DR. CHEN: Our next speaker is going to be talking about I think collagen. Yes, Bob? Let's see. Okay.

DR. ROBERT TRANQUILLO: Good afternoon. So I thank all the organizers and the speakers for this opportunity here to tell you about what we're doing in my lab. So I decided to expand beyond this title so you can see the full picture that actually will end up addressing this particular aspect of my work.

So I'm in the business of making cardiovascular tissue replacements. So some of the axioms that we follow in the lab are to recognize that the tissue function depends on structure as much as composition. And by structure, as we've heard, that means alignment of the cells in the extracellular matrix fibers. And in trying to create one of these tissue replacements, we therefore want a polymer scaffold that provides a regenerative template in the loose sense. We want this template to guide the growth of tissue in the laboratory. So the way that we've been able to satisfy these axioms as shown here, a process that others have termed tissue equivalent fabrication, the basic idea is we take the cell of interest, we entrap it or first we disperse it within a solution of what will form a biopolymer, and I'll talk mainly about fibrin, the fibers that are floating around then getting trapped in this network of native fibrin and fibrils. And then over a period of days through maybe a week or so, the cells exert a very significant force that leads to contraction of this

network. And if you were to anchor it from both sides, for example, the fibers become alive between the points of anchorage.

So we can create something that's a little bit more like a tissue in the sense it has alignment but it's still way too weak to be implanted at that point. But it does have this property of being now a regenerative template because the cells, by virtue of contact guidance, create a network of cell-produced tissue that has the same alignment as the aligned fibrin, and this requires the application of strategic chemical and mechanical stimulation.

And to show you we can actually accomplish this, here are some sections of tissues that we grew for different periods of time. This trichrome stain fibrin – this pinkish red color, the cells are the smaller purple dots. This was actually a model construct that we formed by a process I'll tell you about later. But basically it was a disk-shaped construct. It was initially about three millimeters high and ends up being about 300 microns thick. And so you get a very significant compaction. You can see there's an alignment of the fibrin, which turns out to be transversely isotropic, or parallel to the bottom surface of the plate. And over time, the cells are degrading the fibrin and replacing it with collagen with stains pinkish – sorry, bluish green here. And you can see the collagen that's being formed also has the same transverse isotropy. So this

basically shows that in the lab we can grow a tissue that has a prescribed alignment.

So we've applied this idea to grow various types of cardiovascular replacements, arteries which I'll focus on in a second, fibrillar structures, more recently heart patches. These are electrodes that are pacing the beating of the cells within the aligned ring of cell-produced tissue and also microvascular networks. So these are endothelial cells that are starting to extend and ultimately will form capillary-like structures.

But I'm going to focus here on recent work aimed at trying to create a small diameter artery implant because it will allow me to identify some of our imaging needs many of which have already been identified but you'll get my version of this in a second.

So the way we create our engineered arteries is by carrying out that process I've already described in a tubular cavity. So we have a tube of fibrin gel that's formed with cells entrapped within it – in this case, human derma fibroblast over a period again of days and this is typically about a two-week culture period where we have this fibrin tube formed around a central mandrel or glass rod. You can see it's become much shorter. It's also become smaller in diameter reflecting the cell-induced contraction of the fibrin.

So the trichrome stain shows you that initially it's indeed fibrin. The cells are dispersed throughout. Even

after two weeks, there's a very significant deposition of collagen, but there's still some residual fibrin. What's important to us and that we've really studied over the years is this idea of contraction-induced alignment. So as the contraction is occurring in this particular geometry with the mechanical constraint being this non-intissive tube through the middle these fibers become aligned around the circumference that's revealed by this polarized light alignment image using a method that we published some time ago. So I'm not going to talk about it. But there's a clear anisotropy of the structure, and that's reflected in a anisotropy of the mechanical properties. So if we measure the stiffness in the circumferential and axial directions and take their ratio, we get a value after it's been cultured, which I'll say more about in a second, of about 1.5 and that's not too far below a native artery.

So this is a two-week static incubation process. We do get a lot of collagen deposition, as I showed you. But this is still too weak to implant. So it turns out to be crucial to do some additional chemical or mechanical stimulation. And this bioreactor that Dr. Zeeshan Syedain conceived in my lab has turned out to be very effective for this. So we have multiple samples here melted in parallel between these manifolds and reciprocating syringe pump, pumps culture medium in through a three-way valve. It's distributed among the samples. The medium flows through, and there's a transient pressure pulse so you get a transient stretching

and transmural flow of the culture medium. A lot of it does, though, flow out through the bottom to be recirculated back up and then through another cycle.

So this is typically about a five-week period of bioreactor culture, and I'll say more about this effect later on. But those are essentially – that's the way we make our samples for implantation. So what we did in this particular study that is not yet published is to make these constructs from ovine fibroblasts so we can implant them into sheep without any risk of immune response or any need for immune suppression because we decellularize these constructs prior to implantation. And these are about the size of a human coronary artery, that is, four millimeters ID. And this is following an approach that Laura Niklason has championed, which I agree with in terms of the ultimate commercialization and clinical use of these.

Okay, so let me summarize what we found here, and then I'll talk about the imaging needs. So with these fibrin-based graphs which I should emphasize are completely biological – there's no synthetic components that we've used here, we can immediately entrap the cells and get the cellularity that we think we need. We create this circumferential alignment quite easily by harnessing the cell forces that lead to the contraction around this non-adhesive mandrel that leads also to a mechanical anisotropy. We can very high burst strength in just a couple months –- in fact, supra-physiological -- 5,000

millimeters of mercury using both human fibroblasts as well as the ovine fibroblasts. And I didn’t show you the data, but remarkably you can get very high burst pressures with this system and still have physiological stiffness or compliance.

So for imaging needs, okay, a lot of these things were discussed. But let me just reinforce them. So all this information we got right after explantation of course would be really valuable to get as longitudinal information from each implant in terms of the degree of recellularization, how much proliferation is occurring, the type of cells that are present, white cells and tissue cells, the phenotype of the cells as Gordana mentioned, M1 versus M2 macrophages, as thought to be a very important aspect of whether you get a good or bad outcome.

And from our point of view, for these vascular implants, even if you get spontaneous endothelialization or certainly if you put in pre-seeded endothelial cells, are they still there and what is their thrombogenic state? Are they pro or anti-thrombogenic? Information about the composition of the extracellular matrix is also desirable. Elastin is a key constituent that no one has really been able to create in vitro and it would be good to know whether that’s appearing over time in vivo without having to wait until explantation, and of course the organization and alignment of the constituents.

Now there are ways to get at some of this information no doubt with ultrasound and targeted contrast agents, with intra-vascular methods, and elastography, for example, for mechanical properties. But a lot of this kind of information about the details of the cells and their phenotype as far as I know is not readily available from imaging.

So that's where we'd like to go preferably in a non-invasive way or at least minimally invasive way, so it's not real expensive.

So I'm going to shift gears here now and get to sort of the title of the original talk which is another imaging based approach we've used to try and optimize the growth of the tissue before we implant it. So here's again the process that we're using. I already talked about this. But the point I want to make it here is, as Chris and maybe some others alluded to, that if you look at the histology, clearly there's a very significant time-dependent change in the extracellular environment. There's an increase in cellularity. The composition of the ECM is changing and its microstructure is changing. So therefore when we apply mechanical stimulation, how the cells respond to those stimulations are going to depend at what time you apply the stimulation, right, because how the cell responds depends on its environment.

Okay, so this raises the question, well, how can you maybe optimize the culture conditions certainly in a non-

invasive way by being able to monitor the response of the cells to the stimulation that you're applying? Okay, so we've used the idea of a gene reporter. So in this case, type one collagen reporter cells, using a transgene that was provided by Al Banes at Flexcell International. Basically, we can monitor the transcription of the alpha-1 chain of type one collagen because we have stably transfected cells where that promoter sequence is connected to the firefly luciferase gene. And if you're not familiar with bioluminescence, basically you can apply the substrate luciferin and in the presence of the enzyme that's being produced whenever collagen's being transcribed by measuring the amount of light produced, or photons being generated, you can basically have a measure of the rate of transcription. So David Schaeffer at Berkeley has analyzed this problem and basically if you assume steady state conditions and that the rate of transcription of the gene of interest is proportional to the rate of transcription of luciferase, then the light intensity is proportional to the collagen transcription rate.

So it's good to know that the half life of the luciferase is about two hours. So that means if you're looking at a change on the order of a half a day or day, right, this is a good system to use because the luciferase level will change faster than the time scale of interest to you. But it's long enough because an imaging session may last 20 minutes by the time you add the luciferin and put

it into a Xenogen system and make your measurement. So it’s just about right to have a half life of about two hours for this enzyme for, at least, our applications.

Now the point is, right, we’re trying to maximize collagen deposition to get a stronger tissue by measuring the transcription rate of the alpha-1 chain of Type-1 collagen. Well, even if Type-1 collagen was the only collagen of interest, which it’s not, right, there’s a long way between deposition of fibillar collagen and transcription, right. You have translation, hydroxylation, secretion, et cetera, et cetera. So it’s a bit of a stretch to say, well, if I monitor Type-1 collagen transcription, I’m going to be able to predict collagen deposition. But we decided to take a chance.

So here’s the system that we used. I already alluded to it. We make a hemisphere-shaped construct. You’re looking from the side here at the bottom of a tissue culture. Well, by taking that cell suspension in a fibrin-forming solution and placing a drop in the center of about a one centimeter etch. So here are six of these fibrin disks. Over time, the cells again contract the gel. It changes from a hemisphere to disk-shaped geometry. The convenience of this system is we can easily put into a plate reader or take it down to the IVUS system. It’s not very useful as an implant, although maybe a cornea some day. But for us it’s a model system.

So here’s a typical read out from the imager. So in this case what we did to alter the environment of the cells was to change the inhibitor of fibrinolysis because the compaction of the fibrin is not just due to the cell forces but also the degradation of the fibrin that the cells can create, and we can inhibit that with aminocaproic acid. So at the lower concentration, there’s less inhibition. So the fibrin’s degrading faster, and you see that leads to a higher rate of collagen transcription compared to a lower level of inhibition.

And this plot which is the collagen 1 expression is a function of time and culture. You can see based on the amount of aminocaproic acid that we used, we can either get a relatively high level of transcription over two weeks. But then the situation changes.

So the cellular environment, which again is changing over time because of either fibrin degradation products being formed faster or slower or the fibrin gel stiffness changing as the fibrin is being degraded – whatever it is, the cells are sensing it, and it’s affecting their transcription rate of Type 1 collagen.

So what we wanted to do is to see if we could actually predict how much collagen would be deposited by monitoring the collagen 1 expression rate. So here’s a plot of cumulative luminescence based on these discrete measurements for in this case two different – well, sorry, all three different ACA concentrations. I didn’t show you

the six millimolar case before, this intermediate concentration you can just ignore here in this slide. But clearly over the first two and a half weeks, there's a higher rate or more cumulated luminescence at the lower ACA concentration as the previous slide would have suggested relative to 12 millimolar ACA.

So I should point out this cumulative luminescence is essentially a measure of the cumulative transcription of collagen that's occurred. So therefore we would expect if we make the measurement of the amount of collagen deposited and transcription was a good predictor of collagen deposition, we should see more collagen in the 3 millimolar ACA case which had a higher cumulative luminescence, or transcription of collagen, versus the 12 millimolar case.

And you can see at two weeks in fact that's true. So there was about a two-fold increase here in the amount of deposited collagen, and this [the cumulative transcription] was not quite two-fold but it wasn't too far below it. So in fact the prediction was quite good. By three weeks, you could see from the previous slide, right, the rate of transcription was normalizing. There's no difference here in the cumulative luminescence, and in fact there's no difference in the amount of collagen deposited. So, surprisingly, monitoring the Alpha-1 collagen transcription was a pretty good indicator of collagen deposition, at least in this system.

Now we wanted to make sure that there wasn't some imaging artifact going on because with different rates of fibrinolysis, the thickness of these constructs changes and can be different between the 3 and the 12 millimolar ACA. So what we did here was to create these same fibrin hemispheres. But instead of using the cells that were transfected to bioluminescence, we used non-transfected cells but incorporated fluorescent microspheres. So the hemispheres, they will compact to different thicknesses, but they have the same number of fluorescent microspheres. So if there was a dependence of the detected fluorescence on thickness, we'd expect to see some trend that was not like this. So the fact that there's no correlation between fluorescence and thickness indicates that the changes that we saw with the actual reporter cells was not just an artifact due to constructs of different thickness.

Okay, so we really want to measure the response of cells to something that's more interesting like a growth factor that we can add exogenously and have a more direct connection to the cell response. So we use TGF beta which is known to promote collagen transcription. Here, we actually measured also the level of expression using QRT-PCR. And so, as we expected with 1 nanogram per mill, there's an increase in the amount of messenger RNA for collagen I. This was about, if you convert it to a number, about a three-fold increase, and based on the bioluminescence, it was about a 1.7 fold increase after

three days of TGF-beta treatment. But basically either way there was a prediction of more collagen being transcribed, and we found more collagen deposited after seven days in the case of the TGF-beta treatment. So the basic idea is we know that TGF-beta will work in the sense of being a chemical stimulation for collagen transcription. And so the question now is what's the best TGF-beta treatment to use if you're going to try and use it as your stimulation.

So with this system now, we have the potential of trying what we call an on-the-fly optimization or a data-driven optimization. So we can start out with a lot of samples and start them all in the same TGF-beta concentration, except for this subset where we take some of them and use two other treatment concentrations. And then we can figure out which of those three concentrations given the current state of the cells in those constructs is optimal for collagen transcription. Suppose it's C. Okay, so we take our sample down to the Xenogen system and we measure C. And so now what we would do of course is switch all the samples to that concentration C and repeat the interrogation.

And suppose now B is best because we saw, right, the potential for the optimum shifting with time. We would shift the remaining samples to B and repeat the interrogation. So it's a very rational way to do this, and we started doing this. And if we look at the collagen expression as a function of again the culture time using

three different treatment conditions, what we can see is the optimal concentration which is the lowest concentration that maximizes transcription changes from the intermediate value to the highest value and then essentially to the lowest value. And so hopefully we'll tell you next time whether this optimization strategy works.

So one caveat to this is that, you know, it takes maybe 12 hours to get a [transcription] signal that you can measure based on bioluminescence. Well, that means there's a 12-hour time lag between when you first apply that new stimulation and when you make your measurement. Well, now you're trying to get information 12 hours later and assume that it's still current information. So there's a pretty big time lag. That may be too big of a time lag for this optimization to work.

So we're trying a new approach, systems biology, to reduce this time lag by trying to identify patterns of upstream phosphoproteins after we first apply the new TGF concentration, which occur maybe on a time scale of 2 to 20 minutes after administering the TGF, as opposed to the collagen transcription that occurs 12 hours later. So if you can predict the transcription from this upstream phosphoprotein data, you can reduce that time lag from like 12 hours to 12 minutes.

So how is that going to happen? Well, first you have to identify what the patterns of the phosphoproteins are that predict collagen transcription. And then once you do

that, maybe about 10 of them, 20 – we're working on this using phosphoproteomics, but you'd have to have a way of imaging multiple prophoproteins, i.e., kinases, to be able to use this method. And so how this will be done is for all you imaging people to figure out. It could involve FRET-based kinase sensors and multiplexing measurements.

Okay, so I'm just going to take a real short amount of time to go through the last couple slides here. So back to the bioreactor. The reason we think it works is not because of TGF stimulation but mechanical stretching. And so, well, now we want to use this bioluminescence strategy for monitoring collagen transcription with samples that are being cultured in this bioreactor. Well, it's not so easy. You have to take them out of the bioreactor, put them into a specialized chamber, add the luciferin, bring them down to the Xenogen system. You can do it – it's not so easy. But you get the sort of result that you expect with this pulsed-flow bioreactor conditioning. You see a lot of stimulation of collagen after four days compared to a statically-incubated control sample. These are four mirrors that are showing the bioluminescence from all four orientations. You're looking at the top here of the sample which is centered right below the middle. After ten days, you can see that the collagen transcription rate has gone down quite a bit. So the cells seem to adapt to this constant level of stretching. And so now the question is, right, can you apply this on-the-fly optimization to

identify the optimal regimen of stretching to get the maximum collagen production? And I'll skip the rest of this slide. But it basically tells you that it's not a constant strain aptitude that's going to be best. We found that a periodic increase in strain aptitude is better. But what is best, nobody knows. This approach may answer the question.

So I'd like to thank Drs. Syedain and Weinbaum who contributed to the work I presented and all these other folks as well. Basically, everything I presented was funded by the NHLBI except for the very last bit on the systems biology approach and I thank NIBIB for that support. And this is Minnesota, not in January. It's a very nice place to visit in June. Come see us. Thank you.

[APPLAUSE]

DR. CHEN: Maybe I'll ask you why does the aligned fibrin align with collagen?

DR. TRANQUILLO: As far as I know, no one has really identified the mechanism by which cells respond to aligned fibrins. That's almost separate from the second question which is why is the collagen becoming aligned. Well, one can argue if the collagen fibrils are being extruded through fibropositors and the cells tend to migrate back and forth by contact guidance, they're leaving a trail of aligned collagen, and that's a reasonable guess. But that certainly isn't understood either.

But there's certainly a coupling between cell orientation or alignment of cells and the matrix they

produce. So thankfully it happens, and basically we're exploiting that. Yes.

MS: So the ability to differentiate M1 and M2 macrophages, and when this was originally described, it was a very convenient classification and very polar type, very artificial types of microphage in culture dish which is very different from what actually happens in vivo in biology. So when you say that you want to have the ability to see these artificial polar extremes, do you mean that you want to have the ability to see subsets of human macrophases in the human or subsets of mouse macrophases in the mouse or specifically the N1 and N2 phenotype, i.e., ordinase expression and whatever you find M1 and M2 in.

DR. TRANQUILLO: Well, one could argue that right there's no just M1 versus M2. You have a spectrum of phenotypes, and we're looking at the extremes. So, right, this was just a shorthand way of saying we'd like to know whether the macrophases are promoting regeneration and repair or destruction of the tissue.

MS: Because there's many other markers LY65, LY60 low, CD14, CD16 which are much more predictive of the function than M1.

DR. TRANQUILLO: Right. So as you said, you need to be specific and declare what it is you want to study. So this was just meant to be to point out the phenotype is very important – not just the cell type.

FS: [Inaudible]

DR. TRANQUILLO: The question is does it matter what the source of the fibroblast is. We've mainly used dermal fibroblasts for convenience. So I can tell you that cardiac fibroblasts are much different and not very useful in comparison to the dermal fibroblasts in terms of creating a collagenous matrix. They don't seem to be as effective. What we found is, more importantly, the passage number. So lower passage cells certainly create a more robust tissue than higher passage cells. Thank you.

DR. CHEN: Thank you very much.

[APPLAUSE]