Innovations in Light Sheet Microscopy
Strategies and New Applications
Webinar
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Tianna Hicklin: Hello and welcome to today's Science/AAAS technology webinar on "Innovations in Light Sheet Microscopy: Strategies and New Applications." I'm Tianna Hicklin, the assistant editor for Science's Custom Publishing Office, and I'll be your moderator for today's event.

In this webinar, we'll be discussing light sheet microscopy and how it is transformed to microscopy field by offering a faster, less phototoxic technique compared to conventional methods.

This technique uses a unique illumination approach to achieve high penetration depths, fast imaging speeds, and subcellular-level resolution, which makes it ideal for observing living organisms and the cellular dynamics of biological systems.

Because a specimen is illuminated with a sheet of light rather than a focused laser beam, only regions directly exposed to light will fluoresce, and this enables minimal photo-induced tissue damage.

We have with us today an expert panel who will discuss their own uses of light sheet microscopy and provide insight into its different applications, including Open Source Selective Plane Illumination Microscopy (SPIM) and the dynamic behavior of subcellular components within live specimens.

It is my pleasure to introduce Dr. Thai Truong from the University of Southern California, Mr. Pete Pitrone from the Max Planck Institute of Molecular Cell Biology and Genetics in Dresden, Germany, and Dr. Orla Hanharan from Andor Technology in Belfast, Ireland. Thank you all for joining us. We're happy you could be here today.

Before we get started, I'd like to share some important information for our online viewers. At the top right of your screen, you'll find photos of
today's speakers and a "View Bio" link, which you can click on to read more details about their background and research.

Underneath the slide viewer is the "Resources" tab where you can find additional information about technologies related to today's discussion, and a link to download a PDF version of the slides.

We'll begin today's webinar with a presentation from each of our speakers and end with a Q&A session, during which our panelist will address the questions that are submitted by our online audience. If you're joining us live now, you can submit your questions at any time by clicking the "Ask a Question" button below the slide widow, typing your questions into the box, and then clicking "OK".

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

It is now my pleasure to introduce our first two speakers for today's event, who have weaved together a joint presentation. Dr. Truong will be giving a brief overview about optical imaging, and Mr. Pitrone will then discuss light sheet microscopy in more depth before handing it back over to Dr. Truong.

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Dr. Truong is a research scientist in the Translational Imaging Center at the University of Southern California where he is developing novel imaging technologies associated with quantitative analytical tools for applications in developmental biology, neuroscience, and regenerative science.

Dr. Truong obtained his Ph.D. from the University of California, Berkeley and received his postdoctoral training at both the Biological Imaging Center at the California Institute of Technology and the Physics Department at the University of California, Berkeley.

His recent research includes developing and applying light sheet imaging technology to the study of dynamic biological systems, such as the dorsal-
ventral axis patterning in Drosophila embryos, and the interplay between form and function in zebrafish embryonic hearts.

Mr. Pitrone has been a microscopist and optical designer at the Max Planck Institute of Molecular Cell Biology and Genetics since 2011 where he is responsible for building Open Access Selective Plane Illumination Microscope systems.

Prior to his current role, Mr. Pitrone worked as a microscopy and imaging specialist at MPI’s Light Microscopy Facility for over five years. He provided training and technical support on a wide variety of light microscope and laser scanning confocal systems including spinning disk confocal, multi-photon microscopy, structured illumination microscopy, aperture correlation microscopy, and total internal reflection fluorescence microscopy.

He has earned both a TechRMS certification as well as a Diploma of Microscopy from the Royal Microscopical Society. His focus is on developing OpenSPIM systems to characterize the changes in gene expression patterns over time in developing Drosophila embryos in 3D.

First up, Dr. Truong, welcome and thank you for being here today.

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Dr. Thai Truong: Thank you, Tianna, for the introduction. Thanks to Andor and Science for having us here today.

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Optical microscopy is the use of lenses and other optical components to aid the eyes, to see things smaller than what we can normally see. On this slide, I am presenting a very brief history of optical microscopy. It first started more than 2,000 years ago when people started to realize that light is refracted when it goes through droplets of water.

And then people invented the first eyeglasses, and fast forward to the 17th Century is when the next big breakthrough happened, is when the first microscope was invented by a group of people like Jansen and Hooke, for example.
1665 is an important year because that's the year when Hooke published his Micrographia where he recorded the first observation of the first biological cell. Arguably, this is the birth of modern biology.

So of importance to the kind of optical microscopy that we are going to be talking about today is the invention of the first fluorescence microscope at the early part of the last century. And then with the advent of fluorescence microscopy, now we can label the cellular components of biological samples and then we can use lasers to look at not only the structure, but also how they move about and carry out their functions.

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Before we focus in on to light sheet microscopy, I want to talk a bit broadly about what I see as the three important and competing performance parameters that one has to think about when thinking about biological optical imaging, and those are resolution, depth, and speed.

Most people understand and emphasize resolution, and that's understandable. With the recent Nobel Prize in Chemistry for super-resolution microscopy, that's definitely so, but if one wants to follow biological processes in vivo, biology is 3D, is moving, so one has to also think about depth and speed also.

The kicker here is that there usually is a trade-off between resolution, speed, and depth. Now, if you want an image with high resolution necessarily, it's going to be slow and necessarily it's going to be only at the shallow depth, up to tens of microns at most.

If you want to do deep, usually you use some kind of single point standing approach either with one-photon or two-photon excitation, but then that's also slow because you have to rely on imaging things one point at a time.

If you want to image fast to follow biological dynamics, then usually you use some kind of parallelization to allow you to interrogate multiple points at a time, but usually then the cross-talk between those points will reduce both your resolution and your depth, so one have to really be careful about these parameters in thinking about optical imaging.
But then the 800-pound gorilla in the room is actually photodamage. Often times, everybody has to deal with it, but often times I think it doesn’t get talked about enough because as we know, signal to noise ratio is at best equal to the square root of the number of photons because of shot noise, so in order to do well in any of these parameters, be it resolution, depth, or speed, you have to use a lower laser power. You have to increase photon noise, your number of photons. Hence, one really has to worry about photo damage.

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With that sort of background, we think that light sheet microscopy is actually a technique that allows you to be able to flexibility navigate those three parameters -- resolution, speed, and depth. Light sheet microscopy at the core of it is very simple.

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You illuminate the sample from the side with some kind of a sheet illumination. And then at 90 degrees, you'll do wide field detection, your image, what your illumination light sheet excites in your sample.

With this simple twist in geometry, we think it gave us all of the advantages that would allow us to tackle the next level of biological imaging.

With that, I will pass it on to our next speaker, Pete, so that he can give more details about the history and the practice of how to build a light sheet microscope. Pete, you're on.

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Pete Pitrone: Thank you, Thai. I'm going to be talking about OpenSPIM. It's an open source light sheet microscope that we developed here in Dresden, in the depths of Tomancak lab for observing gene expression patterns in the Drosophila embryos. First, I want to quickly talk about the history of light sheet microscopy.

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It started with the Ultramicroscope in 1903, so here you see another microscope with a pretty funny-looking apparatus here. This is the light source and it goes through these optics, these lenses, and it goes into the sample perpendicular to the detection axis. The detector, the human eye
and the mind behind it, Henry Siedentopf and Richard Zsigmundy built it for Zsigmundy's research on colloids. He also won a Nobel Prize in Chemistry for that work in 1925.

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Dr. Siedentopf went on to create the fluorescence microscope with August Kohler five years later. These microscopes look very similar to one another with a big, huge lamp source on the side and this is sending the light perpendicular to the microscope, but this fluorescent microscope used the same light path as a normal transmitted light microscope.

They sent the fluorescence excitation light through the sample into the objective and they collect it with a camera because it was using a lot of UV and it would do a lot of damage to the eyes, so they collected these images using an old camera system.

Why didn’t Henry Siedentopf look at these two designs and figure a way to put them both together? They didn’t have the correct technology at the time.

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So it wasn’t until the '60s and '70s that they had the technology available to be able to create that. You need a light source like a laser, digital detectors such as CCDs or sCMOS chips, and computers to store the data that these chips collect.

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That was on a technical side. On a biological side, you needed to have fluorescent markers such as dyes and fluorescent proteins to be able to specifically tag the objects of interest. Later on, we're able to take these proteins and express them in living samples pretty much in vivo. There's a beautiful quote from Victor Hugo that says, "No power on earth can stop an idea whose time has come."

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Well, that time came ten years ago with Ernst Stelzer and Jan Huisken and other people in Stelzer's lab at the time. They took all those components and put them together to create Selective Plane Illumination Microscopy or SPIM.
As Thai was saying earlier, light sheet microscopy uses a very novel way of the illumination by decoupling it from the detection. You can illuminate a very thin section of your sample and collect a very crisp and contrast the optically sectioned plane.

Some of the benefits of SPIM -- because the optics are horizontally oriented on the same plane as the ground -- is that you can rotate your sample in a column of agarose or some other type of gel.

Here, you see two Drosophila embryos rotating around, being detected by the camera. There's no light sheet on this particular sample, but it gets the point across very, very, very clearly. You have 360-degree rotation. And so, you can position the sample at exactly the optimal place for any particular Z stack to be able to collect depth information.

And because you can rotate it 360 degrees, you can also collect information from different sides or views, we call them, in light sheet microscopy. You can do multi-view imaging and use all that information to register and reconstruct this data in a more isotropic, true 3D image.

Some of the benefits of light sheet microscopy is it uses cameras for detection, and cameras are very fast and very quantum-efficient, meaning that a lot of the photons that come and hit the surface get transferred in the electrons for them to charge. It can take one shot, the total field view. It works really, really good in light sheet microscopy because that view is in focus and only the information from that plane gets detected.

Here, you see a zebrafish heart beating at 150 Hz. This data was collected at hundreds of frames per second, but it has to be stressed that this was a processed movie. This is the only technology available at the time that can image so fast. I'd like to move on to the next slide here.
Here, we see light sheet illumination. The benefit of this light sheet illumination is that it's very friendly to the samples. Here, you see a data set that was collected with Zeiss Lightsheet Z.1. It's a developing fruit fly and we've tagged all of the nuclei with yellow fluorescent protein. We collected the imaging for 18 hours at 1 minute 20 seconds.

It turned out to be 715 time points and 4 terabytes of data, which is a very large data set.

You see that over the time, it has no photo damage. In fact, it's even starting to twitch right now. The muscle contractions are happening and you can see that it just hatched right there, so all the beads that you see around the sample are used for reconstructing the views and registering them together for later processing, like you see here.

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Then I'd like to talk to you about OpenSPIM. OpenSPIM is a microscope that we're using in our lab for the most part. It is a do-it-yourself microscope. It's a much simpler form of the light sheet compared to other commercial systems, but I'm going to quickly run over the -- if you can see the mouse, you can see that this is the laser and it gets bounced off some mirrors and goes through this cylindrical lens, and this cylindrical lens creates the light sheet.

It gets sent through this illumination objective, which creates the light sheet here in the sample, and the sample is rotated on this motor system that was very inexpensive compared to some of the other companies. It just moves the samples through the light sheet and you can rotate the sample, and the detection lens here takes pictures on the camera. Of course, we need to have some filters to block with the laser light, but all the fluorescence comes through to the camera and hence detected.

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Why do we call it OpenSPIM? Well, I just want to say what OpenSPIM can do. OpenSPIM can image large samples. Here, you see a zebrafish at two days and it happens to be 1.2 mm in length.

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We've taken six overlapping Z stacks and stitched them together using a plug-in in our image processing program called Fiji, and put it altogether. This is a stitch of Z stack and above, you see the maximum intensity
projection, so it can take large, large images or it can take imaging of large samples.

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The next thing I'd like to say is that it can image fast processes. Here is similar zebrafish, except for instead of expressing in all of the nuclei, it's only expressing in the heart muscles. We've parked the sample in the light sheet, so we're not doing any Z stacks. We're not rotating the sample. We've just parked it and we've let the camera go for as fast as we could collect.

It turns out to be here at 10 frames a second. On the left hand side, you see transmitted light with the fluorescence, and on the right hand side, you see just the fluorescence.

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OpenSPIM can also do multi-view imaging. This is what we do for the most part. This is our bread and butter in the Tomancak lab, so we actually are interested in the gene expression patterns -- I should say cataloging gene expression patterns in fruit flies. Here, you see the central nervous system, a gene of interest that's tagged with green fluorescent protein.

Although we've taken very similar views, it turned out to be about six-minute time points. We took it over 14 hours, so it gives us enough temporal resolution to be able to see what we need.

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Why do we call this OpenSPIM? It's because we have a Wiki. On the Wiki, we have very detailed descriptions on how to put the system together. It's an open Wiki that anybody can edit and make contributions to.

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So on it, we have Parts lists. Some of the Parts lists use self-made parts, but we have plans for those, so that's also open. A lot of the components that you would use are purchased from other manufacturers.
We also have assembly videos, and the assembly videos just show you how to put things together.

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OpenSPIM can be built in about two or three hours. It takes about 19 steps. When I say two or three hours, it's alignment and everything. So by the time you're all done, you can start imaging within about a day, although the thing is without software, it's pretty much deadweight.

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We use μManager. μManager is an acquisition software that is an open source software that we run in Fiji.

**Slide 27**

We had to create a plug-in for it, and this plug-in gives an extra degree of movement. Normally it was just X, Y, and Z, but now we throw in some rotation and we can put in position lists that will be able to take Z stacks from different views to be able to put them together, to be able to collect information from all around, and also be able to image one particular position as fast as the camera can allow.

**Slide 28**

Some other benefits of μManager is that it supports a lot of different hardware. During an EMBO practical course here in Dresden earlier this year, we were given the opportunity to test a whole bunch of different cameras and lasers. Here, you see a group of cells in a spheroid and all of these cameras were used to collect this data on OpenSPIM.

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With that, I'd like to say that the OpenSPIM has a very small footprint. It's only a foot by foot-and-a-half, or 300 mm x 450 mm. It's small enough to be put inside a carry-on container, which has been done. We've taken it to different practical courses to be built by students at these practical courses. Like I said, since it can be built in two hours, anybody can really do it. It really is a fun thing to do.
With that, I'd like to say a big thanks to all the contributors, to OpenSPIM. I'd like to also mention that this is not just the only people involved. Since OpenSPIM is a large community, there's a lot of input. There are seven different labs that have put their OpenSPIMs on our Wiki. They're from all over the world, a lot of them in Germany. There are some in Australia and in England.

I'd also like to thank people at the Max Planck Institute for helping us out, especially the light microscopy facility and the IT department. Like I said before with the Zeiss Z.1, we had a 4 terabyte data set, so you can imagine that light sheet microscopy has sometimes a data bottleneck.

With that, I'd like to switch back over to Thai. Thank you.

Dr. Thai Truong: Thank you, Pete.

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For the second part of my presentation here, I'd like now to go a little bit more in-depth, a survey of the latest developments in light sheet microscopy so that the audience can have a feel of what's out there, what's available, and whether it'll be useful for their own research.

I'm going to break these developments into three sub-areas -- excitation, detection of light sheet microscopy, which is sort of a natural separation because in light sheet microscopy, the excitation arm and the detection arm are separate. And then I'm going to talk about a very brief survey of the applications that people are doing out there.

In terms of excitation, basically what is the best way to create a good light sheet or a thin, large enough light sheet so that you can image your sample?

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Pete has described the OpenSPIM project which uses the classical and really a very simple, but very effective way to create the light sheet, which is just to use as cylindrical lens to focus the light only in one dimension, and hence you have a real static sheet to illuminate through the sample.

In 2008, Phillip Keller and Ernst Stelzer and other people in the Stelzer group, they came up with another clever way to actually create
illumination light sheet by focusing the light using a conventional objective, which then gives you good corrections and so on with the Gaussian beam, and then using galvos [galvanometer-based scanners] that can scan this focus beam across the sample and hence, creating a virtual sheet. Doing it this way actually gives you better light throughput and gives you better control overall of the special and temporal delivery of your light.

Now, when one is thinking about designing the optimal light sheet to illuminate the sample, one has to keep in mind the trade-off between if you focus your light with a high NA, then you have a thin sheet and hence you have better resolution, but it also means that you never really have a true sheet, so the sheet part is really just the middle part of the focus here.

If you have high NA, the field of view is going to be small. If you have low NA, the field of view could be large, but then your focus is bigger, and hence you sacrifice some of the resolution, especially the X dimension, so that's something that one has to always keep in mind in designing these light sheets.

Now, there have been a lot of developments in recent years on trying to find better kinds of -- different, perhaps better kinds of beams besides the classic Gaussian beam for the illumination. In my opinion, the approach using the Bessel beam is the most useful approach so far, so that's been proven to be very useful.

In that approach, you use what's called Bessel beam so that you can create a thinner sheet with a larger field of view, but the trade-off is actually -- this picture doesn’t show it all too well, but you have these extra rings around your central focus, so you have to then devise ways to get around that.

Actually, just last week, Eric Betzig, who actually was one of the first people to do the Bessel beam illumination, he published a work that he came up with a way to actually get around those out of focus illumination using what's called a Lattice-light sheet, and that's a whole different webinar to get into. It's suffice to say right now that it creates a lattice of light instead of a focus of light, and with that, then you can illuminate your sample.
All of these strategies to illuminate your sample have been using one-photon excitation, which is very nice because it gives you high signal rates. You can do a lot of multicolor imaging, and one-photon lasers are, relatively, not too expensive. But as we know from conventional meeting, if one really wants to push to get the best and the highest penetration depth into scattering biological sample, two-photon is the gold standard.

As a reminder to the audience, two-photon excitation is a process where a shining light, usually a pulsed light -- most of the time, it would have to be pulsed light -- about half of the energy of your transition, and then absorption up of two of those will give you fluorescence.

And because you need these two-photon absorption events to happen at the same place at the same time, it only happens at the focus of the spot, of the laser spot, because that's the only place where there's enough high intensity.

A couple of years ago in our group, we actually set out to test to see whether two-photon excitation would work in light sheet mode, and the quick answer is it does work and it's got the advantage. It brings the advantages to light sheet similar to the advantage that two-photon brings to conventional microscopy because you have nonlinear process, so you can see much less background of fluorescence, and also because it's infrared light so you can penetrate deeper into your sample with your illumination.

So we're going ahead and actually use that to demonstrate the benefits of two-photon excitation in light sheet mode. Here, I'm showing a lateral slide, an X-Y slide to the middle of the Drosophila embryo image with two-photon light sheet. We used the acronym 2P-SPIM or 1P-SPIM, or the conventional two-photon LSM, laser scanning microscopy.

First off, these images are very good. Now, if you work with Drosophila especially when you look into the middle of the embryo, all of these techniques give you a very decent contrast and resolution in the middle of the embryo, but you can see then two-photon SPIM outperforms one-photon SPIM in terms of penetrating deeper into the sample.
It doesn’t quite compete with conventional two-photon in terms of giving you the X-Y resolution, but if you look at the axial slice, this is on the right here, slices in the Y-Z plane, then you can see then that two-photon SPIM actually -- again, it outcompetes one-photon SPIM in terms of reaching deeper into the sample, but if you compare that with the two-photon SPIM with two-photon LSM, you can see that even though it doesn’t reach as deep, the depth that it can reach could actually give you a much less aberrated image as demonstrated by the still circular shape of the nuclei that we’re showing here, so this is an embryo that has histone GFP ubiquitously expressed in the entire embryo.

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So then the bottom line of using two-photon light sheet is that it maintains the diffraction limited resolution, which gives you subcellular details. In terms of penetration depth, it is about two times better than 1P-SPIM for challenging samples such as the Drosophila embryo. It’s less, but starts to be competing with conventional two-photon microscopy.

In terms of imaging speed, because of all of the benefits that Pete has described with you in the previous slide, in the previous part of the talk, you can image much faster than conventional 2P microscopy.

**[0:35:04]**

The video on the right here shows you that indeed we can follow the entire development of Drosophila embryo with two-photon light sheet microscopy, unlike one-photon where you are sort of limited to about 10, 20, 30 microns on the surface with two-photon light sheet as demonstrated on the cutout picture in the middle of the slide that you can see very well all the way in the middle of the fly embryo, so that is our lab's contribution to improving the excitation of light sheet microscopy.

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Now, let's move on to the detection side of light sheet microscopy, what are the important developments to know about.

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Light sheet microscopy in the detection is wide-field detection. You illuminate with your light sheet and then we get an image with the
camera, and that's why it's fast and it's convenient, as Pete has described, but because of that, it also suffers the same kind of drawbacks that wide-field detection would suffer.

You have cross-talk between different pixels. You have scattering and you have the aberration because it's an imaging mode. Aberration will hurt your image. Hence, the first thing that one should think about and should plan to do when you do light sheet microscopy is one should deconvolve the images. That's straightforward deconvolution. Even blind deconvolution would already help with the images.

Secondly that actually Pete has described in his part of the talk, with light sheet microscopy, usually the setups are built, so people usually build into the system a way to rotate your sample and do imaging from multiple views. If you do that, then you can actually fuse the images together, not that simple fusion, but you actually fuse them using the so-called joint deconvolution process, and several labs have published open source protocols in the Paco lab.

The Tomancak lab where Pete is from is actually one of those leading labs to publish on that, so you can do these protocols to actually do the joint deconvolution so that you can get better images from all of the views and you can get actually reaching isotropic resolution all around your samples.

The third point that I have on this slide here especially when you're imaging with a scan sheet -- because basically, you scan a line focused across the sample fast, but at any given time, actually that sample is illuminated by a line only, so you can think of ways of trying to do confocal line detection, and hence, getting back some of that -- removing the scattering and the cross-talk that is inherent to wide-field detection.

You can do that with a physical slit and there are a couple of groups that have demonstrated that they could do that, but you can also do that pretty elegantly with the rolling shutter mode of an sCMOS camera as demonstrated in the graphic at the bottom of the slide there.

If you can synchronize the readout of your camera to the extent of your focus line as you scan out your sheet, then you can read the pixels, only the pixels that are supposed to be light up and not the other pixels. Hence, you can regain that confocal line detection. I believe Orla from Andor will tell you more about that in her part of the talk. That's about detection.
The last part here, I'm going to give a brief and by no means complete survey of the recent applications that people do for light sheet microscopy. The purpose is not trying to be complete again, but this time, to give you, the audience, a sense of the kinds of samples and the kind of biology that people are doing out there using light sheet microscopy so that hopefully the audience can make a connection and realize, "Maybe light sheet can be useful for me also."

It's a diverse and a growing set of applications. I grouped them into a couple of subgroups. The first one is developmental biology. It's reinvented by -- light sheet microscopy was reinvented by the Selzer group in biology focused on developmental biology.

It's been a very nice tool for the development of biology because you can follow processes of morphogenesis and down to the subcellular resolution to see exactly the cellular dynamics and so on.

I'm showing an example of a work from the Huisken lab where using light sheet, they can follow very well the endodermal migration in the zebrafish embryo there. Other animals, people have done flies of course, sea urchin, mouse, and a whole host of other animals.

The next subgroup of applications would be in cell biology. With light sheet, with the fast imaging in high resolution low photo damage, you can really peer into the subcellular dynamics, the intracellular dynamics that's going on in single cells. The work from the Betzig lab really pushes that to the limit as demonstrated in the recent science paper from their group, and then taking it up to a couple of orders in magnitude in terms of lens scale.

Light sheet microscopy is also very useful for looking at 3D cell cultures, spheroids, and classes of cells that start to resemble more in vivo arrangement of biological systems. I'm showing here a represented image that was obtained in the Stelzer group that they can do live imaging and analysis of spheroids.
The next set of applications I would say that's always been there actually with light sheet microscopy, but it just got a recent boost --

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And if my slide can go forward, I hope it will show the slide where I'm showing the imaging of large samples that are fixed and cleared, so the 3D version of histology. For these samples, they're dead. They're fixed. One could argue that you don’t need to image it all that fast, but on the other hand, they're large.

So just to be able to go through microns or sub-micron resolution, go through all of the volume that's available in these samples, if you image them conventionally with the point scanning system, it will take literally weeks and months to actually go through the sample, and hence the high speed of light sheet microscopy, and actually the fact that you don’t bleach out the sample becomes very important.

I'm showing two examples, the first one from Santi’s lab at the University of Minnesota. His group was able to image the entire mouse cochlea and that is fixed and cleared, showing you a very nice contrast and resolution there.

The recent boost to this effort of light sheet microscopy is clarity and a couple other new clearing mechanisms that then allow people to actually look at a lot of different types of organs. I'm showing us an example here from the Deisseroth group doing mouse brain clearing and imaging of the entire brain of the mouse.

That's a very brief, but hopefully an informative survey of the applications so that the audience can have a sense of what could be done with light sheet microscopy.

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Before I go into the applications that are in our lab, I want to first -- there’s another set of applications in neuroscience. The fast imaging of light sheets in low photo toxic is I think going to become an indispensable tool for neuroscience.

I'm showing here an example coming out of the collaboration within the Ahrens, Keller, and Freeman lab showing that they can capture the entire brain of the zebrafish larvae and the activity, mapping the activity of the entire brain with light sheet microscopy.
This next slide, I want to comment just briefly on sample mounting because with light sheet microscopy with all these advantages and so on that we've talked about, there is one disadvantage, which is you have to be able to have optical access from the side of your sample. That's a new challenge. Hence, it makes sample mounting a tricky business.

Secondly, in a sense, the second challenge is that now that the imaging is mostly non-toxic, it allows you for long observation. Hence, you actually want to come up with new ways to keep things alive.

With conventional imaging, you could say most of the time, the cells can only survive for half an hour or one hour, so you don't really care about keeping it alive beyond then, but now with light sheet, it actually would be alive hours on end, so now you want to be able to keep the biology going so that you can continue to observe it.

So in my view, sample mounting is actually sort of the secret sauce to successful light sheet application if one really wants to push through to look at the intrinsic biology that's going on there. There are various graphics shown, the various geometry of light sheet, the various ways to mount the sample.

For example, one conventional way that people would use in light sheet mounting is the use of agarose embedding, but one has to keep in mind that often times, that actually affects the biology. It slows it down. It does things to the sample, so one has to be really careful about the strategy that one would use to the other sample.

In the next few slides, I'll just breeze through the specific applications that we are pushing within our lab at USC that we're trying to use light sheet to understand the development and the physiology of the vertebrate heart. It requires no argument to say that understanding how the heart works is very important.
Now, the way we look at this though is that usually -- and that's a long history of cardiac research, but usually, there is this multi-scale challenge in studying the heart. There's a lot of work that's been on the structural side, looking at the structural component of the heart that's a lot of work on the physiological microscopic scale side using MRI, ultrasound, to look at the whole organ system.

But we think if you want to understand the heart, you really have to look at the mesoscopic regime where you can look at the individual cell's behavior, but you can capture the entire heart as a whole system.

**Slide 48**

The embryonic heart of the zebrafish offers you actually that nice system where you can get down to the single cell level, but you can see the whole organ that is functioning and still developing. If you think about the heart, as Pete has alluded to earlier, it's a 3D organ that's actually moving. In its beating cycle, cells are moving for 150 microns per second. That's the speed at the fastest space.

Any imaging is intrinsically a 2D imaging, so it's actually quite a challenge to use imaging to capture the 4D motion of the live heart. That's the effort that we're pushing. The light sheet imaging essentially is the only game in town if we want to push these limits and image fast enough so that we can capture the heart dynamics.

In the next few slides, I'm going to show just examples of the imaging results that we have enabled to achieve using two-photon light sheet microscopy and imaging the heart.

**Slide 49**

Here, I'm showing a reconstructed view, a 3D view of a heart, a gene trap heart that has the fluorescence labeling cardiomyocytes. You can see that we can capture the full 4D beating of the heart and the panels show single slides through the heart at different orientation.

So you can see from the graphic there that we can reach essentially almost subcellular resolution and we can identify, as you can see, the negative contrast, the little black dots. Those are the nuclei, which excludes these cytoplasmic label, fluorescence label that we have in there.
The resolution, if you notice, both in X, Y, Z are quite close with each other, and that is the advantage of light sheet microscopy. It’ll give you this quasi-isotopic resolution, so you can follow your 3D sample in great detail.

Slide 50

In the next slide, I'm showing you a different -- or in this one, you can see that we can actually do two-color imaging so that we can follow the blood cells flowing together at the same time with the cardiomyocytes.

Slide 51

The next slide will show you a movie of a beating heart again, but now with essentially a membrane label of the cells in the heart, so we can capture the dynamics of individual cell behavior.

In the movie, we can see that we can resolve the membrane between the heart, the heart cells, and between the cardiomyocytes, and see how they move over the cycles of the heart. At the bottom, we see a panel there where we show that we can segment and track a good number of these cells over time in 3D.

[0:50:03]

And then from these kinds of analysis, we hope to then go through this understanding between the form, what is the shape of these cells and the function of these cells, how do they work together to give you this chaotic beating of the heart.

So with that, I'll end this description of the applications that we do within our lab using light sheet, and in the next slide, I will just show a recap of the advantages of light sheet microscopy.

Slide 52

We talked about high speed, but I think we haven't really talked about one thing, which is to give you a better contrast using light sheet as compared to other modes of imaging that give a conventional co-linear geometry.

And that is because of a sheet illumination, the cross-talk which blur out the image, the in-plane cross-talk is restricted only along the focal plane, so there's no illuminative planes above and below your focal points and
hence, everything else being equal, light sheet would actually give you better contrast then, for example, compare with spinning disk microscopy because spinning disk microscopy, you end up illuminating both above and below the sample.

And then we talked at length about the low photodamage of light sheet because it irradiates only a focal plane at the time, but I think also very important is that because excitation in light sheet microscopy is spread out in space either in a true sheet or at least in an extended line, it reduces the peak intensity, laser intensity at the sample. That turns out actually is very critical for reducing the photodamage because photodamage usually scales non-linearly to the laser intensity of the sample.

Slide 53

With that, I would like to end my part of the talk and I want to acknowledge my PI, Scott Fraser, for providing a very nice environment to work with, and the teams in the Fraser lab. I won't go into the details of who they are, but you can see their names there and the various collaborative that we've had over the years, so I'll end my part of the talk right here.

Slide 54

Tianna Hicklin: Thank you, Dr. Truong. That was very comprehensive. It's a great overview of the applications of light sheet microscopy. And thank you, Mr. Pitrone, for the talk about OpenSPIM.

Now, rounding out today's presentation is our final speaker, Dr. Orla Hanrahan.

Dr. Hanrahan has worked with Andor Technology as an Application Specialist in Life Science for the past three and a half years. Her role brings her in touch with all the latest developments and innovations in both camera technology and microscopy applications.

Previous to this, Dr. Hanrahan managed a microscopy facility in the Biochemistry Department at Trinity College in Dublin where she worked with a variety of microscopy set-ups including wide-field fluorescent microscopy, Point Scanning Confocal, and Spinning Disk Confocal Microscopy.

Thank you, Dr. Hanrahan.
Dr. Orla Hanrahan: Thank you, Tianna. Okay. I'll just go straight into this. I won't spend too long, but I'm mainly going to talk about detectors for light sheet microscopy.

The main thing I'm going to talk about is basically how to choose the right camera. Now, light sheet microscopy has become very, very popular over the last couple of years and it became popular at the same time as the sCMOS camera came into production.

The sCMOS camera has become the camera of choice for this particular application. Now, EMCCD cameras were used in the past, but sCMOS cameras have now become the quest camera or the camera of choice.

The key parameters when you're looking for a camera are really the quantum efficiency and the noise floor. When you look at the spec sheet of a camera, you look for the quantum efficiency graph and the noise floor. The noise floor is made up of read noise and dark noise, and these specifications are all outlined in the specification sheet of a camera.

Now, with the quantum efficiency, it's basically how efficient the emitted photons from your sample can be converted as photoelectrons, so how sensitive is the sensor of actually converting these photons that are emitted into photoelectrons and then giving you an image on the computer screen.

The noise floor then is made up of read noise and dark noise.

The read noise, the general rule of thumb, is that the slower you read out, the lower the read noise; the faster you read out, the higher the read noise. However, with sCMOS, you have a very, very low read noise sensor no matter how fast you read. So you can read it very fast and still have very low read noise.
In terms of quantum efficiency, this graph here shows the two QE curve, the green curve, which is quantum efficiency of about 70%, and then the blue curve, which has the quantum efficiency of about 60%.

Now, the difference of 12% in quantum efficiency is to do with the number of transistors available on these sCMOS sensors. So again, these are showing quantum efficiency curves for sCMOS and you can see the 12% difference.

Now, as I've said, it has to do with the difference in transistors, and transistors are basically the electronics which surround every single pixel on the sensor. With four transistors around every pixel, you're enabling a rolling shutter exposure mode, and with five transistors, you're enabling an additional mode called global shutter.

So having a global shutter exposure mode will reduce your quantum efficiency because essentially, you're covering over the photosensitive area of the pixel with these electronics, and thereby allowing less photons to enter the pixel. So by removing this additional transistor, you're getting 12% additional quantum efficiency and you're getting a rolling shutter exposure mode.

In terms of sensitivity then, as I said, it's quantum efficiency and the noise floor, which are the two key parameters when you're choosing a camera. The read noise is your usual camera detection limit. Now, if you're using a CCD camera, with the CCD camera, if you read a CCD camera very fast and get high acquisition frame rates, you'll also get a very high read noise. However, with sCMOS, as I mentioned, you have a very low read noise, which is inherent in these particular senses and you also get benefits from very fast frame rates.

The dark noise then is dependent on temperature. Now, with sCMOS, sCMOS cameras are ideal because they're fast. Generally, you're using quite short exposures. The shorter your exposure is, the faster your frame rates will be. So if you're using short exposures, you're generally not concerned with dark noise. The cameras themselves can be cooled to a zero or -140, depending on what model you have.

Shot noise is really dependent on the sample itself, on the photons that you're emitting. You can control the read noise by reading it slowly if you
have a CCD. With sCMOS, it's low anyway, so you're able to control that with the camera itself.

Slide 60

Again, as I mentioned, sCMOS have become the detectors of choice for light sheet microscopy.

Slide 61

The reason behind this is because they simultaneously offer extremely low read noise, very fast frame rates, a very wide dynamic range, an excellent resolution, and a very large field of view.

So with sCMOS, you can get essentially down to sub-electron in terms of read noise. You can get this level of low read noise when you're reading extremely fast, so whether you're using 4.2-million-pixel sensor or a 5.5 million pixel sensor, which are huge since, you can read these both at a very fast frame rate, so you can acquire 100 frames per second using the full frame.

Obviously, if you use smaller regions of interest, you can increase this acquisition speed. The dynamic range as well is ideal for light sheet microscopy in terms of seeing very bright signals and very weak signals in one frame. The resolution as well with 6.5 micron pixels, you get excellent resolution with very large images.

Slide 62

They're the key points, I suppose, as to why the sCMOS camera is the camera of choice, and again, the quantum efficiency difference between 4T and 5T.

So because of the 12% increase when you have the 4T transistor, this is being deemed the camera of choice because of the higher quantum efficiency, the higher sensitivity. It means that you have a better photon collecting power with the 4T. That 4T chip, as I mentioned, have four transistors, which enable a rolling shutter mode, whereas 5T has a rolling shutter on a global shutter mode.

Slide 63

Now, in terms of the difference between these two modes, I'm just going to go through it now. These pictures in the slide here, the top part, the
top panel shows you three images. This is explaining the rolling shutter of the sCMOS chip.

Now, when you're exposing or reading the sCMOS chip, everything happens from the center of sensor outwards in both directions, so you have the exposure happening in a pro-row basis, so the first row on either side of the sensor that will receive light will be exposed to light.

And then ten microseconds after that exposure, the second row on either side of the sensor will be exposed and will move up on either side with a ten-microsecond delay from row to row. This is how it works when you're exposing the camera and also when you're reading out.

The global shutter mode, which is not available in the 4T sensor, is analogous to a snapshot mode, so everything happens at the same time.

[1:00:05]

All pixels are exposed to light at the same time and all pixels will read out at the same time, whereas with rolling shutter, it's known as a transient mode of operation. That's really just describing the exposure loads.

Slide 64

Now, what we've done is we've taken advantage of the rolling shutter exposure modes of the sCMOS camera and we've applied this feature, which we call LightSheet PLUS.

Slide 65

Now, with LightSheet PLUS, Thai mentioned this during his presentation and it's really for using a beam of light that you can synchronize. If you can synchronize your light sheet to the rolling shutter exposure on the camera itself. You can do what is similar to a confocal line detection.

So with this LightSheet PLUS, you can actually find the number of rows on the sensor, which will be exposed to light, so this can be a small as one row. So if your light sheet is looking at a very, very low a portion of your sample, you can synchronize this part of the sample using your light sheet on the camera and the rolling shutter exposure mode.

What you'd actually do is you can define the slit width of the number of rows that you're going to be exposed. You can define the scan speed of
how fast or slow you want to move this slit width across the scanner or across the sensor.

You can also control the exposure times. If you're shining a very narrow beam of light on your sample and it's not extremely bright, what you can do is you can increase your exposure time to get more light and more signal, so higher signal to noise ratio.

The benefit of really this concept of slit detection type functionality on the sCMOS chip is that your image quality will be improved since the scan row height acts as a slit detector, rejecting scattered light and improving contrasts and signal to noise ratio, and thereby providing a sharper, a more resolved image.

This is really ideal when you're looking at quite thick samples and looking quite deep into the sample as well where scattering of light is one of the drawbacks, and you're looking at deep imaging. LightSheet PLUS is really ideal for this application, as well as light sheet microscopy. It's really using the rolling shutter of exposure mode of the sCMOS chip which allows us to do this.

Now, as I mentioned in the previous slide, I discussed how the rolling shutter exposure works. I said it moves from the sensor outwards in both directions. Now, this is quite limiting, I suppose, for a lot of you. What we've done as well is we've allowed people to be able to control the way in which the rolling exposure moves on the sensors, so now you have multiple readout modes.

You can use the full chip from top to bottom, from bottom to top, so you can scan your light sheet across the sensor, the whole width and the whole lens and height of the sensor, or you can move it from top to middle, from bottom to middle, so it basically allows you to have a lot more functionality over the working of the rolling shutter.

In the dual readout mode where you can use both sides of the chip, it essentially would allow you to do multi-wavelength applications. There are multi-wavelength light sheets, so if you have two light sheets using two different wavelengths of light, you could essentially lose both half of the chip to image this.
Thank you very much for your attention. It's a very brief overview. You can go to our website if you need more information on any of the topics that I've discussed, but thank you very much for your attention.

**Slide 68**

Tianna Hicklin: Thank you, Dr. Hanrahan and thank you for staying with us since we're a bit over the hour, but I'd like to end here with at least one or two audience question.

Dr. Truong, perhaps we can start with you, but hopefully each of you can maybe comment on this question. What are the limits of the sample sizes on both the high end and the low end that you found you can image with the systems that you discussed today?

Dr. Thai Truong: Okay. On the lower end, you can look at single cell. You can look at subcellular components. If you combine actually light sheet illumination with fluorescence correlation microscopy, you can actually look at single molecule detection also in this typical sense, but actually you can also look at single molecules. Just track them over time.

On the lower end, it's really just to a single molecule. On the larger end, I would say you have live samples. That's because of the penetration that's the issue even if you go to the two-photon application. It probably would be -- realistically be about 500 microns or so would be the limit if you want to see through the whole thing, through the middle.

[1:05:08]

Of course, we can go larger and just look at things on the surfaces like with spheroids and so on. If you have a fixed and clear sample, you can go really, really large.

People are showing their entire mouse brain and other kind of organs. At that point, actually the limiting factor is actually the camera. It's the size of the camera, so that's work for the camera companies. Of course, beyond that, you can also tile your imaging so that you can get larger and larger samples. If you want a number, I would say 10 cm you could still do if you clear your sample well. Light still penetrates through.

Tianna Hicklin: Thank you. Mr. Pitrone, do you have anything to add for the limits of the OpenSPIM?
Pete Pitrone: Actually, I would also add to that statement about the working distance of the objective that you're using, so the working distance of any particular lens might be anywhere from a three -- maybe up to 8 mm depending on whatever lens you're using. That's what I would add.

It all would really depend on what you're using, so if you choose a particular lens with a long working distance, you might be able to get better, bigger samples, but then you might run into resolution problems. You want to have on the detection side as high of a numerical aperture as possible, but also a long working distance is possible.

Tianna Hicklin: Thank you. Dr. Hanrahan, do the detectors you discussed today play a role at all in this sort of limitation?

Dr. Orla Hanrahan: Well, as I mentioned, sCMOS have the resolution, the speed, the low read noise, so they're really the ideal detectors for this particular application.

Tianna Hicklin: Well, unfortunately, we are out of time for this webinar. That concludes the question and answer session, but I'd like to thank each of our wonderful panelists once again for being with us today and for the great talks and discussion they've provided, Dr. Thai Truong from the University of Southern California, Mr. Pete Pitrone from the Max Planck Institute of Molecular Cell Biology and Genetics, and Dr. Orla Hanrahan from Andor Technology.

Thank you to all of our online viewers for your great questions. I'm sorry we didn't have time to get to all of them today.

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For more resources related to today's discussion, please visit the URL now listed at the bottom of your slide viewer. This audio webinar will be available to listen to on-demand within the next 48 hours. We encourage you to share your thoughts about the webinar by sending an email to the address now in your slide viewer, webinar@aaas.org.

Thank you again to each of our panelists and to Andor Technologies for generously sponsoring today's educational seminar. Thank you for taking the time to be with us today. I hope you'll join us again to the next Science/AAAS technology webinar.

[1:08:14] End of Audio