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DR. CHEN: So I just wanted to remind the audience to use the microphone when you ask questions because I think that it's being recorded. And our next speaker is Dr. Toomre, and he's going to be telling us some of the emerging technologies that he's developing.

DR. TOOMRE: Okay, just pull this slide to a different orientation. That's okay. So I'm going to have to make a little bit of a jump back in space and time. We're not talking on our scale but on the millisecond scale, and I'm afraid that I'm really talking on the nanoscale – not the millimeter scale.

And I would actually contend to you that this range of scale – I actually – I'm an outsider, but I'm also an insider. What do I mean by that? I'm an outsider here, but I'm an insider for the cells. I like to look inside the cell, and I'm a microscopist and I'm a cell biologist. And so really the point that I would contend to you that's really important as we're looking at this and talking about the cell is we're talking about not only how it's happening at the level of the tissue and between cells but at the individual cell as it decides how it's going to respond to different signals, to secrete some of the very matrix that you need to deposit and to do that in a particular manner to control cell structure and function.

And so I'm going to tell you a little bit about sort of my view on breaking and controlling the barriers. Now what barriers am I talking about? So one challenge that I would propose to you is how do cells sense and respond the nanoscale to the micron scale environment? And, you know, at least one not forget when one's talking about it adhesions, you're talking about conversion of adhesions from focal adhesions to stronger adhesions. And so you have this whole process of how the cell has to locally deposit material, change things, integrate signals.

And we have to understand if we're going to create this in a tissue level how it's actually happening in the cell, how the cell is responding and doing that. The second part I would say on that is the need to see at that level – the organelle level, also the macromolecular level with techniques such as what is called super resolution microscopes or nanoscopes which some or maybe all of you or none of you have heard about, and I'm going to give you sort of a brief synopsis on this just to sort of bring you up to sort of what I would say where we are with this technology and where it's going.

And then I'll talk to you really about what I view is really important and that's just not seeing. We want to be actually able to control it, and there's different ways of controlling. And so one that you heard quite nicely this

morning was on micro patterns, and I'm going to show you micro patterns from a different perspective, a perspective of not the cytoskeleton but the perspective of membrane traffic and talk to you a little bit about how we're using ontogenetics to control membrane traffic.

And so this is simply to remind you that if we're looking at this piece of coal and this is being focal through it with fluorescent microscopy that you have organization on the tissue level. But of course you also have organization on the cell level and the subcellular level as we go in and zoom it – in this case, looking at vascular traffic in the cell. However, if you really confess, you know, we would say, oh, that's high resolution But it's not. You know, this is what a cell looks like inside. This is tomography, right, electron tomography EM, and you can see it's just absolutely chalk full of cytoskeleton, vasicles, organelles, there's the Golgi to give you an idea – I don't have a – here's basically – this is a microtubular, it says 25 dana years wide. So this is really the environment that you're dealing with. Okay, that's what a cell is.

However, they're not only fixed. As you obviously know, they're alive and dynamic, and they have dynamic ranges of different scales. As you can see, this is over minutes as a cell goes through in this case a cytokinesis.

We're interested in the fate there. But also in the case of seconds and minutes as in this case vesicles traffic out along these microtubular highways to the periphery and in some cases in polarized fashion. So what you kind of see – at least what I'm trying to hope to make you realize is that you have this sort of tradeoff between sort of the high spacial resolution of electromicroscopy versus a live cell imaging with specific probes of light and fluorescent microscopy.

So now this is the slide that jumped. We're going to jump backward to do something on this, okay. So really the dilemma as I see it is you would like to see the level of resolution of electromicroscopy but in live cells, and is that possible and to what extent. And this is simply to remind you of the cartoon and really only focusing on this end as you go across these spacial scales there's really a disconnect between what you can do with light microscopy and electromicroscopy. And this sort of missing gap of what can be resolved is really the focus of the gist of microscopes or nanoscopes called super resolution microscopy. And this is really a pretty hot area, and it's only come about in the last decade and is actually advancing very rapidly.

So this is – you're going to hear about and you have heard about all sorts of imaging modalities. Practically

speaking, when you're talking about subcellular imaging, you're almost – I would say almost always the majority of time right now talking about fluorescence imaging. This could be with organic dyes. It could be with organic dyes which are targeted, quite nice -- obviously, the fluorescent proteins and this is largely due to the following reasons. You have the specificity, right. You have a molecular handle that you can level. You have the sensitivity. Why? Because you can separate the excitation and the admitted light.

You have multiple colors. You have this sort of palette there. Obviously, you have optical sectioning in live cells. But I would say you have that for the others, too. Now I know that many of you know this. But I just want to remind you that you are limited by diffraction, and this is nothing new. This is Abbe's law, and it tells you sort of what is your expected resolving power. And so really sort of the barrier to light for light microscopy and the sort of wavelengths we typically use would be in the order of sort of 200 to 250 nanometers which basically means that's the wall. That's sort of the wall of what you can really go after. And really the trick with the super resolution microscopy is breaking that barrier.

And so then you say, okay, if you can break that, how can you break it. What are the sort of approaches you can

use to get past that limit. So one is near field elimination. That barrier really, as Abbe's Law describes, is for far field. If you're locally exciting something, then that does not – the rule no longer applies. The second one which is really a very clever technique championed by Steffen Hale(?) is non-linear effects and point spread function engineering switch dyes between two states, on state and off state, and I'll give you an example of that. And the other one is to actually say to hell with that. We're just going to have to apply if you don't have to resolve, you just need to tell where the center of your object, in this case the localizing the center of single molecules are.

So in the first case -- this is something that we do a lot of and I'll get back to this in a second – is total internal reflection for us microscopy, turf microscopy and it really just basically relies on having a difference of refractive index of two mediums. We come with a laser light much akin to how you might come with this laser pointer at a certain angle, and this – beyond a sort of certain angle, the light will undergo total internal reflection and actually generate an evanescent field which decays exponentially in the other medium.

So this is really doing is it's optically sectioning the cell right at the very bottom. And we can make actually

variance on this, and I won't talk about it really here. But we can change the angle, and we can change the penetration depth and actually do this in 3D. But to give you sort of an example because I think, you know, we're talking about imaging and so at some point it's useful to actually see what we're talking about. So this is microtubule. It's tubulin YFP. On the left is epifluoresence. You see what you expect – the dynamic stability which is occurring over seconds and tens of seconds. And what's really remarkable is when you start looking with a different modality, you're seeing something quite different, okay. We're looking at the microtubules interacting with the cell cortex. What you're actually seeing in this case, this movie is the microtubule tips. Now they're the same cell as that one, okay – not the exact same cell, but the same cell line. And what you're actually seeing is the microtubules coming in, targeting the cortex, retracting and actually can build the microtubules up in 3D based on that.

Now the other modality – and I want to jump back. I'm really apologizing. I'm going to jump back very quickly to actually go with the stuff that goes with this slide is point spread engineering. So here's the trick on it. So basically you take a fluorophore, you excite it and then you come at it with this STED laser and you drop it back

into the groundsite. Well, that's kind of a switch, and that in itself is not too exciting. But you do a second trick, and that is the stimulated emission, the one that you're pumping back down is shaped like a donut. Now if it was just let's say low levels of power, it really wouldn't do anything. You'd still be limited by diffraction. But now you have a second trick, and that is actually you come with a very high intensity donut. So the shape of the donut has an edge. So what really happens effectively is you've taken your point spread function which is going to be your normal 200-250 nanometers, and you've timed the thing up and there's really no limit, okay. The only limit is more sort of practical ones. You can go smaller and smaller and smaller. And so you've actually changed the shape of what your scanning probe is, and then you scan this over the sample.

Now I jump forward for one second to where we really should be and show you the example. And so this is what you would see with confocal, and this is what you're seeing with STED. Confocal – STED. The resolution that you can get down to on this is as low as – you can report lower. But in the tens of nanometer range is really quite remarkable.

Now the other approach which we call pointalism microscopy does not actually resolve upon resolving the object. It is actually being able to locate the center of

the object very well, and people have been doing this for a while. And I'll just give you kind of one example.

If you take a quantum dot and you put it on the surface of the cell, it's bright and you can tell where the center is and you follow it around. You can see that you can actually track it with a – the center of that with a resolution much better than a diffract limit. So basically you have a Gaxalian, and all you really say in this case is where is the center and really it's a question of fit. You've got enough photons there. You can fit it well, and you can fit it with a 10 nanometers. And so this is what you'd have. You'd have a point spread function. This is what it would look like at a diffractional object looks like that. But you can make the Galaxian fit and the localization of that with a much higher precision.

Now you have to do a switching trick. And so how do you actually – that tells you where one single molecule is, and really it's kind of a painting by dots, okay. And so the way you would do this is you have your dye and you illuminate a sparse data set of that. So you light up a few of these and you would localize them, and then you would switch those off and you would do it again and you do it again and you do it again. And you do enough cycles of this, you can create an image.

Now I said enough cycles, and this may be in the order of 10,000 or 50,000. So you would have to take 10,000 images or 50,000 images to generate one image. Okay, we're talking about a lot of data. But it's really powerful, and here's some examples of this, and this would be by epifluorescence of microtubules and pointalism or polym(?) or storm. There's all sorts of variants of these names, and you can very clearly sort of see the resolving power. And I think probably for you folks, you'd find it quite interesting that you can actually do this in 3D as well using some fairly sophisticated set ups which is using anatometry getting down to an axial resolution of 10 nanometers. And here you can see the surface. Those are the focal adhesions. You can see the top and the bottom of the cell. It's absolutely remarkable, and this can be done in live cells.

So to sort of surmise these two technologies, instead it would be similar to thinking how a confocal microscope might operate. You'd still have to scan, but you're scanning with a much smaller pencil, if you like, and I sort of describe that end. Although the Palm does a different sort of scanning, it's scanning by turning individual molecules on and off, localizing the center of it and building the image up.

So let me just tell you about some of the themes on that and the challenges. Well, they all sort of rely on photo switching probes between two states. In one case, in the case of STED, this is very much controlled. In the case of the Palm or Storm, it's stochastic. But a key point here is that the probe or the dye really does play a central role. So it's not only just the optics and the microscopy analysis but really getting the right probe is really essential.

It also involves scanning. This can be in the case of sort of the raster scan as instead or, in the case of Palm, Storm or Pointalism microscopy, lighting up and detecting these individual dots. And there's no free lunch, you know. There's no free lunch here. There's no free lunch there. You gain spacial resolution at the cost of lower template resolution.

So on that part, you really need advances both the optics and the probes. Now seeing is great. We love seeing. But you know, we like to micromanage, right, and we like to micromanage our cells and we like to control them and there's different ways of doing this. Chris gave a wonderful introduction on this. I'm going to tell you a little bit on our view on some of the micro pattern surfaces in the case where we can see it having an effect on the cellular process.

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So this is a micro pattern. Let me just sort of as the movie plays tell you a little bit about what we're looking at. I'm sorry for the phallic nature. I didn't control this. It is a cross bow shaped situ plate, and what you're looking at is that transpheron receptor fluouren, and you're looking at this by tirf microscopy, and it's being played at 10 points per second.

And what you would see if you were to look carefully whereas you see all the circles, those are actually individual fusion events. These are single vesicles which are arriving and going onto the surface and tethering dock and fusing with the surface. And we actually have developed software that we can actually analyze these events because you can actually see from one movie we're going to get hundreds of these events and mark them as they go along. This is just sort of an overlay to show you what we're looking at.

The other question is, well, how are they distributed, how is this changed, these sort of questions. So I'm giving you sort of a case study. Now in the course of these studies, we actually design novel software to quantify the data. So this is if you took all the points and the events, this is what you would see. But the question is if you see something, if you see it being clustered, if you see it's not being clustered, how do you know that. I mean you're

looking with your eyes is great, but it really is not a quantitative approach.

So we developed an approach to do that, and the way we do this and I'm just going to go through this just really super briefly is that we go through and we would scan and we're scanning over a region and we're counting the pixels and we're looking at how many objects we count in a certain radii and we'll get a certain value for that. However, then we'll take the same number of objects and randomly place them there and do the same thing again and again, and we do that – these Monte Carlo simulations a thousand times, and then we'll compare the real image set compared to the simulations.

And what we can then do is then get a confidence interval at each one of the points to say if it's clustered or non-clustered. And to make it easy to visualize, we then basically show the blue and red as the different confidence levels, and the red being where things are clustered and the blue is where things are being excluded. But we don't actually see something too striking here. It's pretty random.

Now you can obviously see that we have different – these are different cells. We can put them on different patterns. You may or you may not see something. You know, this is very subjective when you look by eye. But we can

run through and we do it with our software and then put it in a quantitative way. We say things. Now in this case you would see some variation between the cells. But as we can micro pattern and put the cells on micro pattern and do this again and again, we could tell you, well, it's actually not meaningful because we actually have the control of looking at many cells which are under identical conditions.

Okay, so you say, well, that's kind of neat, but so what. What are you seeing in other cells. Well, if we take a cell in a petri dish, this is what we see. And you know, you look at this and what you might see is what I do, and that is you've got this big old blue ring on the outside, right. That's a – and it's really clear when we start to do this analysis, right. You see a big blue ring, you see another big blue ring except for right there. You don't see that, right. And why is that? Because actually there's another cell in contact there.

So where the cells contact each other, this zone of exclusion is lost. And this is interesting because I think what you're talking about when you're talking about micro patterns is this ability to mimic some of this contact inhibition that we see, so depending on the type of the pattern that you.

Okay, so that's one way of controlling things. We could control the environment by how we put the cells and the matrix and the way they're attached. But there's another way of controlling things if you really like to micromanage, and that would be acutely manipulating the environment. What do I mean by that? So I don't know, most of you or some of you have heard about optogenetics. Now when you hear of optogenetics, you're probably thinking more of calcium channels and other things and neuroscience. But there's also optogenetics about controlling protein-protein interactions. So let me explain this one.

So you basically have a partner and a bait. And so the bait in this case is targeted to the plasmembrane, and it has a GFP in this case and it has this motif CIBN. Its binding partner is CRY-II. Now what's interesting is if you shine light on this, you switch the confirmations of these two interact. So then we can basically recruit this red protein onto the plasmembrane.

Now on this protein we have basically a kinase or in this case a binding partner that recruits the kinase that is then gets recruited to the plasmembrane. So what we then switch on is that we basically by shining light on it, we bring the kinase to the plasmembrane, and we take phosophoinositides and we generate PIP-3.

Now we've done this in adipocytes which we think is kind of an interesting one to study. So if we turn the light on, I think you can – this is looking by just confocal. Here we see a cytostolic. We turn it on. We're going to see it light up the membrane, and actually the thing will revert as well. Let me just show you one movie. The light's going to come on. I think you see it nice. We go up to the plasmembrane, we turn it off and then it comes back. And so here's another example. So we're turning it on, off, on and then we use an inhibitor for the PIP-3 ward nano, right. So if you quantitate the fluorescence is on, it gets recruited, turn off the light, it disassociates slowly and goes back. Turn it off, it goes slowly back, forth, add a drug, it shuts down, right. Pretty cool. You can now control phosophoinositides in a matter of seconds on the cellular level.

Now we're interested about membrane traffic and wanted to see if actually we can use that as a switch instead of using insulin in adipocytes to cause the transporter to get recruited to the surface. If we could actually use as a system to basically drive boot for or a reporter thereof to the surface.

And I think you will see this as we're triggering with the light. We're getting more and more recruitment to the surface. And actually what you see here is likely

accumulation and Catherin-coated pits. It's a little fuzzy. My students let the focus go. But I think you get the point. So you basically see it go up and up and up, and we can see it with other markers.

Now the nice thing with these sorts of approaches is you can do imaging wise. But you could also do it biochemically. It's actually important control to see that if you're turning the light on, are we getting what you would get from insulin or not. And again, we're basically triggering this signaling pathway, and there's some predictions of a number of proteins which you get phosphorylated. So in this case if we're looking just by adding insulin, we trigger AKT phosphorylation, PS-1 phosphorylation. Now we turn on the light, and now I should say that only a subset of the cells were double transfected. So the reason why it's low is because you're only looking at a subset of the cells.

But you can clearly see that we do get the phosphorylation, and it's actually kind of interesting that PS-160 is phosphorylated to a greater extent than the others. So it's kind of a two-part message: we turn it on, it works. But it's also working a little bit differently. That's telling you that there's some other levels of regulation which are there.

Now the nice thing about these sorts of approaches is that we can turn it on not over the entire cell but locally or focally over a region. And I think you can see there we've activated it just at this specific region of the cell or in this other cell the same. So we have this control which is pretty fine temporally and spacially for action limited it should be by light and temporally the thing happens in less than a second.

So let me just go, as I conclude here, to a few talking points. I say more as talking points than pure summary. So nanoscopes can resolve down to tens of nanometers. But you know, the reality is live cell imaging with that is actually still quite challenging because you're scanning because you're getting this very high temporal information – sorry, spacial information, there's sort of a trade off on the temporal.

So I think these are the things that one has to improve. There is a challenge of seeing something that you've never seen before, and that is sort of the question of how do you know what you're seeing is right. You know, this is something that's sort of inherent if you're seeing it new. Of course, part of the solution is you can say, well, maybe you need to cross-correlate it with something else like electron microscopy. But it is a bit of a challenge.

There is a very large data size. Some of these cameras that we are working with, we can get up to gigabytes in about a second. That's a lot. Optogenetics provide a very flexible mean of control tissue cells, but it's not easy. You have to be especially in some of these systems, we have to – we're talking now about getting three or four genes in. It would be nice to be able to control some of these micro patterns in more ways and just sort of emphasizing the point that was made before of really what I believe is the importance of new tools analysis for quantification.

So acknowledging the people who did the work, Felix Inco, Vladamir, Kresho and Brian and close collaborators Petrodik Mullen and Uri Buridorf, and I have an imaging center called the Cinema Lab and you're welcome to drop by. We have all the super resolution modalities there. So okay.

[APPLAUSE]

DR. CHEN: We have time for a few questions.

MS: So Derek, on your next to the last slide, you just said that, you know, with super resolution you have a trade off between getting lots of information and doing it in real time.

DR. TOOMRE: Right.

MS: You suggested that, you know, this needs to be improved. But really is there – there's a fundamental limit, right. You've got bleaching of your fluorescent

probes, and you've got fluorescent life times that you've got to deal with. You can't – so my question is do you see a way forward in really significantly speeding up some of these nanoscopic methods?

DR. TOOMRE: Yeah. I mean just to give you an idea, I mean the first Palm microscope, it took an hour to acquire the data set. The most recent ones have been in the case of tens of seconds. A lot will come with the dye. I think the dye is an area really where there's a lot of power for improvement. For STED and Palm has a little bit different challenges. So I think – I would say that the probes will offer a lot of possibility of where you can go. There's been some things interesting on image analysis of how densely you can pack things and be able to tweeze apart the information. So you could if you didn't have the photo damage of STED think about parallelizing the system. Right now, it's kind of like the difference between a confocal and spinning disk. Confocal standard one has one beam. And the reason why you can't use multi beams with STED is you may not have the power to have the principle instead. But if you could, then you could think about doing that. So I think there's ways. And you know, if you just sort of step back, if you had said to somebody can you go past the diffraction limit ten years ago, nay, can't do it, right. And what's the speed. So we're getting close to some of

these boundaries. But we've pushed the boundaries so much that it's still hard – I would be inclined to think that we can find ways past some of these other ones. Yes?

FS: Hi. On one of your earlier slides, you showed vesicle trafficking.

DR. TOOMRE: Yes.

FS: And some of those vesicles look like, oh, trains. Several vesicles together, traveling together. Is that what's really going on, or is that an artifact of the imaging modality?

DR. TOOMRE: Uh, I don't think it's an artifact. I mean, well, so I guess is it really going on. So I think – I'll tell you what I think, then I'll give you the evidence. I think that you do have some microtubular highways where you potentially have lots of vesicles trafficking along a subset of these.

How would you look at the artifact? Probably the other way would be to look at some other imaging modality. You know, I would say a CRY-EM to just verify that you don't have that. From what, you know, we're not going with that high intensity in everything else that we're inducing an artifact. At least, I don't think we are from the imaging. So I don't think it's that.

And actually, it's actually more likely to get artifact in the fixed cells in these cases than the live

cells. And you could actually also, if it is an artifact of the light illumination, then you should see something different when you're going at it for a long time than the first second or two. Yeah?

DR. CHEN: Okay, thank you very much.

[APPLAUSE]

DR. CHEN: I believe this concludes our session.

[Recess]