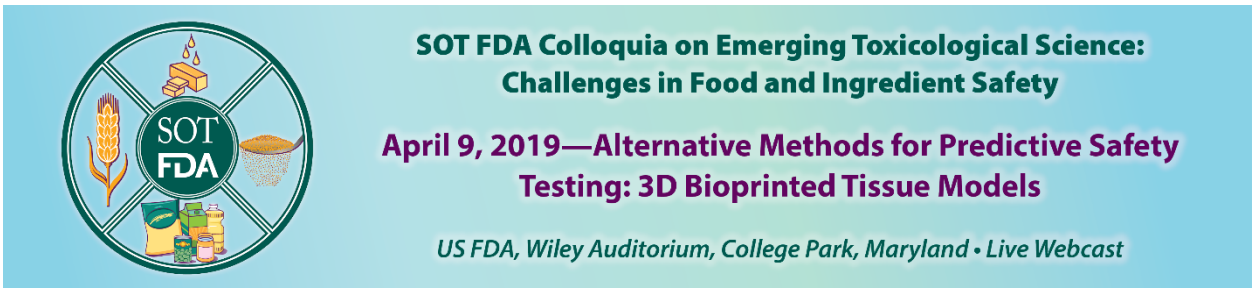

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Note: this is not a transcript.

April 9, 2019

Alternative Methods for Predictive Safety Testing: 3D Bioprinted Tissue Models

Chair: Edward L. LeCluyse, LifeNet Health, Research Triangle Park, NC
Co-chair: Margaret Kraeling, US FDA, CFSAN, Laurel, MD

8:30 AM – 8:40 AM	Welcome and Overview Mary Torrence, US FDA Director of the Office of Applied Research and Safety Assessment, Laurel, MD Speaker Introductions Margaret Kraeling
8:40 AM–9:20 AM	Overview and Challenges of Bioprinting Sharon Presnell, Amnion Foundation, Winston-Salem, NC
9:20 AM – 10:00 AM	Putting 3D Bioprinting to the Use of Tissue Model Fabrication Y. Shrike Zhang, Brigham and Women's Hospital, Harvard Medical School and Harvard-MIT Division of Health Sciences and Technology, Boston, MA
10:00 AM – 10:20 AM	Break
10:20 AM – 11:00 AM	Uses of Bioprinted Liver Tissue in Drug Development Jean-Louis Klein, GlaxoSmithKline, Collegeville, PA
11:00 AM – 11:40 AM	Biofabrication of 3D Tissue Models for Disease Modeling and Chemical Screening Marc Ferrer, National Center for Advancing Translational Sciences, NIH, Rockville, MD
11:45 AM–12:45 PM	Roundtable Discussion Moderator: Edward LeCluyse All speakers and Margaret Kraeling

Mary Torrence: It is my privilege to welcome you to the third SOT FDA colloquium in our annual series titled Alternative Methods for Predictive Safety Testing 3-D Bioprinted Tissue Models. I look forward to hearing about our 3-D priorities and 3-D printing. We use these tissue models in a lot of different ways across the sector of government, academia and industry.

We are in the fifth year of this colloquium and partnership between SOT and CFSAN. The main goal is to provide a forum to discuss the latest toxicological science in the areas of food and chemical safety. And really to stimulate a dialogue among experts about future oriented toxicologic approaches to food and food ingredients and safety assessments.

Over the years a wide range of topics have been covered, again across multiple sectors. I think one of the most valuable outcomes is the fact that it really is a global outreach across many sectors and topics and reaches globally. The other thing I think is really important is the recordings and slides are available on the website. If you missed the first series, you can go back to it. I found that very helpful depending on the topic.

The advantage of doing an introduction is I get to throw a couple of slides in for own office. OARSA is one of the research offices that provides science-based data and support to our program's mission around food protection and cosmetic safety. OARSA is comprised of biological and toxicology research, focused to fill data gaps and provide necessary scientifically based data for various program offices. One of our goals is to provide critical research for the detection and characterization of foodborne pathogens and improve methods and collaboration across research. Also, to apply toxicological approaches to support regulatory needs.

Our division within the Office of Spplied Research and Safety Assessment is focused on prediction. And the importance of prediction in understanding of the effects of chemical toxins. The center focuses on prediction, but we have the support and expertise in three approaches. In vitro, in silico, in vivo. It allows us to look at each approach and decide which is the best. We are trying to develop a concordance database which I find exciting so we can say which approach would be the best without doing animal studies if you don't need to. For the future I think this has also driven our future toxicological work in that we broaden it to organ on a chip, 3D bioprinting of skin, and *C. elegans* as alternative animal models. Again, I am pleased to open this colloquium and I look forward to hearing the presentations and I will turn it over to one of your co-chairs.

Margaret Kraeling: Thank you, Mary. I am here to introduce the speakers. My name is Margaret Kraeling. Today we have four very distinguished speakers who have extensive knowledge and expertise in development of 3-D cellular models, particularly 3-D tissue bioprinting. The first speaker is Dr. Sharon Presnell. She is president of

Amnion Foundation and she will give us an overview of bioprinting and some of the challenges that come with bioprinting.

Our second speaker Dr. Shrike Zhang, who is an assistant professor of medicine at Harvard Medical School. And a bioengineer at the Brigham and Women's Hospital. He will give us a talk on strategies of bio printing. “Putting 3D Bioprinting to the Use of Tissue Model Fabrication.” We will have a break and then after the break we will have another talk, Dr. Jean-Louis Klein, a scientific director at GlaxoSmithKline. We will talk about uses of bioprinted liver tissue in drug development. And the advantages of using three-dimensional models.

Our last speaker will be Dr. Marc Ferrer, who is the director of the 3-D tissue model laboratory at the National Center for Advancing Translational Sciences at NIH. He will talk about biofabrication of 3-D tissue models for disease modeling and chemical screening. After the talks we will gather for a panel discussion, which will be led by Dr. Edward LeCluyse, the chair of this colloquium. He is a principal scientist at LifeNet Health. He is also very experienced in development of 3-D cellular models. I'm going to hand it over to Dr. LeCluyse. Thank you very much.

Edward L. LeCluyse: Thank you, Margaret, and Betty Eidemiller, by the way, for assisting to set up and organize this event. I think personally for my own experience this is a timely technology at this point. I don't know how many of you actually have heard about bioprinting or know what bioprinting is about. I know the first time I spoke to a crowd, an audience about this topic, almost everyone had a misconception about what it involved. I think we have some of the best speakers to come here and represent everything from the development of this technology and provide an overview of this field for you. But also, to address questions about potential uses and what it has to do with the field of toxicology, especially in food science and things like that.

A four-minute break I understand from Betty. Betty, you will let us know when we are back in action?

There went our break, by the way. Is my pleasure to introduce our first speaker, Dr. Sharon Presnell. I had the privilege to collaborate with her on a major project that involved 3-D use of bioprinting to study liver fibrosis. I think she will introduce the topic during her presentation. She can provide a great overview, by the way, of the field in general but also where it's headed.

Overview and Challenges of Bioprinting
Sharon Presnell, Amnion Foundation, Winston-Salem, NC

Can hear me okay? I really appreciate the opportunity to come and be part of this. My role has changed recently and just to get this part out of the way I am a former employee of Organovo, who is probably the first company that began to make tissue from bioprinters. I'm still a shareholder of Organovo.

I thought we could start with some very basic definitions. Of bioprinter, bioink, and bioprinting. I don't know if there is an official working in this area, but I think for me, I still see a lot of wide variation in the interpretation of what is bioprinting. Sharing my perspective on what these things mean. And bioprinter to me is an automated instrument for the spatially controlled deposition of biological materials. Bioink are the biological materials formulated and configured to be dispensed from the bioprinter. Bioprinting is a practice of generating biological outputs through deposition of one or more bioink with a bioprinter. I think the question still hanging in the field is the word biological and whether that infers that live cells must be included to be bioprinting or its simple use of a biomaterial that maybe was derived from a [inaudible] sufficient to call it bioprinting? I see a lot of wide variation in how we name it. That is not as important as what you do with it. But I think as the field continues to grow rapidly, standardizing around definitions would not be a bad place to spend time.

In the history of tissue engineering which has been around for a long time, there are many ways to get three dimensionality. You can take a hydrogel and put cells in the hydrogel and cast that in the shape you want. You can take a scaffold that is either synthetic or decellularized native tissue and create that in a first step and then in the second step you had cells to that. The question arises for many of us, why would I print something instead of doing the other things? There are some important reasons why you might do that. Some are more exciting than others but they're all very real.

The first one that I look at is the unit-to-unit reproducibility. A printer applies the same pressure every time even if it's doing something as simple as spreading out a hydrogel onto a surface. It does it the same way every time. That can get you something biologically or keep you from having to use 10 replicates to see a signal. That would be true in single lot of tissue that you would make and also could be true from run to run to run. If you do it on a Tuesday with operator A or on Friday with operator B, you're more than likely to get the same outcome. I think there is a lot of value in that, independent of some of the more intricate things you can do.

The thing that gets us excited and probably was part of the vision of the pioneers in the early days when they started doing these things out of necessity is, they can incorporate living cells and void spaces which are equally important in the structure, three-dimensional as you’re building it up. This is going to give you a more uniform distribution of cells throughout which you are making, more so than say taking a pre-existing scaffold and putting cells onto that. And you can do things like the components of the tissue can be different in one compartment versus another. That is the spatial patterning piece. For me, that was the incentive to pick up what I was doing and do this other thing which was bioprinting and to be able to make the things you dream about and never could make before. So, hybrid things, and things with patterns, and being able to have features in a three-dimensional structure that you have controlled in your design. And I think that is what engaged us and got us all excited in the beginning.

The field is quite dated at this point. If you go back to 2003, Thomas [inaudible] took an inkjet printer and replaced the ink in the cartridge with cells and demonstrated you

could spit these out in a pattern, and some of them would live. He published this and patented this and this was the beginning. And then everyone said could you do it this way or that way? There was a group out of the University of South Carolina and University of Missouri working together under a [inaudible] grant [inaudible] very interesting, they took an old in script printer and started making things in 3-D. They were focused on printing just cells. They weren't interested in the biomaterial. They were studying chicken wing development and want to place cell aggregate close to each other. We're going to look at a couple of examples of this historic work. And Sue Williams' lab made the first excursion bioprinter in their lab where you take a hydrogel, put cells in and spit that out and it would retain the shape you printed it in. These are the things that were happening about the same time.

If we look at the inkjet this is a couple of simple examples of what was done with that in the day. The ink cartridges over here, this was replaced with a printhead that had syringe pump-type mechanisms on it and could dispense the cells. This is a thin layer of cells that have [inaudible] out onto a [inaudible] gel surface and this is a stripe of smooth muscle cells that have been printed out onto a collagen gel. The cells can survive, they would be patterned, and you could begin to imagine the things you could do with additional three dimensionality.

The aggregate work was a little bit different. The opposite direction. You see these tiny steroids of cells loaded into a glass capillary and this instrument which actually is the root of the Organovo instrument used today, it will have this basic design although the mechanisms by which it dispenses are quite different now, because glass capillaries are not [inaudible]. You take these and you could print these aggregates of different cell types and over time they would merge, physical forces would bring the cells together and you could take little aggregates of cells that would fuse into a tissue where the only thing holding that together is the cells and the [inaudible] that they make with each other. I thought that was fascinating. That was beginning of bioprinting that I came to do it.

One of the important points, we're going to talk about this later, these aggregates were printed onto a bed of collagen. Collagen is a very cell interactive material. If you trying to get cells to stick to each other, when you put them in something like collagen this nice ring structure forms. But if you look around the edges, what I will tell you is if you are trying to get cells to stick with each other, it may be beneficial to use a gel around them that is not adhesive because it keeps interacting with each other. The cells are attracted to their milieu. They will crawl out of the structure you are making. This is something you can leverage in the design of these tissues.

On the extrusion printing side, this is a stripe of hydrogel printed five layers thick with endothelial cells in it in this design here. It's interesting, if you add media too quickly tissue won't come together, if you let it rest it gives itself time to adhere to each other you get these nice structures with little vascular cords running through them, if you look up here they say this one didn't work too well but if you look at the connections forming between these vascular stripes, this is something we have seen in the field over and

over, if you get two components close enough together with each other they will build connections with each other and you can leverage that and understand the spatial reasoning behind it.

As bioprinting came to be, there were all of these basic 3-D fabrication technologies that existed in the world for rapid prototyping. Some of those were not well suited to including cells even though they can make beautiful very intricate structures. You will read about these in the 3-D printing space but the high temperatures and the exposure to certain types of lasers preclude you for being able to integrate cells into those structures as they are being made. However, there are teams of people including the group at Wake Forest that are using technologies like this to build very intricate structures by laying down a polymer layer and then topping that with a cell layer and coming back in with another polymer layer and cell layer. You can't discount these as being involved, it's just a matter of whether you can directly involve the cells during fabrication or not.

Stereolithography is very commonly used in rapid prototyping. In this case selectively, depending on the cells' ability to tolerate a particular type of UV or laser, I think you're going to see some examples in the next talk, you can include cells in these types of structures and get to a degree of intricate feature size you would not be able to get to with one of the other technologies. This is emerging and being modified to be more applicable to cells. This to me is one of the most exciting areas to see things come forward.

There is another laser-based called laser initiated forward transfer. This is more indirect. Cells are coated onto a metal oxide surface; the laser causes them to separate indirectly but doesn't come in direct contact. Pops them off the other side into a pattern. The most common, if you look in the literature, and who is bioprinting and making things, the extrusion-based method, you have a cell and a gel and basically a syringe pump and pneumatic or physical displacement that pushes it out, those are the most common means by which people are fabricating in 3-D today. And the droplet-based printing which would be the early inkjet and modifications going forward where the shear forces going to be higher, the resolution will be finer. The tolerance of the cells might be less.

This is just simply a look at visibly the most common forms of printing. Extrusion-based where you have material in a pump, and you might have a printer that has six heads on it that all different materials and you can interchange those. It is being directly extruded and the important thing here is whatever material you're using is able to retain the shape you give it when it prints, if it puddles out you may be better off casting a gel in the shape you want. Inkjets are doing droplets which usually have more than one cell and some can be quite large depending on technology. You are pattering those down. Under much higher shear forces and the viscosity has to be lower. With the stereolithography you are using a pool of cell-laden material and [[inaudible] that selectively with a laser in specific spaces. That allows you to get a tremendous amount of intricate architecture. I think you'll see some of that today. One

of the challenges you have to fill the well with all the cells and the cells can be precious. There is some raw material considerations on that. And this is laser induced forward transfer. You have the lasers interacting with the metal oxide surface which pops the cells off below and there is no direct contact. That is also good for making a very fine pattern structures.

If we look at the most common modes, the extrusion-based and droplet-based, I thought it would be neat to compare. You want to make a tissue and say the tissue has a feature that is this size. It's good to understand where the limits are with the technology today. At least for me in the beginning my imagination was far ahead of the practical realities of the instrumentation. You can imagine making all kinds of things. You get in the lab and you say it's too big for this and too small for that. Resolution of extrusion printers today on the low end, the fine end, the finest feature size is about 150 [inaudible]. The droplet-based technology can go to the 50 micron. You can't hold the vision that you're placing individual cells and building something like a [inaudible] with every cell in place. The viscosity of the ink for extrusion-based printers, you are printing something pretty thick. Anywhere from 30 to six time ten to the seventh [inaudible] compared to less than 10 for droplet based. The impact, what you print it onto, isn't going to stay put or is it going to keep the shape you gave it? On the shear side of things, with droplet it is pretty high. I would say you can create a tremendous amount of shear and it's widely variable and is a good thing to measure. While some cells like fiberglass are used to being squeezed through small spaces, it's kind of what they do, something like a large hepatocyte is not too happy about it. Measuring mechanical forces like shear help you be successful.

On the ink side of things, there is a bunch of different ways to think about the bioink and the most common ones are really the cell laden hydrogels. That's what most people are using. If you have some kind of biomaterial, [inaudible] coupled with something to make it adhesive because it's not naturally adhesive, people are making hydrogels out of decellularized tissue matrix from particular organs of interest and of course there are synthetic ones the [inaudible] and things like that [inaudible] with salt or chemicals. There are polymer based ones, like [inaudible] those are going to be more the hybrid I was telling you about. They're going to be dispensed and made into a layer and maybe spray cells on top of a layer, but they are not compatible from a solvent perspective and temperature perspective on working with live cells directly. And then there are the more unusual but still very interesting people working with just cell suspensions or aggregates with very little if any biomaterial involved. In those applications the driver is to get the cells to interact with each other and touch each other which in a lot of solid tissue that is what you have *in vivo*. There still quite a lot of interest in that.

As we think about, you're going to make a tissue, what are the bioink considerations in doing that? You want to think about how cellular does this need to be when you use it. Are you trying to mimic a tissue function that requires all the cells to be touching, is there an ECM that needs to be deposited and maturation time has to be considered and will the biomaterial you're using withstand the time for that maturation to happen

functionally? The physical properties. You going to make something and use it immediately and you need to pick it up, you're going to have to work with the material the gives you the stiffness that allows you to move it. If you're going to apply any kind of pressure to it then that strength has to be there at the time it's manufactured, you won't have the luxury of waiting for it to get there on its own. Whether something needs to be leak tight. If you're going to print something and use it immediately as a barrier testing or to put it into a place where can't leak, like a blood vessel or bladder, then you would want that barrier function to be there immediately. Is that attainable or would you have to look mature it over time?

I think something all of us, at least the speakers that I know today, we spend a lot of time talking about physiologic relevance. I say when you bioprinted something you haven't finished, you just got something started. You can make all kinds of things but if they don't do what you set them out to do, it's a demonstration. I think that is really the interesting and fun part and where I believe we are getting to finally in the field. The papers are getting to be not about, I made something, they are getting to be about, I'm using this and look what it does, and I couldn't do this before. This is when the chills come over.

I thought it would be good to talk a little bit, we saw the pedestrian examples, the beautiful examples of inkjet printing. Here's what people are doing today. Very sophisticated system, mostly lab build, not on the commercial available side. They are doing things like this example they have taken a [inaudible] of fiberglass with or without that [inaudible] of fiberglass it printed cancer cells on top of it in an aggregate form, I'm checking my time to make sure I'm running on time, printed cells on top of it and they are looking at important things like the [inaudible] activity which is so important in cancer cells for their motility and they are showing when they print these aggregates on the wall as opposed to not doing that, they can see a great increase in the amount of activity in the [inaudible] cells. There's a lot of examples in oncology coming forward people are pattering vascular elements with tumor elements, patterning tumor with stroma, tumor epithelial with stroma. This is really an active area in breast cancer and other cancers.

On the extrusion printing side, I would say this is the area of printing that has gone wild. New products every day. New printers, new bioinks. Lots and lots of very good labs working in this space. Now we are beginning to see is the mode of making something, what you choose is becoming, the lines are getting more blurry. People are getting very creative and I see this the slope of creativity and you're going to see a lot of that today. Some mind blowing things where it is not just about making a three-dimensional structure initially but in a second step coming in and using cross-linkers that are already in the bioink. You can create additional fine structure and additional things in that three-dimensional space. That enables us to do things we couldn't have imagined before. Okay. No worries.

I will go as fast as I can. Is this ready? There is a little message box on top of my side on the screen. It's not there for you. Okay. We will go and I will ignore that.

I was going to talk a little about the example on the right because the kidney is something near and dear to my heart. This was so exciting to see. This came out of Jennifer Lewis's lab. What they did was take a material, they called them fugitive biomaterials, they're there to create a [inaudible] space basically, and then they're going to go away as soon as you put it in, you can use things like make something out of alginate and digest it away [inaudible], there are ways, to put something there and take it away so you can have a void space which is super clever. I think this is [inaudible] 127, they make this intricate proximal tubule and then they cast a gel around that and then the [inaudible] goes away and leaves this space in the gel they then seed with cells. This is a hybrid approach that allows her to get to something so fine in resolution and intricate that you could not do it printing it the other way. I think this is brilliant. The references here and on the slides at the end. Okay.

I thought I would show you a selection of the extrusion based printers on the market today. The way I see the printing technology developing, because these are mechanically somewhat easier to deal with. The operators can learn how to operate it. A biologist is not afraid to use this. It's not intimidating. I think that is important. They are out there in a very wide variety of costs and complexity, probably the more complex is this one over here, fully contained. And advanced solution system. And the smaller systems will fit into a safety cabinet and use them in the safety cabinet. Bioprinters exist only through technology partnerships like the Organovo center, you can get access that way or it's used in-house. The extrusion printers are winning the race. But I think in the future we will see a lot of combinations of the extrusion printing and some other technologies that helped overcome some of the limitations of extrusion printing. Everyone is on the job trying to make that happen because the potential is too high to let it be.

When you are thinking about making a new tissue, I thought it would be helpful to go through some thoughts on requirements in thinking about the tissue design upfront and figuring some things out before you actually buy the printer or get the ink and start. It is helpful to know if you're trying to make a piece of liver, a tumor, what are the target tissue functions you are trying to achieve? Because if you can let go of the idea that you're going to make a fully formed organ with all the bells and whistles, what is the one thing you're trying to get at and measure? And then get that down to its simplest components. That is where it is good to start. And what cell types would be required to achieve that? We can talk later about cell lines versus primary cells and that types of things. It does matter. Is there an existing bioprinter and bioink combination that would already be compatible from a resolution or geometry do you want to achieve or tolerances of the cells you need to work with the instrument and the materials you're going to use? And what is the requirement for future size? If you know you cannot measure or get what you need in a resolution of 150 microns, and extrusion printing is off the table for you unless you start combining it with new technology. And what you printing onto? That matters a great deal. Unless you're going to print something and then scoop it up and use it immediately and implanted. The way cells and materials

interact with the surface they are printed on to have a huge impact. Even as something simple like the dash you are using can make a difference.

You also have to think about how finished does this product have to be when I make it? Is it the example where the minute you make it you want to use it within the first 24 hours? So, needs to be complete. Or can you make it and then take care of it for a while and let it get to where you want it to be? The answer is you can and want to do that, now you have another order of things to deal with on the back end of how you care for the tissue, how do you feed the tissue, how do you analyze it in the end? If you are making something, putting a commercial hat on, if you are making something and you're going to hand it to someone to use as a product, what does the end-user really expect? What can you hand them that they can use? Because you can have a beautiful biological system but if they can't measure what it is a need to measure, you may have struggles ahead.

Thinking a little bit more about the print on demand, some additional considerations, for print on demand you're going to want to use that shortly after production. I would say less than 24 hours. Over 24 hours you have to think about how you're going to care for the tissue after the printing. Because they can consume a tremendous amount of nutrients when you're dealing with that much mass or cells. If something has to work immediately, you may require more cells of a particular type than you would if you could put some in and let mature over time. Physical strength might need to be high if you need to manipulate it immediately after printing. And the use of biomaterial I would say is much more common in these types of applications, you have control over the biomechanical strength of the tissue, you can make things that can be handled and manipulated well, whereas some of the other things have to mature and lay down the extracellular. For the sake of time I won't go through the other things.

I thought I would show a couple of highlights that I think are relevant in the food safety space. When we ingest anything, it goes to the liver, pretty much first. Anything in there. Because of absorption and extrusion through the gut I thought I would show a couple of examples there how bioprinted tissue are being used. This is the one that Ed eluded to the we did in collaboration with Ed and a graduate student, very talented Leah [inaudible]. What was done here is three different cell types that are important to the liver. They were used to make this core metallized tissue that sits inside a regular transfer plate. This format was chosen so someone could handle afterwards on a format they were used to using. For 14 days exposed to methotrexate or thioacetamide or vehicle and then some liver functions were measured and general toxicity markers and then importantly, histology was done on these after-the-fact. The bottom line and references are here if you want to dig in and read this, there are a couple of papers that have come out related to this work, the general toxicity of this until you get to the high concentration is pretty mild in terms of injury and what you're looking at here at the longer time points is starting to show with lactate dehydrogenase release.

What was interesting to us, because these have mass and you can pick them up and look at them and you can process them histologically, these are tri-chrome stains

where the blue you see, this is the cross-link collagen deposition happening in the tissue in response to the drug. This is a vehicle treated tissue and you see the large nodule of fibrosis, all these [inaudible] hepatocytes adjacent to that, and you see the pericellular fibrosis running through the tissue. These are phenomenon that until this work was done, you could really only see in the animal models and the animal models were not good recapitulaters of what was happening in the human situation.

This started a flurry of activity I would say on using three-dimensional models and trying to model not just overt toxicity but some of the more complicated endpoints that can occur when you ingest fatty food or have a fatty diet or eat too much McDonald's or eat a lot of things that are encased in plastic. There's a lot of work in the space. Cheryl Walker gave a beautiful talk at SOT about the epigenetics reprogramming that predisposes someone [inaudible] related to the exposure to BPA. That is worth looking at and having a look at as well.

You can imagine taking these printed liver tissues or other things like them looking at the compound effects of a normal liver. You can imagine taking liver and making it fatty and having it represent a different population and testing the safety of a drug or contaminant in that regard. You could also imagine integrating now with the coming forward of the chip system, the multi-tissue system, you can think about integrating things like this together and getting the metabolism by getting tissue together, liver, gut and kidney. There is work going on, our people are taking the pieces of liver and moving them toward the clinic as patches to facilitate metabolism and things like that.

I thought I would show the intestine as well. This is recently published work. Here you have a layer of intestinal myofibroblasts and then intestinal epithelial cells on top of that. And you see it develops this really nice architecture. The epithelial cells stay on top and are layered on top. You can see the staining here. There polarized as they should be. They will make [inaudible] and lysozyme. The green dots are [inaudible] cells in the layered structure.

These can be used for a variety of applications. Here they are looking at transepithelial resistance. This is what normal intestines range would be for TEER measurements. Caco-2 which is probably the most common cell line used is up here, if you put it in 3-D it gets better. If you make a printed tissue it gets better. The 3-D printed tissue is really beautiful here and in line with native tissue. And the fibroblasts only, [inaudible] they did directional transport looking at the movement [inaudible] through the [inaudible] transporters. And showed you get directional transport. Things like this are useful for transport, they're useful for tox, and we see here with exposure [inaudible] you see a biochemical response, you see loss of the transepithelial resistance. An uptick in LVH. A complete loss of prostaglandin excretion. Also, just overt histologic damage. This is how you can think about using things like this. In the basic safety arena. I would say the uses of intestine are pretty similar to the liver. You can look at toxicity, barrier function, and screening for things that repair or prevent these types of injuries. It would be very fascinating, especially with things like the study to see how they differ in the presence of a micro bio flow. You can start thinking about integration here.

We talked a lot about the good stuff and exciting stuff. And asked if there were challenges in the field. Certainly, I think the ones I would cite is the biggest problems or challenges is the raw materials access. You can have a beautiful tissue design and make a tissue but if the raw material going in, if the cells were not right, were not good quality of the right type of cell, it won't turn out well. You can't bioprint something into being good that wasn't good to start with. We have all done those experiments. You have to think about the compatibility of the hardware and chemistry you are using and cells you are using. Whether they can tolerate the mechanical forces or shear forces of the instrument. One of the things about [inaudible], it makes beautiful structures, but its epithelial cells get coded with it and they will stick to each other anymore. You have to think about things like that, the interface with the biomaterial and cells of whether that contact is going to prevent them from doing something later that they need to do for you to get function.

I think there are things like scale. It's challenging to make things with find tissue and challenging to make things with large tissue. If you think I'm going to make a whole liver implant, that's a challenge, how do you keep it alive while you fabricate something that large and how you take care of it after you make it? Those are the things we think about down the road and probably a good topic for the panel discussion.

I want to hammer home the starting material. I pull this out of the intestinal paper. This is native human intestine. These are three bioprinted primary cells. The one thing, this is expression profiling, things are really important. You see easily misalignment with the native cells. If you focus on these, you see important in the transporters in particular you see some good heterogeneity between individual donors. I think one of the things we have to overcome is that primary cells are great to use because they give you function, but the donor to donor variable is very real. It doesn't necessarily mean we shouldn't use primary cells, but we have to have mechanisms by which we characterize them and understand them and use them wisely.

For the future, we talked a little about the printers and the inks as we go along. Since we are short on time, I'm going to leave you with what I think is a new frontier. The bioprinting technologies are going to evolve, the bioinks are going to evolve, and I think the new frontier here is maintenance and analysis. We make these tissues and they are beginning to do some amazing things. Figuring out how we get the information out of that, but still allow the complexity to be there, I think that is a new frontier. That is something maybe Marc will speak to as well from his perspective. I will give a shoutout to my collaborators and also the pioneers that started this a long time ago.

Putting 3D Bioprinting to the Use of Tissue Model Fabrication
Y. Shrike Zhang, Brigham and Women's Hospital, Harvard Medical School and Harvard-MIT Division of Health Sciences and Technology, Boston, MA

Thanks for the introduction. [Inaudible]

I have interest in these companies [inaudible]. Our lab is more interested in developing *in vitro* models of human tissue and organs for two applications. The first is for drug development making drug screening more accurate and the second one is for personalized medicine, the second one is for personalized medicine for integrating with materials.

So, it's been interesting making *in vitro* models of human tissue for drug and personalized medicine purposes. Here is an example of a system we are having built in the past few years which is the automated multiorgan-on-chip system which primarily consists of three different components. The first one is device designed by housing the microtissues. And being able to put them together for integrated introductions and those kind of studies. The second part is bioprinting and biofabrication which is something I'm going to talk primarily about today. It's good now. The third part is, once you have the microtissues in place, we want to know how they behave or interact with the molecules.

So, we have some average and some different sensors that allow us to do continuous monitoring of the microtissues. The complexity involving the tissue, we know that human tissues or organs are complex and each of the organs have their microscale units which are complex involving multiple cell types coming together and often times you see them come together for function at the end. The question is how to really mimic the complexity of human tissue and how to rebuild the interactions between the multiple cell types in our *in vitro* models.

One of the earlier ways that people used this is for pattern techniques. You have a mask which selectively penetrates to light. If you shine light on the mask there is a light pattern going through it and if you have a photo active hydrogel it's going to put in different shapes. For example, the structures. These are easy techniques but the issue with that is, if you want to build three-dimensional structures [inaudible]. As Sharon laid out in the first talk, we have different types of bioprinted technology because they are a fully automated process allowing us to deposit fabrication tissues in three dimensions in a controlled matter allowing us to have more ability over the different processes. We have interesting exclusion bioprinting, we also have some other endeavors for example stereolithography and other techniques which I'm going to briefly lay out.

One of the earlier examples going to give is the multi-material rapid extrusion by printing system that was built a couple years ago. In this case we combined the outset of the multiple bioinks to a single printhead. What happens here is by controlling how they are extruded in the movement of the printhead, we can very nicely control how the different bioinks are extruded in a simple process. Each term we add a different material. So eventually it can simultaneously extrude all seven types of materials. Here is an example it printed the material in a single printing process. As I mentioned, it is a single nozzle multichannel system which is very fast because you don't have to switch. In a conventional sense where people use multi-nozzle systems, where you have to switch nozzles and materials then the printing and timing for switching the nozzles can

be as much as the printing process itself. In our case you don't have to switch nozzles meaning you can deposit the different materials in a fast way. We can print the different materials 10 times faster than an existing multi-nozzle based platform. Then you can print more complex structures, for example these slabs of multiple colors and you can print the human tissue or organ like structure with essentially as many materials as you want. From this movie we can see that the extrusions of different bioinks is pretty fast and continuous without any delays when you switch materials.

We can also tune the bioink we can [inaudible] as an okay level all the way from 75% to 100%. Here are some examples, a heart like structure and kidney like structure composed basically of multiple cells coming together with different color. We can see pretty nice demarcations between the cells in the printed structure indicating the multi-material single nozzle printing system. We can go a little beyond that for example in print now structures to mimic certain tissues. In this case actually printing the concentrations of the HAP to model a steel structure. It would show that if you see pretty osteoblast on top of the structures, they were able to respond differentially to the content. One of the recent examples in collaboration with a colleague, we were able to further print more complex structures for example in this case bone muscle interface. You can deposit for example different materials in different sections, or different particles in different parts to differentiate.

Here it shows some examples of the multicellular printing system and different ratios. For example, nanoparticles. You can get more complex structures essentially in the shape of the area for modeling purpose. After a certain time and culture, you can have a multi-material bone muscle. These are also demonstrated by nice genetic profiling or gene expression profiling with different expressions relevant to bone or muscle or interface.

We have also been printing by electronic devices for modeling tissues that are bioelectrical active. In this case in collaboration with a colleague we developed we mix the gels and based ink and were able to cover the tube with this gel matrix very nicely. Using this multi-material bioprinting system we can pass these electorally active [inaudible] depending on the application. Since its bioactive component and gel component we can put cells on top to basically allow better intercellular interactions providing the electrical conductivity.

One of the recent examples is you can also print these patterns in different ways and while you print these patterns you can see so the [inaudible] can align according to the patterns that you print. They can form this by [inaudible] and you can use it for printing purposes and how they are affecting the behaviors or other parameters. Another example of multi-material extrusion bioprinting is that we have partnered with a partner in Europe in producing a bioprinted model. We used the material printing system with two. We printed a mini brain model which contains [inaudible] inside. Into the cavity we printed another cell type and that we can start looking at how the glioblastoma cells are interacting in this mini brain model. We first showed with three-dimensional were able to promote the gene expressions of microfiche and also glioblastoma cells. You have

different models and look at how the different cells come together to interact with each other, for example we can show the brain tumor cells are attracting into the mini tumor region and being able to prime these macrophages into phenotypes. And in turn promote the proliferation of the brain tumor cells. We can use the mini brain tumor model to explain what chemotherapeutic agents and other agents as well. So, that is the extrusion bioprinting.

There's also a technique which is very [inaudible] in this case we can shine a pattern of light on this solution, a layer of the gel. What we do is we can move this up to expose another layer of the solution that is on crossing. If you repeat the process you can imagine you can eventually form a nice 3-D structure of a complex shape. However, the issue with the conventional stereolithography means the can only be one reservoir. Can only print one material at a time. We had [inaudible] strategic way to do this. [Inaudible] now you can infuse different bioinks into the chip device to achieve multi-material printing.

Some examples here, we start with one bioink and you can do a cross-linking procedure, you can lower down the stage for the next layer. Now the second material, if you repeat the process again and again, we can very nice print structure three-dimensional materials. Now more complex tissues, for example model a tumor model which contains complex [inaudible] or disordered structure within the material model and further created more complex multi-material for multicellular type interface structures we can model for different applications. That is the multi-material printing applications that we have been able to try to develop in the lab.

We have also interest in generating vascular models because they're one of the most important components of essentially any tissue type. As Sharon mentioned it's a technique called [inaudible] you first bioprint the fiber structure that is serving as the fusion template. On top of the fiber you either print or cast hydrogel matrix. By cross-linking hydrogel matrix and then by removing this template, you can create hollow channels to model hollow tubular structures. This initial technique we were working on was based on active templating which could be physically removed after bioprinting to create channels. We have also adapted technologies based on [inaudible] templates, you can basically [inaudible] the liquid out to create channels inside. You can put cells inside. Here what we did is we can seed endothelial cells in the channel and allow them to attach and proliferate on the channels to eventually form a [inaudible].

Here a couple images showing that they conform nicely on the surface of this microchannel. If you culture these channels long enough, they can also form secondary project forming this multi-tissue model. It shows [inaudible] if you don't have the layer but only a channel, [inaudible] if you have the same channel the fusion of the same molecule was much slower to demonstrate the functionality of the cells to basically flow down the fusion molecules.

Not only can you create a vascular model, also you have the other cell types, now it's creating a vascularized tissue model. One example that we have established is the

vascularized liver model. If you infuse for example nothing into the microchannel, we can see how the molecules are affecting the integrity of the cells but also affecting the [inaudible] and importantly, diffusion-dependent manner. You can see an effect. This is very important because you can never do this in a conventional 2-D culture. If you put it on the plate every single cell is going to receive the same dose at the same time. Which is not really the case in the human system because in a human if you have [inaudible] molecules flowing the going to fuse out. Depending on how far away the cells are from the base cells that have the fusion factor. Farther away the cells, our model can [inaudible] more accurately recapitulate how they are acting in the system potentially. [Inaudible]

Not only can we model the vascular structures we can also model vascular disorders. Here we created a model of thrombosis. What's we have created the microchannels through bioprinting through clotted blood in, we are forming a very fellow type model of human vascular thrombosis. We can see that they're very similar to each other. Model has the tissue and cells and replicated cells in the middle which are similar to both structures. As I mentioned not only can you have the cells or the clot in the channels, you can also have a secondary matrix, in this case we incorporated fibroblast into the matrix. We can look at how the fiberglass is interacting. Now you can look at more complex interactions between the different cell types [inaudible] there are more vascular structures that are hollow in the human system.

As Sharon mentioned in our case the models for breast cancer are sacrificially bioprinted structures. In our structures we see the breast cancer cells into the microchannels, and we did show the cells in the microchannels which proliferate and once they become [inaudible] they were able to invade from the channel into the surrounding matrix. And invasive process. Here a couple of images showing the process. The outward invasion of the cell from the channel, we also show the inward aggregation of the cells in the microchannel which is very similar to where you have inward aggregation of cells and outward migration of cells in the surrounding matrix or tissues. The cells were able to keep the viability of this culture period and we did show in a preliminary example that the cells seem to express more [inaudible] to capitulate the process. More recently in process with a private space company we were able to send some models into the suborbital flight and hoping we can study for example breast cancer expression and space conditions for example microgravity and other conditions.

So, an example I showed so far are hydrogels. These are structures that we used two bioprint. The advantages of hydrogels is they are very [inaudible], but they are not stable. You have to use them pretty much immediately. Recent example, we thought was interesting, we created these paper-based tissue models based on bioprinting. Optimizing bioink formulation and the matrix formulation we were able to print, for example, these paraffin-based bioinks into the cell of matrix. To create different structures of the bioinks. By doing this, it's like a sacrificial printing, creating the biomatrix, we can create this matrix-like structure resembling paper. These papers are not only paper, they actually have bioink in the middle. These are [inaudible] by

heating it up we can liquefy the bioinks and remove them from the paper devices to create channels and the channels are becoming usable. The advantage is there low-cost. Cheap. They have essentially very long shelf life. Once you create the devices they can be stored for as long as you want. Once you use them you can rehydrate them and put cells inside. You can rehydrate the device, and infuse, for example, endothelial cells into the microchannels and put a second cell type, for example breast cancer cells, into the surrounding matrix so you can create breast cancer models. So, these are very interesting low-cost tissue models that we can created using bioprinting processes.

So, another printing strategy that's [inaudible]. So, what I mentioned before are those [inaudible] bioprinting based tissue-like structures having vascular channels inside. So, those are more amenable for creating vascularized tissue models. But now we are creating standalone vascular or tubular models that are mimicking different tissue types so in this case actually create our own novel system which is [inaudible] and if you infuse or deliver the middle [inaudible] interior and exterior in a single step create hollow structures using bioprinting. So. these are hydrogel of [inaudible] to eventually create biologically relevant tubular structures that are standalone.

So. some examples basically by tuning the sizes of the models or layers or different flow rates of different layers you can create hollow tubular structures across a wide random sizes [inaudible] to emulate the different tubular structures that are present in human system across a wide range and using bioprinting of course you can pattern these hollow fibers in two dimensions and three dimensions and essentially any way you want. And here is a printed structure and the structure is hollow and composed of single hollow fiber that we were able to print and [inaudible] you can put cells inside so this is actually [inaudible] tube that was cultured for 21 days, so we are able to create biologically relevant tubular structures and vascular structures in this case as well.

So, another example that we have recently found is hollow tube that are able to be chilled for permeability and we have optimized our bioink practice and showed that by choosing properly the bioink, we can actually choose a permeability of the hollow tubes in the way that we want so depending on if you're using [inaudible] as a bioink, you can have different permeability profiles. For example, if you use linear [inaudible] as a component of bioink, you can form very dense structures of the tubes so it's giving you very low permeability. But if you do the same thing but use the [inaudible], then it's much porous wall structure, giving you much stronger or higher diffusion profiles. And by [inaudible] all these different parameters, we can essentially model all the permeability profiles of different vessels or blood vessels [inaudible] in the human system for modeling purpose.

And my example is that we can not only model the vascular structure, I mean, the blood vessels, but also the [inaudible] vessels, because in human tissues it is not only [inaudible] blood vessels but also the [inaudible] vessels that are not [inaudible] but they are [inaudible] in a single direction. So, using this bioprinting strategy, not only can we create hollow [inaudible] structures but we can also create the tubes that are

[inaudible] on a single and now we can apply pressure and can [inaudible] from the tissues.

So, one example with that we can create these tumor on a chip devices by bioprinting a pair a single [inaudible] blood vessel but also a single and blinded [inaudible]. So, we did show that if you have we knew have one channel that's the blood vessel versus if you have two channels, blood vessel and [inaudible] you have very different diffusion profiles of the molecule as we would see in the human system and you can actually incorporate, for example, breast cancer cells into the matrix to model the breast cancer. And then we did show a difference between 2-D and 3-D cultures. 3-D gives you [inaudible], but more importantly, there's also a difference between single-channel and multichannel. With one channel and two channels you have very much difference diffusion profiles of the molecule. With two channels you have a [inaudible] getting you potentially different dose [inaudible] same model. These are more basic and more [inaudible] models that are creating these circulations to allow you to accurately screen [inaudible] the tissues or cancers *in vitro*.

So, now we can also, sometimes we'll combine this hollow tubular structure like systems with the [inaudible], as I discussed in the beginning. And the rationale for that is for a lot of the tissues in the human system they are not uniform. So, take vessel as an example. Coming from the arterial structure, so having endothelial cells in the middle, and muscle cells on the outside. Going to the capillaries a single structure only having the cells as monolayer. And coming back to the multilayer structure. So endothelial cells plus the smooth muscle cells. By combining the novel system with our multimaterial printing system we can actually now digitally tune how many layers of what you can put into the hollow tube structure and turn on or off out layer to switch between single-layer and multilayer structures. So, for example, you can start with multilayer structures in the beginning and at some point you can turn off multi layers and now it's becoming a single layer structure and at some point you can turn on and become another multilayer structure. So, you can switch between one single, one and two layers.

So, a few examples would be basically the construction of multilayered complex tubular tissues *in vitro*. So, for example, we can create ureters that are having [inaudible] cells on the middle layer and smooth muscle cells on the outside or can create vascular structure having endothelial cells in the middle and having smooth muscle cell on the outside, giving us the capacity to model the complex tubular structure in the human system *in vitro*.

And another application for the vascular walls is you can create vascularized tissues in this way as well. For example, what the bioprinted vascular [inaudible], so there's an interstitial space between the microfibers. And you can see another cell type and now it's becoming a vascularized tissue model as well. So, as an example, we created the vascularized myocardium *in vitro* by fitting cardiomyocytes into the space of the vascular [inaudible] so we can also by tuning the alignment of the fibers, we can also tune the alignment of the cardiomyocytes, so we can eventually form a volume of

aligned cardiomyocytes interspaced with the vascular structures, very much mimicking the [inaudible] structure. So, here is the movie showing that process. Basically, the endothelial cells are [inaudible] labeled and you can see their alignment and the fibers, and the cardiomyocytes are actually long labeled and you can see their synchronized and spontaneous contraction of this little microscale vascularized myocardium model. And cardiomyocytes were able to express their biomarkers; for example, [inaudible] and eventually can for example place these little bioprinted vascularized myocardial model into the bioreactive system for continuous tox screening.

I think that's going to be the last scientific slide which is our [inaudible] and bioink and bioinks are important because they have to first support the bioprinting process, so being able to form shapes with bioprinting, but at the same time they have to support cell function after bioprinting. So, we have been really interested in developing multiple bioink formulations to do that. So, for example is the shear-thinning bioink which allows us to [inaudible] bioprint the tissue structures using [inaudible] bioprinting, and more recently we have developed technologies for printing [inaudible] molecules in an indirect way. So, for example, we print some molecules that are of interest, for example collagens or [inaudible] those molecules that are important in the [inaudible], they don't have enough mechanical strength, so we can't [inaudible]. So, what we can do here is we can combine them with the molecule called alginate to form [inaudible] bioink. And if you use the [inaudible] bioprinting system [inaudible] the middle and cross [inaudible] from the exterior and initially form the shapes of the printed structures in a single printing process to basically structural stability. Once you have the structure you can print and actually do a second cross-linking for the [inaudible] molecules and their structures and what you can do is you can use [inaudible] to wash away the these cross-linked, physically cross-linked alginates, so now, if I shake hands the matrix almost made up entirely your [inaudible] in an indirect manner. So, more recently, we have done this for example [inaudible] other [inaudible] bioinks which gives you the capacity to promote cell stretching in bioinks that you print that are forming [inaudible] after printing processes.

I think that's pretty much it and then we have websites which have all the papers I discussed today. If you're interested, you can look at the website for more information and of course the agency and the members.

Edward LeCluyse: We have time for one or two clarifying questions. Thank you so much. So, Betty, do we want to adjust the schedule because of delays at all because we are at a break point now. Be back here at 10:30 a.m.

Edward LeCluyse: It is my pleasure to introduce our next speaker, Jean-Louis Klein. And by way of additional background, I've known him for a number of years. Jean-Louis has been involved for most of his career in development of novel physiologically relevant human-based systems and more recently in early drug discovery and development human disease relevant models and so some of that will become evident during his presentation as well as how they've begun to apply or insert this new

technology into their drug discovery and development processes. Thank you. Jean-Louis?

Uses of Bioprinted Liver Tissue in Drug Development
Jean-Louis Klein, GlaxoSmithKline, Collegeville, PA

I will talk today with about how we will incorporate this new technology into the drug development. My title mentions in fact the talk would be much more general than that but what we learn a lot of the new model 3-D printing model using the liver. So as mentioned I am an employee on the GlaxoSmithKline.

I will start by explaining the process of drug discovery and why we need this new model and when how we use. The drug discovery is a long process and started with targeted discovery. We need to identify which target we want in the disease and we do that by either looking at the what is published or are now more and more using genetic data. This we call *in silico* approach. Then when we identify the target would need to validate that because is not because someone published a paper saying how that is involved in the liver disease [inaudible]. In addition, a target in the drug should be a target that not only is involved in the disease but manipulating this target will [inaudible] phenotype. And when we are committed to the target, we go to what is called the lead ID and that's where the high-throughput screening takes place. I mention that because when I talk to people they focus on the high-throughput screening and think the development process is high-throughput, then clinical trial. A lot of work before and even more work needs to be done after. So, even if the model is not fitting for high-throughput screening, we can still use it.

Once we identify a lead with the screen or whatever method that's not close to a drug so we need to improve the quality of the molecule to become a drug. We need to improve the potency of the molecule and improve the selectivity of the molecule and need to improve the solubility and so on. So, it takes a couple of years to really improve that molecule to become something that looks like almost a drug.

And of course, it is the ADME and the toxicology. When we have something like a drug, we have to make sure we have to know the half-life of the molecule. We have to make sure we know how this molecule is metabolized and so on. And you know a lot about toxicology in PKPD.

And that's where the clinical would start. We can divide the clinical aspect in three phases. And first phase is to focus most of time on the toxicology, do we know is the molecule we want to put in is toxic or not and that's phase 1. And if the toxicology is acceptable, we go to phase 2 and three and start to address the potency of the molecule, is it free walking and [inaudible] not only compared to a placebo effect no treatment that sometimes there's a drug in the market for that application is to compare to what is in the market and has to be better. And better sometimes can be as good as the molecule in the market but much cheaper. So, that gives you an idea of the drug

development and as you can see also on the bottom it takes a long time, between 10 to 15 years to start from the target ID to a drug.

And not only it takes a long time but it's a very inefficient process; in fact, most of the molecules we never finish as a drug. As you can see on the table, the candidate molecule in phase 1 the toxicology molecules 10% of them would become a drug. 90 percent will fail. So, it's a very inefficient process. And of course, when we move up the different phases, it's a little bit better. Phase 2 16% [inaudible]. And even at the phase 3 [inaudible] large clinical trial when we [inaudible]a lot of people [inaudible] two or three years to do that costing more than $100 million, still only half of these molecules would become a drug. So, as you can see it is an inefficient process. Most of the molecules will fail and when we looked at why this molecule would fail, why it's not working, that is the second table, phase 2 or three, the main reason is because of lack of efficacy. The molecule is not working as we expected and still have the problem of toxicology or toxicity is a problem of safety and phase 1 is mainly toxic aspect. So, you can see, the main reason why the candidate molecule will not become a drug is because in clinical trials the molecule is toxic and not efficacious.

What does it mean? It means that all the work we did before with clinical trial don't translate into a human. Then, in the introduction, you hear about predictions that is a problem during the preclinical phase. The models we use don't tell us what will happen in human and we blame the mouse model. mainly because it's a kind of last step, and that is not good, their heart is not good, but I'm told the all models we use before including *in vitro* is not the proper model. We use them because it's easy to use and convenient and cheap but is not predictive. We need to change that is a requirement. We cannot keep going trying to develop drugs that will fail most the time. And a lot of people, and probably rightly so, blame the rodent model model that we use and you can take almost any kind of disease you like, all type of toxicity, and I'm sure you'll find a paper saying that the mouse model is not predictive or that rat model is not the best model to use to try to guess what will happen in humans. So, we know the mouse model is not working and we need something else but right now we have nothing to replace it, so we keep using it and we need something much better. And we mentioned time to time this model we are using are not really predictive, we also too often don't have a study in the very vigorous way. And I'm sure that also plays a role in the fact that we don't predict what will happen in humans.

And again, if we look at what I call historic model and we keep using them and as I said animal models fail and we know why, in fact, if a mouse is a mouse the physiology of the mouse is different from the human. The metabolism is very different from a human. We use the mouse or rat to try to predict obesity or metabolic disorders. Why do we do that, who knows, if the animals are so different? We got misled by this information.

We know also that most of the disease or toxicology is different in human and rodent. Again, we know that, and we keep using it. And even worse, when we use cells, [inaudible] cells, when we use is not a surprise [inaudible] 40 or 50 years on plastic

tubing and nothing to do with where they are coming from. They are called human cells, human liver cells, and it just come from the liver, from tumors, and so many [inaudible] even at the chromosome level, but in the past completely different. These cells have been selected to grow fast on plastic in 2-D and not been selected to be predictive of what will happen in humans and again we also picture this into a medium that was developed for convenience. [inaudible] developed this beautiful medium called [inaudible] that most people use because it is convenient. You don't have to change it every six hours. It is convenient because it pushes the cells grow quickly, but again, physiologically it doesn't make sense. How man people do you know that have [inaudible] of blood? [Inaudible] when you do a test you don't have that. [Inaudible] developed this medium because he wanted the cells, to change the medium only twice a week and he wanted these cells, these tumor cells, which are known to be dependent on glucose to grow fast. That was the purpose, and he did a very good job. But again, it's completely irrelevant.

And the way sometimes we design runs or analyze the study can be much better and we need also to push for a much more vigorous scientific approach, kind of going back where we were doing 20 years ago. I think we tried too much to cut corners, to save money, to go fast, we pay a huge price for that, because I think we neglect the quality of the science. Again, and as you know, most papers now that are published, it is very difficult to reproduce what is published and from academia or business. That, we need to do much better on that.

So, when we look at the model for we would like to do again, I say my ideal preclinical model, of course, my ideal model would be have a small human [inaudible]. That will not happen. So, try to be also realistic. So, as I said, one of the first requirements, we want human cells and human primary cells because we know cells that are on plastic are different than tissue they are coming from, so we want human primary calls. We also want or would like the cells we put in this model keep the phenotype they're coming from and that's where you have to be careful because it's not because the cell is coming from a diseased liver or diseased tumor that in that model that cell has the diseased phenotype. Because again we know [inaudible], so yes, it may come from the diseased liver but in that condition, in fact, the cells may reverse to a more healthy phenotype. So, that's where we have to be careful.

3-D organization [inaudible] we so many papers show 2-D versus 3-D is two different inaudible], even if exactly the same [inaudible] populations. The [inaudible] affects so much the phenotype of the cell that's a fact for most. Multicellular model is what we love because we know that even if we look at the phenotype of the hepatocyte [inaudible] that, in fact, may be caused or [inaudible] by the cells in the liver. So, if we have only hepatocytes, we may miss only the signal pathways that in fact are coming from [inaudible] cells. So, if we can have that, we will be very happy. And as you can see what I'm trying to describe here looks like 3-D printing model.

And we mentioned the flow, also, is critical, because again, it's what happens in humans and the cells are so different from the recreation. You can see on that

histology slide the heterogeneity of the tissue. When you look at a slide like that with liver, would liver, you can see that even hepatocytes don't always look exactly the same. So, when we have a 2-D culture, when all the cells look the same, we know we are not recapitulating what is going on in the liver. And one critical aspect, and Sharon mentioned that, the technically reproducibility. And I want to mention here that it's a source of variability of the technical aspect that we want to minimize and then the first bullet point about human, that we want to keep that variability. We want to keep the donor-to-donor variability in that assay. And that's what I mean also by keeping the original phenotype. We want to keep the human variability; we want a minimum variability from the technology and even more important that variability has to be able to be possible. Again, that's what we will compare, we have to make sure when we do a screen on day one it would be the same or similar data on day two.

Multiple readout, also. I will elaborate a little bit more about, but Sharon also mentioned. A phenotype is just not one gene going up or down. It's just not one enzyme that activity decrease or increase. It's a result of multiple effects and if we just use one readout, we miss a lot of information. And again, I'm sure that Marc will tell you much more about that. But to me it is critical, the complexity of the model goes with the complexity of the readout. Try to get information as much information as we can when we use this model. Again, these models are sometimes expensive and gave us a lot of information so we should try to get more from that.

So, I'm talking about the ideal model, but we know the model is a model, and has a limitation. We know that as a fact. So, the question we have at GSK is how we will know the model we [inaudible], one part of the organ, one part of the disease, and how will we define that, how will we know that? Again, we try to develop a strategy that will be based on a science approach, not because the model looked good because it's a beautiful picture behind the model. We want to be sure we have the rational approach to say, here is the model we want, and we take that model and it does what we need or doesn't and if not, we don't use it. And in fact, that concept is called domain of validity and I like it and it was hopeful for the biology by Scannell and Bosley in 2016. I think it's a brilliant idea. The domain of validity came from physics. If you, I know almost nothing about physics, but I know some law in a classic physic would not apply to the [inaudible] physics. [Inaudible] will have a different [inaudible] than electrons around the nucleus.

Similar in statistic when I know more. I'm sure you all know that you can use some calculation method only when the data are normally distributed. That's the domain of validity. You can use that approach that formula only if the model is of abnormally distributed. So, we want to do the same thing with models in biology so the domain of validity of a model is the type condition when the model will predict what will happen in humans so the model would help us to delineate when can be used, when it can predict what will happen in a human and when should not be used, that's equally important. Don't use a model because you have it. We have to use a model because it will predict what will happen in humans. That's what we are after.

So, it’s easy to say. It’s a little bit more difficult to do. So, we developed that strategy to say the approach will use when we evaluate the model. Here what we should do is here is a validity of the model to consider and can we use it or not. So, we start with the toxicology and that's what you want to use the model for and that's a starting point. And then we have the disease, and that needs to be very defined, I’ll tell you on the next slide what I mean by that. We look at what are the characteristics of the disease. And again, I talk about human, not rodent, not any animal. In human, what will call that liver a fatty liver [inaudible]. And same thing for toxicology. Once you have these characteristics, then, you can start talking about models.

So, we don't start the with model, we start with the disease, but based on the characteristics you want to mimic, you can already have an idea of which model you want. But then if you want to talk about a hepatocyte, it would be a good idea to have some hepatocytes in your model. then when you have the model, you have the characteristics, you start testing your model. Can you induce the disease, or can you reverse the disease? That would be the ideal for us when we try to identify drugs. Again, we have to reverse the disease phenotype. At at least we have to show that the model will induce a disease, because when we work the target identifications, we have to show that the model will induce the disease. Because when we work many of the target identifications, we have to show that these targets are involved in the disease.

You [inaudible] the model, then you compare the data you obtained from, again, your second bullet point there, from the characteristics that is not in human. And only then you decide if this model can be used or not. You decide your domain of validity and what is critical here is you define further targets you want to look at. Too often I saw papers when they use the model and [inaudible] would can be common to the disease. And they said oh, I have a liver model because my cells make [inaudible]. No, is it enough to say that [inaudible] make a liver? No, if the characteristic of the liver you want is [inaudible] cytochrome, you define that first, and now you look at what looks like a liver. And very often it’s not one characteristic, but it’s a set of one. There is something absolutely required, and something would be better. But you define first what you want, you don't define after you look at what the model can do.

When I mentioned to define the disease of toxicology you want to look at, that's what I mean here. If you go to [inaudible], you'll never be able to find a model that is predictive all of that. If you want to model that will predict all the human toxicity good luck with that. You will not probably find it. If you want a model that will recapitulate any type of tumors, you will not find a model like that. So, narrow down as much as you can and what you are after if looking for a model that will be liver disease is more refined, you know the characteristics of what you are looking for. If you look for a model of the triple negative breast cancer basil like subtype, you narrow down that a lot because this type of cells are very specific characteristics. That's what you can do that, use that to define the [inaudible] characteristics.

If you narrow down also enough, you already have a suggest what type you need in your model. Again, liver [inaudible], you need hepatocytes. But you probably need also

other types. Again, maybe [inaudible] may not be a bad idea, [inaudible] also may be useful to have. So, again, based on the disease you narrow down you already have to guide you what kind of model you will use.

And talking about characteristics of the disease, what kind of characteristics. Again, you can talk to hepatologists, of course, and if I look at the slide here, what I look at, and you will have probably some good ideas of the characteristics. But it's not always the case. There is still a lot of data that will help you identify the key characteristics. And most of the disease or toxicity have probably gene expression profiles by now, and this can be very useful you just have to be careful about the quality because sometimes people call a liver, a healthy liver or a diseased liver without checking for the pathology. This is data that are the most available around any disease.

Pathway. If you talk about fibrosis, liver fibrosis, we know that [inaudible] is involved and that could be useful in your model, can you induce the fibrosis if you [inaudible]. If that doesn't work, you probably don't have a fibrosis model. And histology. I love histology and it gives you a lot of information and gave you information at the cell level or even sometimes at the organ level. It's a powerful source of information you have a lot of information there and if you look carefully [inaudible] heterogeneity of the tissue. Even if you have a fatty liver not all hepatocytes are full of fat. If you have an [inaudible]-infected liver, not all hepatocyte may be not affected by [inaudible]. It gave us a lot of information and very useful information that too often we ignore because we don't know what to do. And I put on that list we take a look and I have probably a bias about the liver as you can see. But whatever model [inaudible], you can look at [inaudible] activity and so on. Then what characteristic you are after?

As I say you will have to challenge your model. You will have to test it, so you need tool reagents to do that and being a pharmaceutical company, you can expect that the first thing we put there is chemicals. We have of course a lot of chemicals and unfortunately we have also chemicals that went into humans and we know what this is doing in humans because of toxicity bad for us but it can be very useful to build a model because we know exactly what kind of toxicities develop in humans and you can use that same molecule to test your model to see if you could recapitulate that toxicity in the model you are after. And again, sometimes you're working on the model that there is already at least one drug that works on it. And you should test that drug in the model and see the model is efficient in your model. It's a good way to test models.

But chemical is not the only way you can do and right now genetic tools [inaudible] the gene and have to be careful when you do that and you can [inaudible] gene, you can now modify the gene by itself using CRISPR or TALEN. And that's a huge advantage of 3-D printing model you have access to the single cell first and can modify maybe before you put them in the 3-D printing model, so that is a very practical aspect we use 3-D model for. And it's not limited, you can use anything you like, bacteria, virus, and so on. And I mention again the medium. It's a critical path and you can change the composure of the medium and if you take a piece of liver, if you add a lot of glucose hepatocyte should react. Most of the cells will react. And that may be [inaudible], but

also, it’s what is going on in humans and there is no limitation on the way you can test the model.

Readouts. I want to mention, and Sharon mentioned, for the readout it's critical, so the first thing, the readout should be based on the characteristic of disease or toxicity are looking for. I put the example of fibrosis and one of the major characteristics of the fibrosis is collagen deposition and the first thing we measure is the amount of collagen because we try to correlate with what is going on in human with the disease. Same thing with mitochondria, some compounds will affect the mitochondria, in this case will be part of the readout. We would measure the mitochondria activity number and so on. And again, as Sharon mentioned it's really to me it's useful to go not only after one readout but multiple readouts.

And now, with the computer and so on, here it mentions an example which one image you can measure more than 200 features, it is amazing the amount of information you can get from one image and I put an example not the liver, it’s muscle cells, we just stain with four different reagents and measure the features. And we gave it to a statistician and [inaudible] back to 20 features and with each feature, 20 features, he said if you give me that profile, I will tell you which cells you put on your [inaudible]. It's a very powerful, again, readout and interesting when we tested for [inaudible] that compounds had on mitochondria we [inaudible] mechanisms of actions [inaudible] with these molecules because they can induce and change the mitochondria activity and do much more of that and it can affect the size of the cells or affect other organs. But also, we can also group molecules that have the same mechanisms of actions by looking at the signature. Just 20 features of one slide, it is really powerful, and again, when we do something like that, we can also measure what was released into the median and add that and do gene expression [inaudible] on this slide to see some change and so on. So, again, we use complex model, we should use complex multiple readout because there's a lot of information there, very useful information.

So, now, when we have a complex model what we try to do is to use it and I want to stress also that the cell phenotype is a result of the genetics and the environment. I know the technology [inaudible] genetic on [inaudible] and so on, we can do it but a gene expression is not equal to phenotype. So, it’s part of it, the genetics affects the cell phenotype, but the environment, also, is equally important. We talk about 2-D versus 3-D, and we heard about the ink used has a strong effect on the cell phenotype. Even one paper showed even you just change the amount of one molecule, like collagen, and you can change a phenotype using the same molecule but because of the structure [inaudible] is slightly different more or less water may affect it other cell types. Again, often it's enough the medium composition has a strong effect, and flow so also affects the cells.

We are very excited about 3-D printing because some advantages. Don't need to compare 2-D versus 3-D, and the fact we can reconstitute also a model using primary cells and I mentioned reconstitute because it's an opportunity to modify the tissue. If we want to know if a cell [inaudible] hepatocyte you can make a tissue with [inaudible].

Or you can modify also, the genetic [inaudible] affect the hepatocyte. So, you can play with the system, that's the beauty of the 3-D printing you can play with the primary cell and depending on questions you want to ask, long-term [inaudible] but if we compare 3-D printing tissue primary tissue, the precision-cut tissue slides, most of these 3-D printing will live and be functional much more longer than the tissue slides. It's an advantage and we need that because toxicity you can see in 24 hours and some toxicity would take a week before seen something and same thing compound activity for disease. The fact to be able to modify the genome of the cell, the primary cell, and reproducibility is a factor as Sharon mentioned because you can put the cells [inaudible] same force and was a good model, a very reproducible model.

The challenge, on the other side, we have for us, we don't have yet a choice of a lot of models and again when I mentioned the domain of validity, one model, one question, if I have a second question maybe I don't have the model to answer that, and of course the pharma, I would love to have the choice of 20 liver models and it will come I'm sure. And Sharon mentioned that it's a quality of the primary human cell that's absolutely critical, as Sharon mentioned if you use poor cells, you'll never get a good model. That's a fact. These cells are not well defined, and we have example in the lab when we have hep C liver and of course when we look at when we did some histology that was in fact [inaudible] was clear and it was mislabeled. And of course, if we use that as a hep C model of the liver, we would be completely wrong, and again, the quality of the cell is important.

We mentioned to have [inaudible] ideal one, now, the challenge there is to get the cell from the cell [inaudible], not from different donors, because if you do that you add another level of challenge. And I mentioned live-cell imaging, I love imaging, it's a powerful system, a lot of information from there, but of course you can imagine now we are talking about the a piece of tissue and we can image the cell with that tissue without destroying it and this tissue involved change with time and it is important to be able to see them changing. And that in fact is why we like the 4-D, 3-D plus time, is critical but it's challenging right now. And the technology is coming here.

So, I will end by taking that slide to express where and when we should use new models like 3-D printing tissue. I would say almost everywhere in the pre-critical phase maybe an exception right now because of the throughput, is the high-throughput screening. Again, we need to screen 1 million compounds, very often the screening models are not yet there as a throughput. But to me I would say it's a detail as someone that works [inaudible] and it is critical to identify targets, and for that I don't need to screen 1 million conditions or 1 million targets. I need a few targets, but I need to show that if I manipulate these targets, I will reverse the disease phenotype. If we start with the wrong target, whatever we will do after that, even if we do a fantastic job in developing, identifying, and developing molecules, if it's a wrong target, it will never do a good drug because it's a wrong target. You can see I need predictive model before the high-throughput screening and after. Again, if we identify leads from the throughput, I want to make sure it is a correct lead. So, if we cannot use predictive model like 3-D printing model for high-throughput screening [inaudible]. If we can use

this model to validate the leads, and use this model, this predictive model, to optimize the leads. We want to make sure that it will work in humans.

That said, also, I don't have to use the same model from the beginning to the end because the question may change. Of course, I have probably different questions about target ID and validation than talking about toxicology. It doesn't matter. We can have several models for the liver I have no problem with that as long as we are predictive. And that is a key: we need predictive model. The throughput, to me, is a question number two. The first one is, is it predictive and can I trust the data that I will get this model, can I trust this data will tell me exactly what will happen in humans? That is what we are after. And I will stop here, and again, thanks a lot of people, mainly Ed and Sharon for the[inaudible] discussion that we will have about these predictive models, and a good number of people at Glaxo when we have similar discussions. Thank you.

Edward LeCluyse: Any questions? Great overviews of the field in general. So, happy to introduce the last but not least presenter for this series Dr. Marc Ferrer, who is a Team Lead in the NCATS Chemical Genomics Center and the Director of the 3-D Tissue Models Laboratory at NCATS. And Marc, I have to admit I couldn't help but notice you went doing your undergraduate degree in Barcelona, Spain, to Minnesota, so I'm just wondering what did the brochure look like? Actually, it's a great university. So, Marc has been involved in the implementation and the uses of these 3-D tissue biofabrication techniques for disease modeling and drug discovery. Thank you, Marc.

Biofabrication of 3D Tissue Models for Disease Modeling and Chemical Screening

Marc Ferrer, National Center for Advancing Translational Sciences, NIH, Rockville, MD

Thank you, Ed, for the introduction and Margaret for the invitation to the Workshop and a huge thank you to Sharon, Shrike, and Jean-Louis for the wonderful introduction to the bioprinting techniques and Jean-Louis especially for really like explaining why we're interested in 3-D tissue models for drug discovery and drug development.

And it was not on purpose, but I have no conflict of interest, and my first slide is actually Jean-Louis's last slide and I'll spend a minute and explain a little bit what NCATS is, which is NIH Center for Advancing Translational Sciences. And within NCATS the intramural labs at NCATS, we have assembled the capability of traditionally having been in pharma companies in terms of discovery and drug development and assemble them to make them available to the academic community so we have several programs that go from the same drug discovery pipeline that Jean-Louis showed and from the target discovery, we have the RNAi HTS group. And we have the in NCGC which NCATS genomic branch which has high throughput screening capabilities and medicinal [inaudible] capabilities. We have another program which is involved in lead opt and IND-enabling studies, and many of you may be familiar with the Tox21 program, the collaboration of many government to model

toxicity using *in vitro* assays. Most recently we had stem cell differentiation lab developed to improve methods for stem cell differentiation and scale up. And very recently we officially created these 3-D tissue models that grew and like Jean-Louis explained, the idea is that we want to 3-D cellular develop these models set up more predictive models of the effect of a drug will be in humans and like Jean-Louis explained the plan is to develop these models and integrate them into the different parts of the drug discovery and development pipeline within NCATS.

And the value in today what I will be talking about is some of the work that we have been doing sort of setting up these capabilities and some of the tissue models that we have developed and some questions that speakers have brought up and focusing on the disease skin models in my talk today will be about.

Really the way we see the cellular models is almost like a continuum from 2-D to spheroids has been a lot of work be done with tumors spheroids as single cell with cell lines and a lot of work now being done with organoids or I'll talk a little bit in terms of bioprinted tissues and mention about organ on a chip. And the idea is if you go from left to right you increase the physiological complexity of these models and hope is that as you increase physiological complexity of the models the drug responses will be more reasonable to what you see in humans and there is a lot of work that goes into making these models and you have to be strategic as to where you invest your resources if you want to get into this area from the application point of view.

We care about throughput because we are in the space of drug discovery and development so we increase the physiological complexity would use the throughput and you can think of throughput in this sense of testing compounds and dose responses. And using some of the machine learning QSAR technologies you can start screening diversity collections and cover a lot of the space and think of doing an HTS with 3-D models, but you can think of throughput to use it for a lot of the reasons that have been mentioned before. Can we embrace heterogeneity of patients? If you want to test cells from different patients, you need throughput and otherwise you will be able to do it. It’s just practically not possible to do it so you can think up testing compounds and think about patients themselves and think of maybe some of the cells will be diseased versus normal. And you want to study within a tissue what are the cells that really drive the disease phenotype? So, you can think of all these permutations and computations in the numbers get big very quickly so having throughput in your 3-D models is something you will need eventually and that is something we are spending quite a bit of time and to see how much we can push the miniaturization of these models into platforms of high throughput mode.

And we get these questions all the time, our model is more predictive of drug outcome in clinic and people ask us for predictability score how good it is and we are hoping eventually will have but right now we don't. A lot of the times we get the question is how much better 3-D models than 2-D models and we get into philosophical discussions as to whether a layer of [inaudible] on some sort of gel, is it 3-D or 2-D? Do you need bioprinting for that? Like Jean-Louis said, if you put cells in a [inaudible],

and that changes the gene expression, is that 2-D or 3-D, or how much value does that give you, just by putting cells in some sort of [inaudible] that I more tissue relevant. So, these are questions that we also try to answer. And finally how much complexity do we need in the model? And some of the work, some of the data, that we are getting that you do complexity helps you but as mentioned it really at the end of the day is what we use and what disease you're interested in and what model you are interested in and include the complexities is a lot of work and so you have to be very mindful of how you spend your time and what is at the end.

Somebody said that validation is the most abused word in this field in the sense of what does validation mean? And it means a lot of things to a lot of people but I think for us validation is critical from the point of view of technical what we call a technical validation and characterization of the tissues in the sense is like what do they look like? Learn early on from our coloration with the histology is critical and following the guys we set up histology lab in our group to be able to do a lot of these because you need to know what the tissue looks like and spend a lot of time comparing your histology to histology of real tissues and how close. Physiology whenever you can if you have tissues with a function you want to know that the tissue are making has the same function as a tissue that are you are trying to mimic. Maturation or time of progression has come out before how long does it take for the tissue to have the right function and right morphology and as critical as [inaudible] how long does it stay mature, stay viable, how long does it stay having the same and relevant physiology? So, that is important because we not only want to make a tissue we want to use this tissue as a screen platform so we need to know if we had a compound and are going to go for a week of treatment that during that week the tissue is not falling apart and we are losing the validation that we have achieved.

Everybody has put up the issue about having functional endpoints that are relevant to the disease that you are studying or it's relevant to toxicity. And on this and what we are trying to do is bring some technologies both invasive and noninvasive that allow us to look inside the tissues and really get information that is relevant to whatever disease we are interested in. And really, we are trying to supply the same robustness criteria that is being applied in HTS to the if you guys are familiar with the Z factors, we want to know the robustness and the high and low signals and how many can we apply the same standards to 3-D models. And whenever you can, can you validate the features from a pharmacologically with compounds. Most of the times it's easier to do than like CRSPR and can we have a set of compounds we can use to really demonstrate that the tissues capture the pharmacology that we would expect?

So, the goals of the 3-D Tissue Models Bioprinting Group at NCATS was then to use 3-D tissue biofabrication techniques to create models of human tissues in multiwell format because we are interested in throughput for modeling the drug discovery and development. And we don't work in isolation; our programs are all collaboration with academic scientists and companies and the idea is that the outcome will be a catalog of both normal and diseased validated qualified clinical human in tissues. And from discussions that we had it's interesting that when we talk about both toxicity and

efficacy testing most of the toxicity testing is actually done on healthy humans and healthy cells so there's a lot of interest in terms of testing toxicity and disease models which really when you give a medicine you give it to a sick patient not a healthy patient peer. Can we do the toxicity study and disease that is and how is that different from doing it in the normal setting?

Just to give you this is our current portfolio of projects. So, we have a collaboration with [inaudible] over at the National Eye Institute on a modeled retina. I'll be talking about the skin model, and then we have another collaboration with [inaudible] over at University of Chicago on model of the omentum, metastatic side for ovarian cancer. And last year with a new initiative at NIH to address the opioid crisis we had been tasked with developing models of the brain and working on a neurological unit and 3-D model of the blood-brain barrier, so I won't be talking about those. Today I'll focus on work that we've done with the skin.

Our process is progressive, you start simple, and you build complexity and for the skin, we basically started with dermis epidermis model, dermis of ECM and fibroblast and epidermis made of keratinocytes and different stratified from the epidermis. Very simple model and that some of the data I will show today and again to investigate how much complexity do we need in these tissues for what? The project we follow is shown in this schematic and there was a lot of work that had been done on developing epidermis models and epidermis to. We didn't reinvent the wheel based on the product protocols we develop and skin epidermis model and not really bioprinted doesn't need to bioprint it you can bypass keratinocytes on a transwell and use the usual protocols of submerging and bringing the keratinocytes a few days into a narrow liquid interface to stratify into epidermis.

And in the audience spend a lot of time like can we actually, this is a protocol, can we do that in 96? It would be a huge advantage to be able to have these epidermis models in 96 and some of the work I will show is work she did demonstrating that you can actually do this in a 96 well plate and get nice histology and epidermis.

And we actually used bioprinting matrix to complete full thickness skin and I will show some work with [inaudible] in the lab. They went to the next level of complexity by adding vasculature into the skin tissue by mixing [inaudible], creating a pattern of vasculature and adding the keratinocytes and creating the thickness, the full thickness skin. I'm going to show you some data from this model and some of the disease models they've done. We spanned a lot of time characterizing the tissue and shown on the right a lot of histology a lot of microscopy and we also exploring noninvasive techniques like optical tomography. These are actually, those are techniques that are used in hospitals in humans but they haven't been really adapted to the lab. So, we are working with investigators who are interested in taking those technologies and manipulating them so we can use them in a plate-based format.

We are trying to develop [inaudible] we can use for screening whether it is measurements. So, this is one slide showing the work they did for the epidermis

equivalent. Essentially, you use keratinocytes and we used existing protocols. It worked quite well with the structure of the skin and using different markers and different layers of the epidermis. You can see as the skin [inaudible] they set up big [inaudible] understanding the variability. We wanted to use this for a collaboration with a group interested in testing the toxic effect of compounds. They have done a lot of work into keratinocytes and they were interested in testing some of the compounds that had shown toxicity in this 3-D model. This was key to be able transit [inaudible] it was good enough instead of doing the typical [inaudible] the effect of some of the compounds. That looked very nice. They are in the process of testing those compounds. [Inaudible] is also in the process of using the protocol because we want to expose differences between the epidermis and full thickness tear the idea is how much complexity, would it make a difference? I want to know how much work you have to put into the systems.

The full thickness work that [inaudible] to sort of streamline the process with thickness using bioprinting, we were interested in making the process more industrialized, more automated, so we have a printer with different methods. You can use extrusion; you can use inkjet. With that capability, we were able to develop a full thickness skin. You will use one of the syringes to extrude the fibroblasts with a hydrogel and we are using another syringe. They do a coating of laminate. Then they let it intubate for an hour and a half and that they submerge for seven days. Then they have skin equivalents that did not contract. One of the issues with the set of existing protocols is contraction. You start with this beautiful skin and you start remodeling and you start contracting and you get good function. It can be nice if you cut it right. When you look at the whole well, it does not have a good morphology. With this protocol, we had a good set of uniform morphology. There were some vertical contractions. Overall, it looked pretty good.

Then we were able to do a barrier function studies from the top [inaudible] the field has developed for function. That looked quite nice. We were looking at the inverse of resistance. You can see there is very little conductance when you have the epidermis. Then finally, you look at the viability of the tissue [inaudible]. This was part of the physiological validation of the tissue.

We were excited about using optical coherence tomography, and this is with the collaboration with [inaudible] lab at Lehigh University. We were interested in looking at the tissue as a whole. When you do slide sets, you just look at certain areas of the tissue. OCT does not have cell resolution, but it lets you look at layers within the tissue. What you see at the top is the corresponding image. You can resolve the epidermis and [inaudible] you can do it, so you can see your tissue, how it looks. You can see a detachment. Overall, the epidermis is covering most of the dermis. That is nice. You can do measurements, the thickness of your epidermis and dermis. It is nice because you can do that with OCT. You can almost use it as a quality control. It is not invasive. You can take the tissue along with measuring the TER, which is not invasive, you can do OCT and see how your tissue is developing and maturing. It is important to know without having to sacrifice half of your sample that your tissue is developing

correctly. Having this technology is powerful. It is nice because you can see the transwell membrane. It is a nice reference point of where your tissue is.

With this tissue, [inaudible] collaborated with [inaudible] is at Rockefeller University, [inaudible] is at NYU to develop a model of [inaudible] cell carcinoma. I show you that this, you see 3-D as a set of different models of different complexities from the spheroids, organoids bioprinted tissues. We are seeing a trend. There has been a lot of work. You can use screens. People have shown that you can screen those [inaudible]. There is a lot of interest in [inaudible] in the context of the well but do it in the context of the tissue in which these tumors are growing. We have two programs where we look at tumor spheroids within the concept of the tissue where this tumor is going to grow.

This is one of the examples. Essentially, [inaudible] they took the fibroblasts, the keratinocytes, they bioprinted, they preform. In this case, they preform tumors with a cell line and included that in the tissue while the epidermis was developing. We did the evaluation. Where we are now is trying to use microscopy to see if we can truncate the tumor formation inside the tissue. The goal is to test compounds to see if this affects tumor formation in the tissue.

He is developing this technology called focal microscopy. Again, it is noninvasive. It is complementary to fluorescent microscopy. The great images, essentially the reflectance from the top, you see tissues and you see how uniform they are; this is the epidermis. He is doing a biopsy, so you can put it in a holder for his microscope. It does not really disrupt the epidermis much. When we get the tumors, which are labeled, you can see these tumors inside the tissues. We were interested in testing compounds that affect the tumors, but do not affect the tissue. We spike [inaudible] what you see here is the tumor and you see these green cells, 1% of keratinocytes within the epidermis. We demonstrate the tumor is in the tissue. The tumor cells are there. What seems to be happening as where the tumor is, the epidermis is disruptive. That is an observation. Where we are now in his here is a microscopic image with tumor cells and the 1% keratinocytes. This data shows when we add a compound, we see an effect in the red. Almost 50% and barely any effect on the keratinocytes. This is by counting cells and this is by counting fluorescence. We can now it is on the keratinocytes. This is a standard of care. It looks good that we can see both the effect of the compounds in the tumor, but not in the normal tissue.

I will switch gears to the last model that I am going to be talking about. This is something that [inaudible] did using bipolarized [inaudible] to create a model of atopic dermatitis. What they did was use a bioprinter. Again, they use iPS-derived endothelial cells, fibroblasts and pericytes to create what they called the vascularized [inaudible], the vascularized dermis. After incubation for about a week, they add the keratinocytes on top and they bring it up to the early interface. That is the skin and cauterization using focal microscopy. [Inaudible] it is top to bottom. You are going to start from the bottom. Then it goes through the epidermis with different layers and you see the different patterns depending on the different phase of the keratinocytes. [Inaudible]

keeps complaining about how much histology she has to do. It is a lot of work, but we have to do it. We have to demonstrate that these skin models actually have the right morphology with the right markers expressed at the right place. It looks good. I want to point out how you can see the vasculature [inaudible]. So, we don't have perfusion. We can connect it to these tissues. We have static. That imaging that [inaudible] did showing this again in the different layers of the epidermis, just a little bit of a zoom in and to the dermis. This is the exact pattern. We can look at everything in between. I will show you where you can see it clearly within the printed area. Here is a zoom in of the vasculature and how you can see it.

With this model in place, we wanted to explore how we can use these to develop a disease model. The disease we decided to work on was dermatitis, which is an immune disease driven by [inaudible] cells. There were papers in the literature where people had [inaudible] to induce atopic dermatitis. You start simple and we decided to first go with an induction of the dermatitis phenotype. What I am showing here is part of the TEER of different days of the early liquid interface. You can see the normal vascularized tissue, you see an increase in the TEER. A nice formation of tight junctions. With atopic dermatitis with [inaudible], there is a much reduced TEER. That was a nice illustration of how TEER could be a nice phenotypic measurement of AD phenotypes.

She also did histology showing that normal vascularized tissue versus the AD tissue [inaudible] they are not expressed in the AD. And E-cadherin was nicely expressed [inaudible]. So, these were sort of validation of the disease marker showing that we were capturing some of those in this model. It looked like we were getting a good AD model. So, we knew that [inaudible], too, so we added different [inaudible] inhibitors to see if we could correct these phenotypes. I will start with the middle which is the TEER phenotypes. You have the normal then the disease is decreased. We are excited to see that once you add [inaudible] inhibitors, you actually correct and increase the TEER on that AD induced phenotype. [Inaudible] decided to take the medicine, and anti-inflammatory. It is used for AD. It did not have an effect. It would work that way. This is not just a general correction of the phenotype, but it is a mechanism [inaudible] it was amazing how well these inhibitors corrected the phenotype through the normal phenotypes.

A third [inaudible] inhibitor. That medicine did not correct these phenotypes. Both were up by PR and histology. We see a nice correlation in terms of how these compounds are able to correct the phenotypes. We did a lot of marketing to see whether the compounds between these compounds were able to link the normal marker after the AD phenotype. It is amazing how well it works. What is interesting is CD31, which is a marker for the vasculature. You can see that it somehow disrupted in the phenotype. The inhibitors were not able to correct that specific phenotype. That goes to the discussion before of what am I going to measure. All these phenotypes some of the compounds might affect some but not the others. At the end of the day, it is like well, is the phenotype that we see going to be a liability? It is a potential toxic effect?

Just to go deeper into this model, we cleaned the cells in a zigzag pattern. It seemed better in the phenotype because in the phenotype, we did not see [inaudible] in between the bioprinted areas, you see nice coverage of a formation of micro-vasculature. But that does not happen. When you actually measure across this area, you do it cross-section of intensity, he see it going up and down. These are the areas where, these are the bioprint areas. We do not see formation of vasculature in between the readouts. What happened with [inaudible] inhibitors is nothing breaks the vascularization in the formation of the cubes. You can see it here by measuring monikers how there is a little bit of change of normal versus AD phenotypes. The inhibitors do not correct. They have more of an effect of reducing the vascularization.

Another readout we decided to use is measuring [inaudible] . Measure a panel of cytokines. What this table shows is we measure these cytokines [inaudible] discovery assay. This is day one, day 10 and what is interesting is the black column is normal. The red is AD. We have cytokines that go down and some that go up. Eotaxin is one of them. IL-1alpha is another one. You look at the effect of the [inaudible] inhibitors and they, for the most part, they correct the increase in the cytokine level and bring it down close to the normal level. You can see that with eotaxin and you can see that TARC. You can see other patterns. Nothing is black and white. It is interesting that some of the patterns, you see corrections. It does not correct. This is another phenotype we can use to see if the compound corrects a phenotype or not.

This is my last slide. It is a closing slide in the sense that I am trying to answer some of the questions that were raised before in terms of how much complexity do we need? In this slide here, we see the TEER measurements of epidermis, non-vascularized full thickness skin, and vascularized full thickness skin. Normal, atopic dermatitis, and the JAK inhibitors. What you see is the epidermis has not much effect. We wouldn't have an AD phenotype by measuring TEER in a 3-D epidermis only model. Tell me when you add the dermis. You see a reduction in TEER. You see it both in the non-vascularized and the vascularized. You see that shows the effect of the JAK inhibitors correcting this reduction in the model. This is something you need the complexity of the full thickness skin to see a diseased phenotype. If you look at the toxin, this one is just the 3-D epidermis. We were not able to detect eotaxin. It is only when you go into the full thickness that you start seeing levels of eotaxin. Those increase with atopic dermatitis and then you see the correction with the JAK inhibitors.

This is the statement that [inaudible] said: Why would you test compounds in 2D? When you start doing these comparisons, you start seeing the effect of compounds on different disease phenotypes. You start seeing disease phenotypes with different complexity. For us, this was eye-opening. I think we are on the right track in terms of using 3-D models for the disease modeling and compound testing. So, this is just acknowledgement. Most of the work that I've shown today is from Lucia and MJ and Paige and Kristy Derr. Thank you.

Edward LeCluyse: Thanks, Marc. Any questions before we start the panel discussion? [Inaudible].

Roundtable Discussion
Moderator: Edward LeCluyse
All speakers and Margaret Kraeling

Edward LeCluyse: So, if all the speakers wouldn't mind taking a seat at the table. [Inaudible]. I guess the panel is open to questions.

Audience Question: [Inaudible]. Okay. How long do you think it's going to take for pharmaceutical companies or any type of drug developmental experiment to replace annual testing? Like right now we are trying hard to have a physiological relevant model. However, I still think, or found what I read, is animal models are going to be the step between the clinical trials and the previous testing. So, how much work or how long do you guys think is going to take?

Jean-Louis Klein: I would say right now, we are not ready to replace any animal work with 3-D models because we don't even have one organ on the chip or in the dish, so we don't have that liver or a lung, so, of course, we cannot even connect them to get a full body. Right now, I think what we are doing is we need to add this kind of models, 3-D models or chips, to the animals. It is a more complementary approach. Hopefully, these models will keep developing, we will get better and better. We already know that animal models are not that good. I think with time we will be able to show which model is better. It will be years before we can completely eliminate animals. There are some toxicity that we can see that we cannot predict, we cannot [inaudible] even in the mice. Right now, because we don't have the full body in the dish, it would take years to go there probably. Unfortunately.

Marc Ferrer: I think I agree with Jean-Louis in the sense that they will be complementary for years. I think one of my nightmares is we do all this work with human cells and we are still going to be using animal models. What if a compound works in a human 3-D tissue model and does not work in animals, what would you do? People are asking can you do the same, can you reproduce animal tissues? So, it is almost like a sequential, you start with humans, see if it works in the equivalent animal model, 3-D tissue model, and then you go into animals. But you know already whether it is going to work or not in the animal. I think for years, it will be complementary. We will try to integrate these models in the drug discovery pipeline, try to generate data and hopefully there will be enough data in the clinic that it will fit back into how well have we been doing [inaudible] where we have been developing 3-D tissue models and we have generated data.

Jean-Louis Klein: I will add one point also. More and more molecules are human specific. If you think about antibodies, it has become challenging to test even this molecule in animals because by definition they will not do anything. I think there is strong pressure to get this model to be developed. I think it is coming. And it will get better and better because we absolutely need it. Now, we have to use surrogates, so we have to develop kind of equivalent antibodies that will work in mouse or rat or dog

to assume this antibody would do the same. There is a pressure to get human models for toxicology and for efficacy. I think it is coming. It is getting better, but we are not yet there.

Edward LeCluyse: Bear in mind some of the issues we are able to address with these systems, we could not approach prior to this, like the liver fibrosis data. That particular project was driven by the fact that we could not study that in a laboratory. No animal models recapitulated the events or the effects we were looking for. I think to some degree, that is the driver or the foundation for going in this direction.

Audience Question: I know that some of the speakers had raised the point about standardization of a lot of these methods and these constructs. I was wondering if any of you were aware, because I participated back in the 1990s, a new class of medical devices came on the market called tissue-engineered medical products. These were basically the same types of things you are talking about, which were biomaterial scaffold containing living cells, which was ultimately grown in a laboratory and intended for ultimate implantation into humans to repair or replace damaged organs. The agency rather than develop its own standards, actually went to an outside organization, [inaudible], and there were actually a whole series of standards that were developed by [inaudible]. They had standards that dealt with the biomaterials. Other standards that dealt with cells and finally standards that dealt with the combined product. I was wondering if anybody on the panel was aware that those standards have been out there for over 20 years and have taken advantage of them?

Sharon Presnell: There is kind of a resurgence in the last few years of groups trying to take those types of documents, take the new processes that are going on today and to develop standards around it. There is a standards coordinating body that is working with organizations like, what is their name? The Army Advanced Regenerative [inaudible] Institute. There is a conglomerate of people working around it. I believe they are drawing on the previous work that has been done and trying to build on it. There is hope, I think, in that regard.

Audience Questions: Thank you so much for the wonderful presentations. This is creative, quite new to me. But I was listening to Dr. Klein from GlaxoSmith. My question is on one of your slides, when you had the advantages of the human 3-D printed tissues, you said it does have a long-term culture. I was just wondering when I look more at the food additives and we look more into chronic studies in animals, how long can you go when you say long-term cultures. How long can you go with your 3-D models?

Jean-Louis Klein: It is always relative. I was trying to compare to something as complex [inaudible] that we can do in a 3-D printing model. The most model that we can compare to, it's more tissue slice, precision cut tissue slice model. These are beautiful because you start with exactly what you want. You start with a liver, you start with a [inaudible], you start with a lung, but it is incredible how fast they change. Sometimes within 24 hours, because with the liver, if you don't have the right

conditions, all the hepatocytes are dead. Compared to that, 3-D printing can be much better. It is still relative. And any model keeps evolving with time. As Marc mentioned, it [inaudible] to use it. Do you need time to make sure this model will measure enough so it looks like the organ you tried to mimic? But also, don't forget that it keeps evolving, and as Marc mentioned, they may also die. So, again, each model has to be characterized. And it's part of the validation is to know when to use it. It's not because at one point it's a good model, maybe the day before or day after, it's not anymore. So, it's always relative. But again, because progress are making this model, I think it has become more stable. I really hope we will be able to keep them longer. Again, some studies we can do 24 hours is enough, with some you need a week to be able to see something. Again, it depends on the questions. I am hopeful this model will be better and better in terms of how long it would be stable.

Audience Question: I noted in Dr. Presnell's talk that one of the differences between the extrusion technique and the inkjet is the shear forces and pressures involved and viscosity. I was wondering if [inaudible] gels have been used or tried, which have a low viscosity and high shear forces and high viscosity and low shear forces to kind of compensate for these effects?

Sharon Presnell: Can you give me an example of a specific one? I may know these more by name than the general description.

Audience Member: Well, the classic [inaudible] gel is ketchup, I guess.

Sharon Presnell: Oh, OK. Gotcha.

Audience Member: You have to shake it up to loosen the, to reduce the viscosity. Cytoplasm, clays, there's a number of [inaudible] gels. They have these properties where at high pressure, the viscosity drops to virtually very low numbers and then it becomes thick again when pressures are reduced.

Sharon Presnell: So, the shear-thinning. We did play with a lot of those just to figure out ways to ease the passage through the instrument even though compared to inkjet, there are a lot less shear forces generally in the extrusion-type methods. But still, really fussy cell types like hepatocytes do not care for the process of being squeezed through a small orifice. We did play with some of the shear-thinning gels as what I would consider very, very minor components of the bioink. They did make a positive difference in the survivability of the cells after dispensing. You probably know more about that than [inaudible].

Y. Shrike Zhang: I can add a little bit on that. We have been working with some of the shear-thinning hydrogels as well. Not that much. We are working with gelatin-based hydrogels that are processed to be shear-thinning, so we can directly extrude the hydrogels and of course when you extrude them, then it becomes more liquidy so it's less shear [inaudible] on the cells. I know for example [inaudible] group at U Penn have been doing a lot of shear-thinning hydrogels in their bioprinting processes. So, for

example, [inaudible] Texas University have found something with nanoclays that are [inaudible] hydrogels. I know people have been working around that corner for quite some good efforts. For us, there are other ways you can use for reducing the shear forces that cells experience. So, for example, one way is to do the [inaudible] bioprinting, so you have [inaudible] device so then if you use a low viscosity gel to start with, you can still print in that sense. There are ways to go around that issue of shear force during bioprinting. I know people have been working around the corner as well.

Edward LeCluyse: I guess for the sake of the colloquia and its mission in terms of identifying challenges and emerging technologies for the food and ingredient safety, this could be as much a discussion topic for the audience as well as the panelists. How might we begin to envision applying these new technologies for evaluation of food safety and so on. Part of that might be if not, then what are the barriers or challenges unique to that? Does anybody want to tackle that one?

Audience Member: I have been a longtime friend with Margaret. The Center for Food Safety also regulates cosmetics and there is an issue of dermal toxicity. Some of these dermal models that were presented today, and I know Margaret over the years has worked with a lot of different models. We must not think of terms just food, think also cosmetics.

Margaret Kraeling: If I am allowed to say, we did work with some skin that Marc provided to us to see if it was feasible in the diffusion cell. We are still working on that a little bit. It is interesting. Maybe down the road we can develop, it can be developed into.

Marc Ferrer: What was interesting to us when we started working with you was that we thought this problem was solved because people had been working on skin equivalents for years. We thought we are going to do it because we started working with Organovo and we wanted to have a tissue that we thought would be easy because it is a layered system so it's idea for bioprinting. But as we learned more and started talking to you guys and other people in the community, everyone seems to be struggling having the right skin equivalent. It is a lot of work that needed to be done. There were a lot of possibilities to work together. I was amazed by how unhappy the people in the field were in terms of the skin equivalent.

Audience Member: Just to follow-up on Ed's question, I guess one thing that we are concerned about is being able to predict toxicity for foods, food additives, cosmetics, and supplements. Like Jean-Louis said, in our intro, it is all about predictions. Just to remember since we are a regulatory agency, we are pretty focused on applied research. We almost have to rely on you guys to do a lot of that basic research into the development because we just do not have the resources to do a lot of the R&D to get these things evolved to the point where they would be useful for doing toxicity predictions of different chemicals of interest essentially to CFSAN. I think that is where we fit in. It is very resource intense; I can tell. You talk about your level of throughput which is varied across everybody speaking today. Marc is really pushing for a high-

throughput format and some of the others are focused more on disease modeling probably and slightly lower throughput. I think that is an issue both for pharma and for us. The level of involvement to get these to the point where they can be useful for screening or predictions needs some work.

Edward LeCluyse: On that topic, it seems like would there be some areas in particular, I guess skin coming to mind that seems obvious that everybody, as a place to start and then going back to the topic of validating a system, what standards or metrics or acceptance criteria would we need to see or want to see around these kind of models that might pertain specifically to food and cosmetic safety, for example.

Audience Member: I cannot remember, Jean-Louis talked about validation and it is a tough term to deal with. I'm not sure, for a lot of FDA assays, we talk about qualification. It is tough to go to a point where any of the models, there are very few that are validated, whether for skin sensitization, irritation, absorption and all of these versus some of the tests used for pharma. We look more at the evaluation and qualification and fit for purpose, like Jean-Louis talked about. We need to make sure we have got a model giving us the correct answer for that chemical or series of chemicals. In the foods area, that is more of a drug focused event where they are looking at qualifications. We definitely still need those predictive models that are fit for purpose or can cover a particular chemical space. Does that help?

Edward LeCluyse: That is exactly what I was referring to. I think in a way coming up with performance specs, standards, that would be helpful. And fit for purpose, I think that term is relevant or appropriate here. Is there a top three list of either model systems? Because I am thinking skin, possibly gut, liver. I don't know if you could come up with or the members here could imagine a top three priority list for fit for purpose systems that do not exist in your mind, or you wish existed.

Audience Member: We have studies going on in all of those areas. They are dependent upon the chemical of concern that we are looking at. In our division, we have had some models for most all of the different tissues and organs. It becomes a chemical specific targeted model. It would be tough to give you a top three. Hepatotoxicity is a biggie.

Edward LeCluyse: That seems to be everywhere.

Audience Member: Something that would be helpful, too, is unlike drugs where you are dealing with a single chemical entity, with food, you are dealing with complex products. Remember the dietary supplements are regulated in the United States as food, and these tend to be very complex botanical products. Many times, they contain even more than one botanical. Something that would be useful for the FDA CFSAN would also be validating these systems with complex mixtures.

Edward LeCluyse: Any other topics from the panelists themselves?

Y. Shrike Zhang: I want to add one thing from the engineering point of view. I think one thing I feel right now is bioprinting has been restricted to research labs largely because instrumentation has been a little more complex than it should be. For example, if you want to make something that's complex with bioprinting, [inaudible] may not be translatable to other places. Certain things are more complex [inaudible] and different things. I think one thing that I think people should probably do, from the company point of view, for example, [inaudible] I think I talk to people all the time because I think they have to make printers more widely accessible and operational so people can adapt them for different purposes. For engineers like us, we can potentially [inaudible] doesn't really matter, but for biologists, for example, if they want to use a printer, they have to be very usable in the sense that it's simple, reproducible, and robust.

Edward LeCluyse: I think on that topic, the question as someone who represents more the end-user, are you guys envisioning a future where bioprinters will be in every biology, toxicology lab or do you think they will be able to acquire the bioprinted tissues that are fit for a particular purpose or assay?

Y. Shrike Zhang: Probably both depending on specific situations. There is a research perspective. People want to develop their own different models for different testing, then most likely, they are going to get their own printers for these kinds of things. But for [inaudible], they can potentially work with models produced by some other people that are already validated for the purpose.

Edward LeCluyse: Jean-Louis, what are you envisioning for industry?

Jean-Louis Klein: I think you are right. I am sure industry is willing to buy the end product, the tissue. There is one concern here, of course it is quality. Again, it's the reproducibility, quality, and so on. It has to be very high standard. It has to be more regulated because we cannot say we tested this tissue, toxicity in the tissue, there is no QC associated. [Inaudible] we have to give the data to FDA or the regulatory agency. So, it has to be GLP standard or something like that. We would love to get that. But right now, because there is no regulation of the tissue, we cannot do it. In early phase, having access to a printer, 3-D printer is probably some value, but there is so much technical aspect that as a biologist, I don't know. I am pretty sure I do not want to know. I want to make sure it is working as it's supposed to be. Anything that will help that would be very useful.

Edward LeCluyse: Sorry, Sharon, did you want to comment?

Sharon Presnell: I was just going to say it is the conundrum of the business model for the company looking to do this. I have lived this. You have some challenges. If your goal is to provide the instrument and anticipate your end-user can be successful with the application given only the instrument, I think anybody who has had their hands in this sees the challenge of that. If you are going to provide the instrument and hand it to someone else, you are also going to have two provide them with the qualified raw

materials for a particular application and hand them that as well and all the protocols and everything else. By the time you have supported that, from a financial perspective, you probably would have been better off to make the tissues [inaudible] the tissues. I think the commercial side of this whole thing is in a state of flux because the easy button is to provide the hardware in every agent and to leave the end-user on their own to make things work. What I will say is. the very, very tight partnership between the engineer and the biologist. And the biologist with depth in the space that you're trying to operate, is in my opinion really going to be the critical piece of getting something that ends up being the winner and applicable.

Edward LeCluyse: Related on that topic, Marc, I was going to ask you because a number of speakers addressed the issue of tissue quality, cell quality. I hear this a lot from my colleagues that from their experience, the results are only as good as what goes into these models. From your own experience and the direction you are trying to take this with NCATS, what do you envision are going to be the minimum performance or quality specifications for the use of human tissues? And Jean-Louis, you commented and keep emphasizing the importance of the phenotype and having access to normal and diseased cells and tissues.

Marc Ferrer: I have to start with the normal, the quality in terms of histology and just basic characterization. We learned about from the get-go, working with [inaudible] Organovo how critical that was. The [inaudible] is kind of difficult because every tissue has a different function. Skin is a barrier. For liver, it might be whatever. So, that is the challenge. Then you go into the disease phenotype and how you QC that you get the right phenotype [inaudible] but also, the aspect of reproducibility. It is a huge resource investment to be doing that and scale it up in an industrial way that will probably be required to demonstrate the utility of these tissues, the validation, fit for purpose. What Jean-Louis was talking about, the space, right? If I throw in cells from a different patient, is the whole system is going to go overboard? What is going to happen? What other variables that the heterogenicity of the systems will give you. There was some data that was shown illustrating three patients. What do they have in common, what is different of what I'm measuring? [Inaudible]. Maybe if you are in a disease and you focus on a few readouts, that seems to be good enough, not enough, how you embrace that heterogenicity?

I think to the previous question: I think we actually do purchase some tissues and spheroid models that some companies develop. At the end of the day, it will take us a lot to develop protocols where we can have complex organoids and complex spheroids with different types of cells. That, to us, makes more sense to just purchase and see how they function instead of spending a lot of internal time, [inaudible] was saying, everybody has an expertise. You have a tissue engineer in the group, you have someone who knows about assay development, but you may not have a neuroscientist in your group. You say how am I going to create a tissue that has [inaudible] and different type of cells, [inaudible] know where you start, so you have to partner with someone who is an expert in that particular tissue, in that particular field to work together, this is what you need, this is how you're going to go about it. Sometimes the

best way to partner is with a company that has the expertise. It is not cheap. At the end of the day, it may be the fastest way to have a system where you can start testing compounds. I guess it is not an easy answer.

Edward LeCluyse: I think it relates to another important area, especially these more elaborate types of model systems. That is some sort of quality control metrics and whether the burden is on the manufacturer or on the end-user in terms of specifying what is going to meet their needs and if, like you said, you are the one doing the bioprinting and it is a you to find the right source.

Marc Ferrer: I think if you buy the product, the burden is on the manufacturer.

Edward LeCluyse: Good answer.

Jean-Louis Klein: If I can add something. We talk about healthy or normal and disease. These are not black and white phenotypes. That is a challenge. [Inaudible] we start calling cells healthy enough that we say, OK, it's not a diseased cell. And same thing [inaudible] if it's a progressive disease, so which [inaudible] are you talking, you talk about tumors, same thing. So, that's the first thing, to qualify the cells, the primary cells that we got, it is critical, for 3-D printing but for anything else. When we talk about 3-D printing tissue, it's not because you got a cell from a diseased organ that that disease phenotype stay. You have a level of qualification or validation to do. You need the qualification or validation of the cell you start. You also need a validation of the tissue you try to save. Again, it's a lot of complexity, but we have to go there, because at the end of this, as a pharma, we want to treat population of people that have that diversity and so on, so we have to understand it, and we have to go there.

Marc Ferrer: I think what we're finding, back to Jean's point about the source, cell sourcing, is OK, I'm going to go primary cells because I can buy them, right? So, you sort of get into this nightmare of, oh, they advertise astrocytes that are [inaudible] positive, and then you bring them in house, and they are not. What are these cells, right? And do I get into trying to figure out what they are selling me, or I decide, forget about it, I'm going to go iPS-derived astrocytes. And then you go into the literature and you might have sources, but there are all these different protocols for differentiating. People claim you can differentiate iPSCs into astrocytes. OK, so which one should I use? Either way, it is not perfect and it's very resource intense. The truth is that what they advertise, a lot of times is not what you get. [Inaudible] we just want to make the tissue right, so just use the cells, and then [inaudible] you will have to go back [inaudible]. It happens all the time. This is very time-consuming and resource consuming. We do spend a lot of time trying different sources of cells and talking to people and what they tell us is the best cell to use.

Edward LeCluyse: I am also reminded of another important aspect of all this. If we are going to start defining what is a toxic response or perturbation from normal, do we know what normal is, especially for human biology and pathology? In the field of toxicology, we talk about point of departure. Do we have good definitions around those

responses? Again, for someone who has focused on the liver for most of my career, do we know what normal human liver biology and pathology is? And I would say not clearly. Is the burden on us as part of our joint scientific community to start putting some definitions around that? So, to be fair to these emerging models that we can say where are we starting from if we're going to start saying it's a point of departure from normal?

Marc Ferrer: It is almost like you want to forget about normal. You went to embrace the diversity and learn how to handle it in a way. The question is how do you do that? And whether you have, again, enough throughput to generate all thee tissues with all these different cells and understand where the variability comes from, with what readout that you're going to be using. Right now, we are limited on the throughput that you [inaudible] the heterogeneity of the cell. It is not practical.

Edward LeCluyse: Good discussion. Anything else from the audience, panel? Anything you are dying to get out while the opportunity is here? Personally, I think it is great. I appreciate Margaret, Betty, and the team for putting this together. It is a timely topic. It is the right time to discuss these things and start working through this so the next generation, we will be addressing all these properly and convincingly. Because to me, it is long overdue. We have been having these kinds of discussions for decades, I would argue, these organotypic model systems. It used to be, as Jean-Louis pointed out, that a cell on a plastic petri dish was biology, and it does not mimic much of the interactions, the architecture, the phenotypes that go on in real life in organs and things like that. So, we are on the cusp of some major breakthroughs. It is a good time in the field.

Marc Ferrer: I do feel we are at an inflection point. Some of these bioprinters are hitting the $40,000 mark. A lot of academic labs can actually afford it. Once this technology gets more integrated into biology labs and they realize they can actually do it, I think more and more data will be generated. It has been our experience that once we show what we can do, people come to us, not to screen compounds, but to do biology. People will come to us and say could you actually test these, and it does not have to do anything with drug screening or compound screening. They just want to explore biology in these 3-D models. Some of this basic research scientists, can I bring this technology, they don't realize it is still an art, so it is going to be a lot of investment and resources. I think once these printers get in the labs and get more [inaudible] commoditized, I think generating some of the data we have been discussing, not from one lab, but many labs working on it and generating data, that will be valuable to everyone because we will have more of it. I think we are just at the beginning of it. Some of our collaborators decided to buy a bioprinter. They have $40,000, and [inaudible] you are going to need some time to learn, but I am happy for it because they will help everyone bring these technologies more into the basic research.

Edward LeCluyse: Excellent point, I think, to end on. Anything else from the steering committee? Okay. I would like to think the speakers and organizers again. Thank you very much.