite a lot of crosstalk between the apoptotic pathway and the autophagy pathway, and a very pivotal series of studies have shown that Bcl-2 which is an important regulator of apoptosis also is an important regulator of steps in the autophagy pathway. So look for more of that as the field matures, but we certainly are well aware of the fact that the autophagy regulatory network and the programmed cell death regulatory networks are highly interdigitated.

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And summing things up, I want to thank the folks who have provided some of the images that I used here today, and they are listed here, and thank you for your time.

Tianna Hicklin:

Thank you Dr. Abrams. And just a quick reminder to our live audience, please submit your questions at any time by typing them into the textbook at the bottom of the screen. If you don't see the box, click the red Q&A icon and it should appear.

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Our second speaker today is Dr. Bill Telford. Dr. Telford received his Ph.D. in microbiology from Michigan State University in 1994, where his laboratory developed some of the earliest techniques for flow cytometric detection of apoptosis. He received his postdoctoral training in immunology at the University of Michigan Medical School, and became a staff scientist at the National Cancer Institute, National Institutes of Health in 1999. He is currently the director of the flow cytometry core laboratory in the NCI Experimental Transplantation and Immunology Branch.

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Dr. Telford's main research interests include: instrument development, particularly in the area of novel solid state laser integration into flow cytometers; flow cytometric stem cell detection and characterization; and functional characterization of early apoptosis by flow and image cytometry. Welcome and thank you for joining us today Dr. Telford.

Dr. Bill Telford:

Oh thank you Tianna, and again, thank you to the organizers for having me here.

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Dr. Abrams gave a wonderful general overview of what goes on during apoptosis and I'll show this picture. Again, he already showed it, but this is a picture from John Kerr's paperback in 1971, a long time ago and it says, "Apropos now as it was when it was originally published showing the difference between not necrotic or traumatic accidental death; an apoptotic death that is signal-mediated and has a number of discreet steps ultimately resulting in phagocytosis of cellular fragments."

We do a lot of flow cytometry in my lab and all of these processes, most of them, can be detected by flow cytometry. Flow cytometry has turned out to be a marvelous technique for looking at apoptosis particularly in the immune system.

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And it all started back in the 1980s. This is the town of Pushchino, it's a little scientific village south of Moscow with a number of institutes in it, and an investigator by the name of Samuel Umansky recorded what we think was the first incidence of looking at apoptosis by flow cytometry.

He built his own flow cytometer and he was actually looking at the effect of radiation on chromatin structure, and what he saw was a sub-G0/G1 peak, when he ran his DNA histogram, cells that have been labeled with a DNA dye.

And as you can by the title here, he knew exactly what he was seeing. This was apoptotic cell death, one of the first times it has been seen. And within the next few years, a number of investigators jumped on this, and the earliest techniques for looking at an apoptosis looked at the changes and light scattering, forward and side scattering, where the cell show decrease in forward scatter and an increase in side scatter. And also loss of DNA by using DNA binding dyes in apoptotic cells.

What's really critical about using flow cytometer when you look at apoptosis is that we're looking at individual cells. We're no longer looking at DNA fragmentation and lysates, we're able to see the actual process going on in individual cells, and this is a critical point that will keep coming back to.

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Now, as Dr. Abrams mentioned, the signal transduction of cell death is very well understood now, both the extrinsic or receptor-mediated pathway and the intrinsic pathway resulting from radiation or stress that's mitochondrial-mediated. And there are a number of targets both in the signaling and in the downstream morphological changes that we can see using apoptosis. And as I'm sure most of you are aware there are a variety of probes and assays that we can now use.

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Looking at both the early apoptotic events like Cytochrome C release from mitochondria membrane potential changes, Bax translocation, intermediate changes like caspase activation and then later changes, such as TUNEL, detection of DNA fragmentation, Annexin V binding, and finally the major changes in cell size and optical density in loss of DNA. So we have a lot of choices available too is if we want to look at apoptosis by flow.

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So when I give this talk, I'd like to give four take-home lessons. First of all, remember that apoptosis is a highly variably process even within the same cell type under different conditions. You can have different

morphologies and different signal in characteristics, so don't rely on just one assays. Try several, and if possible, combine them into a single assay, use multiple assays at the same time. This is very easy in flow cytometry and you don't need various sophisticated instrumentation to do it. You end up with a very powerful assay looking at multiple characteristics and try to combine biochemical and morphological assays whenever possible.

Also, let your assay not only measure cell death, but characterize it. And again, go after biochemical targets like caspase activation for example. You can learn very interesting things about your cell system. Finally, take pictures. As Dr. Abrams pointed out, apoptosis is ultimately a morphological process. The gold standard for looking at cell death is looking at morphology, so take pictures whenever you can, and there are some nice technologies for doing that.

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Now I won't go through many of the assays specifically because we don't have a lot of time, but I just want to point out a few major principles in looking at apoptosis. Now, this is a very simple cell death assay here. I have induced apoptosis in EL4 cells and I've done an assay that I'm sure you're all familiar with, looking at Annexin V and propidium iodide. So the viable cells are here, the early apoptotics are over here and the late apoptotics are here.

Now this is a simple assay, but it's actually very information-rich. You're seeing a process where the cells are viable, and then the earliest apoptotic cells appears, these are cells that are Annexin V positive, but they have not yet damaged their membranes enough to let the DNA dye, propidium iodide in. So Annexin V is coming up earlier than PI, and by using these two reagents together, you're not just measuring cell death, but you're measuring the process of apoptosis from viable, early to late. And in fact, you're actually doing four assays, not two. Because you also have forward and side scatter available as well and this can be done on the simplest two-color flow cytometer.

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Also, take a great deal of care when you're looking at the scatter. Most of you will see a change in scatter when apoptosis occurs, and naturally you'll draw a gate around not only the viable cells, but the apoptotic cells as well and that's certainly a correct thing to do. But what I also

recommend you do is draw a gate around the cells that look viable based on scatter only. The cells, that if you just ran these things without a reagent, they would look perfectly okay. And if you do that, you can see those cells are already starting to die. Their Annexin is coming up and some of them are even permeable to a DNA dye. In this case the dye 7-Aminoactinomycin D.

So we like to gate not only on all the cells, but also the so-called "scatter viable" cells. I put scatter viable in quotes because they're not really viable. They're already marching down the pathway to cell death. They've already committed to it. But again, very powerful assays can be done using relatively simple reagents and simple instrumentation. And you can take that further as well.

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In this assay here, I have kept in my Annexin V and my DNA binding dye. I'm using Pac Blue Annexin V and 7-AAD or SYTOX Advanced which is a Life Tech version of it. But I've also added in FLICA. FLICA is a fluorogenic caspase substrate that binds the caspases when they are present inside the cell, and what you can see if I gate on my scatter viable cells, that is the middle series of panels down here, top and bottom and the middle here. You can see that we have a viable population, an early apoptotic and a late apoptotic. So we're able to look at the entire process using three different assays simultaneously, and again, an approach I highly recommend if you're looking at cell death.

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Now to cover the last of my take-home points, imagery, getting pictures of your cells is always an excellent idea. As I mentioned, and as Dr. Abrams mentioned, apoptosis is quite variable and pleiotropic. Imaging gives verification that it is occurring and allows you to even characterize it to some extent, to see the chromatin condensation, to see the cytoskeletal breakdown. It gives you additional analysis options like pixel-pixel analysis that can be useful for analysis as well. And if you're doing adherent cells you don't have to remove the cells from their substrate. You can scan them right on the slide.

Now this is a Compucyte iCys, this is a laser scanning cytometer. It's really a cytometer, but instead of putting the cells in a tube and running them through a stream, you put them on a slide and scan them underneath the microscope. But the data you get is very similar to what you get for flow

cytometry. So we can detect our cells and then segment them to our regions around them and analyze them for their fluorescence.

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And this is a great way to look at cell death because in this case we're doing the same assay we were doing before a similar one, a caspase, DNA dye and Annexin V, and we're able to identify the viable, the early and the late apoptotic cells, but we're also able to get morphology as well. These images over here are actually not photographic images, they're PMT reconstructed images, and you can see the Annexin V and the DNA dye permeability in these untreated and treated fields, top and bottom.

Slide 50

And what's great about scanning cytometry too is that it's all relational. You can go back and look at the cells that are identified cytometrically for their morphology, so I can draw regions around my early, middle, and late apoptotic cells and actually see the cells that correspond to the cytometric region. So cytometry and imagery are correlated; again, a very, very powerful assay for looking at the changes that occur in apoptosis. As I mentioned you can do it here in cells using this technology as well.

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Adherent cells like to be adherent. They don't like to be ripped off their substrates, and doing that by scrapping or by trips, and often tends to disrupt the apoptotic phenotype or even induce apoptosis itself. So you can look at apoptosis in adherent cells by doing your staining directly on your chamber slide or in your flask and running them on a scanning cytometer. Keep in mind that adherent cells will round up when they undergo apoptosis, so you might lose some of the later ones, but again, a great way to look at adherent cells undergoing apoptosis.

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There are also some stream-based imaging systems available now. The Amnis ImageStream is the most prominent of these. In this case we've taken Daudi cells, induced them with a drug, camptothecin; and again, done that three-stage labeling, caspase, Annexin V and DNA dye. In this case the cells are not being imaged on a slide, they're being imaged

actually in the stream, but we're able to identify the apoptotic phenotypes very clearly, and as with the Compucyte iCys we can go back and gate on the cells that we're interested in in the cytometric data. Cytometry and imagery are correlated and you can see the phenotypes very nicely.

So in this case I am able to gate on my viable or early apoptotic, intermediate apoptotic and later apoptotic or necrotic cells and see the dyes correspond to them; a very, very powerful technique for looking at apoptotic cells.

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Now, Dr. Abrams has mentioned autophagy. I'm just going to mention it very briefly because there are not that many flow assays out there for it yet. As he mentioned it was originally thought to be an apoptotic process. Now it is largely believed to be a cell survival mechanisms where cells can down regulate their energy requirements to meet challenging conditions, but without necessarily dying. One of the gold standards for measuring autophagy is looking at LC3, light chain 3, a protein that segregates the autophagosomes that are phagocytizing defunct mitochondria and other intracellular organelles, and eventually bind to lysosomes where their contents are digested.

We were running U2OS cells here. In the control cells in the upper left-hand panel, you see a lot of GFP fluorescence. It segregates to the autophagosomes when these cells are induced to undergo autophagy. So if you can look at that segregation, it's one way to measure autophagy.

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The assay that's actually available out there is an EMD Millipore assay. What you do is take a cell that has been stably transfected a cell line, stably transfected with GFP-LC3, you induce autophagy and then you selectively gently permeabilize the cell. In the cells with no autophagy, the GFP will leave the cell. It will leak out through the pores in the cell. If the GFP-LC3 is segregated to the autophagosomes though, it will be retained inside the cell and by looking at that difference on the flow cytometer between controlled and induced cells, in this case, the upper top panel here, the cells are leaking out a lot of GFP. In the lower panel they're retaining some of it.

By looking at that difference between the two, you can get a semiquantitative measure of whether autophagy is occurring. This is still

a very new area in cell biology and it's a very new area in flow cytometry. We don't have a lot of assays available for it yet, but there are some coming along, and I think we're going to see some exciting developments in the next few years.

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So anyway, just to remind you of my take-home lessons. Remember try a number of different types of assays. Don't just open media catalogue and buy the first apoptosis assay you find. Try several different things. Try to find out that will work well with your cells. Never use only one assay and combine them if you possibly can. I will keep harping on this because it's an important point.

Again, let your assay not only measure cell death, but characterize it. Look at the biochemistry of that apoptosis. You can learn a lot about your cell system that way and take pictures whenever possible. If you don't have a scanning cytometer, that's okay. Put them under an epifluorescence microscope and take a picture or make a movie. It's a great way to confirm that apoptosis was really occurring.

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So I will conclude there. As Tianna mentioned I work at the National Cancer Institute. I run a flow cytometry laboratory there, and both EMD Millipore and Molecular Probes which is a Life Technologies, Thermo Fisher Company have been very, very supportive to us in studying these phenomena, and I'll stop there.

[0:45:17]

Tianna Hicklin: Thank you Dr. Telford.

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And now we're going to move right along to answering some of our audience questions. A quick reminder to our live audience, you still have time to submit your questions by typing them into the textbox at the bottom of the screen. If you don't see the box, please click the red Q&A icon and it should appear. And please stay tuned after the Q&A session for a short presentation from our sponsor, EMD Millipore, who will talk about their cell death detection technologies.

And moving right along to our first question, Dr. Telford since you were just discussing the importance of using multiple assays, I'll direct the first question to you. Since apoptosis, necrosis and autophagy can all occur either independently of each other or simultaneously with each other, is it possible to experimentally distinguish these mechanisms when they occur simultaneously?

Dr. Bill Telford:

I think doing it by flow cytometry is probably not possible at this stage in the game. It's something that could be done morphologically and certainly should be tried. Again, the relationship between autophagy and apoptosis is still being elucidated at this point because a lot of the changes that occur during apoptosis like degradation of intracellular organelles can occur in autophagy as well where the cells do not necessarily die with another.

But I reiterate, I would try to combine, for example a caspase substrate, Annexin V and a DNA dye together, and then absolutely take pictures. Right now EM is actually one of the gold standards for looking at autophagy, to look at the formation of autophagosomes and the dissolution of the organelles within them. So definitely take advantage of imaging technologies. An epifluorescence microscope, it's fine for this.

And at this point, I wouldn't necessarily try to combine the two, but we have done experiments where we've looked, for example, a caspase in autophagy in the same cells in different samples, and it turns out caspases are not actually well activated during autophagy. So it should be possible if it's done properly.

Tianna Hicklin:

Okay, and to follow on to that question. For a heterogenous cell population, is there a technique that can identify the percentage of cell populations undergoing apoptosis during necrosis then?

Dr. Bill Telford:

Well, certainly. One of the big advantages of doing apoptosis by flow cytometry is that you're seeing individual cells. When I first started doing this we would take cell lysates and look for DNA fragmentation and it worked, but you were not seeing individual cell events which is something that as long as the cells don't bleb apart into tiny pieces can be possible by flow cytometry. We often get asked the question, can I do simultaneous phenotyping along with it because flow cytometry is applied primarily to apoptosis in the immune system, and people want to look and see whether they're double positive, 44 positive, 25 positive thymocytes are dying.

It's a little risky to do that. Remember these are dying cells. Dying cells tend to bind the antibody nonspecifically, that's why we throw propidium iodide in with our phenotyping because we want to get rid of the dead cells, we don't want to see them. If you're going to try it, I would use a very early stage apoptotic assay, maybe a substrate for Caspase 8, something that will be up regulated prior to the membrane and cytoskeletal damage that we see later on in cell death and could bind the antibodies nonspecifically.

But with flow cytometry you have the power of gating, so you can gate on scatter and if you're looking early enough you can look at phenotypes and look at the percentage of apoptotic cells that are dying in any particular time point.

Tianna Hicklin: Great! And Dr. Abrams, in your opinion how does apoptosis help a cell apart from aiding and aging?

Dr. John Abrams: How does it help a cell or how does it help the animal?

Tianna Hicklin: This specifically refers to a cell but perhaps --

Dr. John Abrams: Well, I can easily sort of discuss how it might help an animal or a tissue. How apoptosis might help the cell? That will be a little bit more difficult question. Let me just first address the -- generally speaking apoptosis is sort of a noninflammatory or nondisruptive way to remove cells that might be carrying viruses, might have some kind of genotoxic damage that they sustained. So by eliminating and renewing the tissue in this way, eliminating those cells and enabling renewing tissues helps the tissue renew and the animal as well.

[0:50:18]

In terms of helping the cell, you might be thinking about how sort of partial demolition through apoptotic pathways may help to ultimately remodel cells. So classic examples of cellular remodeling are now becoming very well-studied, and what we're seeing in these contexts is that apoptotic pathways and apoptotic machinery is being utilized in these context in order to promote the remodeling of the cells.

So classic examples in the nervous system where dendrites are pruned for example, or synapses are remodeled. And in these cases, we know that there's localized activation of effector caspases, Caspase 3 in particular. And more important, we know that there's functional activation of the upstream apoptosome which is the hollow enzyme of

Caspase 9 that ultimately brings about the activation of effector caspases. In the apoptosome in those context is absolutely required for the remodeling that is seen in those nervous systems, those neuronal cells.

So perhaps that's what the viewer is asking, is does partial apoptotic activation somehow promote remodeling and the answer is, as we're seeing yes, definitely.

Tianna Hicklin: Okay great, and if you could both comment on your opinion on what the best method to use for setting apoptosis in looking to discover therapeutics for cancer patients?

Dr. John Abrams: Bill, why don't you take that one first?

Dr. Bill Telford: I'll take that, okay sure. Well, I think one of the things you should be very careful about in looking at cell death in tumor cells treated with chemotherapeutic agents, and in Dr. Abrams' video showed this quite dramatically. When we look in the immune system, cells undergo apoptosis and they tend to remain discreet single cells even once they die, and this is probably not a situation that ever occurs in vivo because as soon the cell starts to become apoptotic, it will either bleb away into little pieces, or a phagocyte will come over and eat it up and it will be gone.

So what we have seen in a lot of cases in tumor cells, rapidly growing tumor cells is that the cells literally, as Dr. Abrams said, literally boil away. They form these little tiny apoptotic bodies that are eventually phagocytized and that becomes very problematic when you're trying to analyze them by flow cytometry because they're no longer discreet objects and they're often down at the threshold where you can't see them anymore.

So this is one of those situations where flow cytometry actually might not be the best way to look, and you might want to look at morphology, you might want to try a microtiter plate assay for caspase or something where you're not looking at individual cells anymore. So certainly try the caspase assays. Caspase 3 is not present in all cells, but it seems to be present in most mammalian cells. Try those out but get out your microscope, look and see what's actually happening under the microscope, and if your cell seemed to vanish on the flow cytometer, they may be undergoing apoptosis just fine, it's the just the way they're doing it is not conducive to flow cytometric analysis.

Dr. John Abrams: And to amplify on this -- Tianna, can I control the slides here for a second?

Tianna Hicklin: Sure.

Dr. John Abrams: Okay. Maybe I can get to the place if we're asking about sort of high-throughput screening there. I have a slide that I could actually use if I can get there in time. But if you're thinking about screening and screening for sort of inhibitors or sort of modulators of cell death pathways, then really the fundamental pivotal consideration there is the opportunity to do this at very high-throughput and very rapidly, and this also demands that you have the minimal number of interventions in any given experiment.

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So in this particular slide on the upper left, there is a nice sort of schematic that shows how cells can be plated in this particular format, 96-wells or 84-well plates. In each plate in this particular situation has a different RNAi molecule inhibiting a different gene in the genome. But the most important considerations for screening is the most simple assay you can run with the least number of steps because in this particular kind of experiment you're running tens if not hundreds of thousands of experiments in replicate. And so any step that you can eliminate or optimize and minimize in terms of intervention really helps eliminates problems in the assay.

[0:55:01]

In this particular situation what we're looking at in the upper left panel is CellTiter-Glo which is essentially a surrogate for following levels of ATP, but other cell viability measures are possible as well. The nice thing about a CellTiter-Glo is that you squirt it in and you just get one measurement. There's no additional steps in terms of fixing or staining and things like that, so major consideration for screening is minimal numbers of steps.

Tianna Hicklin: Great! Thank you. So we're almost out of time here, perhaps just one final question. Dr. Abrams, perhaps you can answer this one. Is there a way to distinguish between whether autophagy is behaving in a pro-survival versus pro-cell death fashion?

Dr. John Abrams: Yes, there's only one definitive way and that's to intervene functionally. So in order to determine whether -- lots of times we might witness cells dying with lots of autophagy in the cell visible either by LC3 staining or by electron microscopy or both, and we really have no way of knowing

whether or not that death event is occurring by some other means and autophagy is elevated at the same time. So in that case autophagy is associated with the death and that would be considered to be death with autophagy.

The alternative model, and does occur at times as well, is that it might be that the actual lethal event that's killing the cell has something to do with the elevation of autophagy itself. The only way to definitively determine between death with autophagy and death by autophagy is to actually intervene and eliminate one or more of the autophagy functions and ask what happens. So if this even that you're witnessing is truly death by autophagy, and perhaps by mutating or eliminating or silencing one of the critical mediators of autophagy, known as the ATG genes.

If those cells are now kept alive, and they otherwise would have died by removing the autophagy function, then one can make a fairly definitive conclusion that that particular context was death by autophagy. Alternatively, if we eliminate and prove that we've eliminated the autophagy function and the death still occurs, that would more likely be consistent with the model of death with autophagy, and all of these are context dependent. So the answer to your question is you must functionally intervene to determine between those two models.

Tianna Hicklin:

Thank you Dr. Abrams, and unfortunately, we have come to the end of the Q&A portion of our webinar, but we will now have a short presentation from Dr. Kamala Tyagarajan, a senior research and development manager at EMD Millipore. Welcome Dr. Tyagarajan.

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Dr. Kamala Tyagarajan:

Thank you Tianna. I would also like to thank Dr. Abrams and Dr. Telford for their very stimulating and enlightening presentation on the cell death pathways and approaches and practice today, and thank you all of you for staying through this brief presentation on Simplified Solutions for Cell Death and Cell Health Analysis with the Muse Cell Analyzer.

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So as we just heard in the talks by John and Bill, dissection of cell death pathways requires information from multiple steps in the process. So there's a variety of information needed to truly understand what's going on, and many of the barcodes suggested have been arranged from mitochondrial potential changes, activation of caspases, DNA fragmentation, autophagy markers and changes in morphology.

We also learned that multiple technologies can provide different layers of information that further our understanding of the cell death process, and these can range from microscopy, flow cytometry, imaging cytometry, and also technologies for imaging adherent cells. It's also important to note that in such studies, that time and dose response of our studies can provide critical insights, improve the sequence of events in the pathway, and it can allow you to get a more comprehensive picture of what's going on as you study these changes.

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As we look at the variety of technologies available today for investigating cell death, while each of them brings specific advantages and distinct advantages, there are also some disadvantages that limit the utility. For example, a technology such as Western Blot or ELISA enable to provide poor cell-specific changes, although they are very useful to get information on the total change in the process, but poor cell-specific information is lost. If we look at certain imaging methods for example, they can have sudden limitations on whether equivalent number of cells are counted between sample to sample or enough number of cells are counted which might have some impacts on reproducibility or accuracy of the results seen.

[1:00:35]

The other and we've also seen methods, such as multiparametric flow cytometry or imaging cytometry which can be very powerful and provide content-rich data for cell death analysis, but these can also have their own limitations such as access to instrumentation maybe limited. There may be a requirement for a great deal of expertise and training for setup, and also cost-related limitations in order to utilize this platform.

So there's a need for easy-to-use, affordable, accessible methodologies that can robustly provide answers to common every day questions that we researchers are investigating in cell health and cell death, and for us to work quickly, accurately and reproducibly. Now I'd like to share with you one such example of this kind of technology which addresses some of these limitations effectively, and this is EMD Millipore's benchtop Muse Cell Analyzer.

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So what is the Muse Cell Analyzer? Muse as in affordable, low-cost, miniaturized flow cytometric platform that can provide quantitative cellular information on dedicated application. The instrument is a closed platform where the reagents and software are closely paired together for dedicated applications to simplify the process of getting information on cell health and cell death pathways. The instrument has a very small footprint and it's based on the principles of microcapillary cytometry, and this allows for analysis of small sample sizes and results in low biohazard disease, and the system can give information on percentage and counts of population as well as information on fluorescence intensities of populations.

But one of the key transforming features of the instrument is this highly intuitive touchscreen software interface which guides researchers through multiple steps with ease simplifying the process of obtaining cytometric data and getting the exact results and end points they are looking for. And this allows to take away the complexes of performing the cytometry experiment and makes the technology easily usable by people of varying expertise levels.

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So Muse provides multiple solutions of cell death mechanism, and if you look in the slide, we heard about the different early and late processes during apoptosis and other cell death processes, and what is shown in the right is a variety of assays which allow you to probe questions such as oxidative stress, nitric oxide stress, MitoPotential or Annexin V or some of caspase response. In addition, it also gives the capability to study questions related to cell health such as cell cycle of cell proliferation and as well as autophagy. And many of these assays are two-color assays which allow you to look at two markers simultaneously.

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So to show you an example of an assay on the Muse, here's an example of a Muse MitoPotential assay. And in the development of this what we've done is greatly simplified the process of obtaining information on mitochondrial depolarization and cell death simultaneously, and a schematic of the simple process, no-wash assay process as shown, where the researchers can add the reagents which are provided in the kit and quickly get to the highest answer on a Muse using a software which is specific for the MitoPotential assay.

For example, as shown on the right, here's an example of an assay output which has dark clots, there's data in the percent of populations which are exportable in different formats.

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I'd like to show you a quick brief example of how kinetic and time course studies can provide more enriched information. Here is a study where we performed, where we treated Jurkat cells with Staurosporine in the top and Gambogic Acid, an antitumor agent at the bottom. And the cells were treated for different periods of time and then analyzed with the Muse MitoPotential assay or the Muse Annexin V and Dead Cell Assay. As we look at the percent of cells affected, as you see on the top, what we noticed is that for Staurosporine, gradually with time, we see an increased population of cells showing depolarization as well as Annexin V changes almost just trailing right behind it.

[1:05:43]

If we look at the impact below on Gambogic Acid for example, what we see is an almost immediate impact on mitochondrial depolarization and there's a differential in the kinetics of the Annexin V response and the mitochondrial response which we've seen repeatedly under many different conditions. And this is suggesting that Gambogic Acid is having a severe impact on the mitochondria, and in fact publications and the literature suggest that the Gambogic Acid actually targets the mitochondria and triggers the apoptotic cascade from there, and a mitocan drug that is now very popularly used I think also for the treatment of lung cancers.

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I showed you some example of cell health assays on the Muse, the offering span multiple areas. We have dedicated kits which address a large number of signaling molecules which may be of interest in apoptosis research as well as immunology research.

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So to summarize quickly the Muse Cell Analyzer, is a small powerful and affordable platform for quantitative and accurate cell health and cell death analysis. The platform utilizes a combination of simplified sample prep with dedicated kits and software modules for investigating cell death, and the instrument is a very easy-to-use instrument and it

provides results and then easy to use format for the researcher which is application-specific.

We are hoping that technologies such as Muse can help reduce the analytical complexity for performing and obtaining cytometric-based data in cell death analysis and allow researchers to focus on biological questions of interest in the investigation of cell death pathways. Thank you for your attention. Back to you Tianna.

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Tianna Hicklin:

Thank you Dr. Tyagarajan. And this concludes our webinar for today. I'd like to thank each of our wonderful panelists once again for being with us and for the great talks and discussion they've provided. Dr. John Abrams from the University of Texas Southwestern Medical Center, and Dr. Bill Telford from the National Institutes of Health, and thank you to all of our online viewers for your great questions. I'm sorry that we didn't have time to get to them all.

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For more resources related to today's discussion, please visit the URL now listed at the bottom of the slide. And this audio webinar will be available to listen to on demand along with the PDF of the slides within the next 48 hours. And we encourage you to share your thoughts about the webinar by sending an email to the address now in your slide viewer, webinar@aaas.org, and please be on the lookout for more webinars from *Science* which you can find by visiting webinar.sciencemag.org.

Thank you again to both of our panelists and to EMD Millipore for generously sponsoring today's educational seminar. Thank you to our online audience for taking the time to be with us today and I hope you'll join us again for the next *Science/AAAS* Technology Webinar.

[1:09:00]

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