Discovering Disease Mechanisms: Advancing Imaging in the Cell

Hello and thank you for joining us for this Science/AAAS webinar. I'm Sean Sanders, commercial editor and webinar editor at Science.

Today's webinar is the first of three exploring advances in imaging technology from cell to well to animal. Cell-based imaging is now ubiquitous in most academic laboratories and the data generated by modern microscopy systems are central to top quality research. Advanced microscopy applications including high-content imaging and live-cell imaging are now becoming mainstream, but choosing the most appropriate technology, both hardware and reagents, for a project can also be a challenge. This webinar will seek to explore the trends in cell-based imaging, both in terms of technology and application, with a special focus on fundamental disease research.

It's my pleasure to welcome to our speakers for today's event. Dr. Spencer Shorte from the Pasteur Institute in Paris, France and Dr. Klaus Hahn from the University of North Carolina in Chapel Hill in the United States. Each of our speakers will give a brief description of their work, after which we will have a Q&A session, during which our guests will address the questions submitted by our online audience.

Note that you can resize or hide any of the windows in your viewing console. The widgets at the bottom of the console control what you see. Click on these to see the speaker bios or additional information about technologies related to today's discussion, or to download a PDF of the slides.

If you're joining us live, you can submit a question to the panel at any time by typing it into box on the bottom left of your viewing console and clicking the submit button. If you can't see this box, click the red Q&A icon at the bottom of the screen. Please remember to keep your questions short and to the point as this will give them the best chance of being put to our panel.
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Finally, thank you to PerkinElmer for their kind sponsorship of today's webinar.

Now, I'd like to introduce our first speaker for this webinar, Dr. Spencer Shorte. Dr. Shorte was appointed academic research scientist at the University of Bristol in the United Kingdom in 1988, where he studied mechanisms of intracellular calcium signaling using novel optical imaging techniques. He then secured a European Commission fellowship to work in Paris, France, developing multidimensional optical imaging technologies for live cell and tissues in neurophysiology. In 1998, Dr. Shorte moved to the Medical University of South Carolina (MUSC) and then to the Pasteur Institute in Paris. In 2006, he founded and is currently director of the Imagopole at the Pasteur Institute. This is an infrastructure harboring four groups with expertise in microscopic, ultrastructural, and cytometry-based imaging technologies. Dr. Shorte is the author of more than forty research articles, five patents, and editor of the book “Imaging Cellular & Molecular Biological Functions”. Welcome Dr. Shorte. Thanks for being with us.

Dr. Spencer Shorte: Thanks very much for the kind introduction, Sean, I appreciate that a lot, and I also thank PerkinElmer and Science for actually hosting this opportunity to present. I’m very honored to share the stage with Klaus Hahn.

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So, I'd like to begin by giving an overview of some of what we do here in the Imagopole in the Institut Pasteur of Paris.

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Our focus isn't just cellular imaging. In fact, our focus extends far beyond that because our need and our aim of rule is to understand the molecular basis to infectious disease. And of course, infectious disease is founded in the interaction of the host and the pathogen and so this is a complex process that involves both in situ and in vivo visualizations so that means our experimental models have to extend into the in vivo context.
So, we're interested in integrated imaging technologies and to this end, the Imagopole is comprising several different groups that work around with themes of imaging from electron microscopy through to optical imaging in animals and in cells. We try to integrate that information, our ultimate goal being to provide integrated facilities for the Institute itself and necessarily too to provide ourselves a point of view based on the pipeline, if you like, between molecular and cellular understanding of those pathogen interactions.

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Now along these lines -- this is one of my favorite slides. I think anyone who knows me will probably say I probably overplay this slide. It's taken from the Florida State University Molecular Expressions website where they have a good representation of the scale of biology when it concerns imaging. So, as a biologist, we look with our eyes at a macroscopic scale and this can be clinical imaging for example where we see the overall behavior of a tissue, for example, we see whether a bone is broken in x-ray.

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But then of course in fundamental research, this extends all the way down to wanting to image, really write down the molecular level and that means at a single molecule level or necessarily at the structural level where we try to extrapolate the structural context of a macromolecule, a protein, or a virus for example.

And our goal ultimately is to try to integrate the levels of data at all of these different phases. So, of course, this means there's a whole heterogeneity of different technologies and very much we choose the technologies we wish in imaging to try to extend across these scales, so necessarily where we see genomics and proteomics in the fundamental form as 1D data, if you like. They only have meaning once they extend into the functional context and it's here that we see the notion of integrative biology, which is being underlined by imaging giving us a systems biology point of view on the way systems work.

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So in that sense, we really got to raison d'être to be working with imaging at the cutting edge. With state of the art imaging, we can really stretch between the molecular through to the cellular and the tissue and in vivo models.

Now, of course, in the last 20 years or so with the discovery and eventual Nobel Prize for the discovery of GFP, the fluorescent
proteins, it's clear that the value of these genetically encoded fluorophores is extremely high because they give us contrast. So fluorescence is all about giving contrast and everything we use it in is to try to distinguish contrast in the context of an experimental background.

Now, in this way, we can use genetically encoded fluorescent probes, but also we can use organic and inorganic probes, so Quantum Dots for example being a great example of a native particle that gives us the possibility for multispectral readouts that have high contrast. So, not to labor the point, of course, we're all very aware of the enormous impacts that luminescent and fluorescent probes have given us to date. And the very much the emphasis has been until very recently as being very much on fluorescence approaches and I'm going to try a little bit today to give a twist on that emphasis towards luminescent and bioluminescent approaches.

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Now, of course, with the luminescent probes, this will be nothing without the automation of the microscopy, so from the days of 20, 25 years ago, where an automated microscope may have had an electric stage on it for example. Today, a standard microscope in the laboratory could comprise a multiplicity of automated features that allow for much of the mechanics and the optical mechanics to be manipulated with name of scale accuracy. That doesn't necessarily mean so that we can extrapolate by sampling and sub-sampling from a cellular volume, from a molecular volume. We can actually subsample the volume in a rapid and automated manner that allows us to actually make conclusions about the spatiotemporal relationships of the high-contrast labels that we have inside our cell.

So necessarily in this context, we go from a wide-field microscope where even there, there is some degree of sub-sampling that goes on through to very, very complex microscopes, confocal microscopes, spinning-disk microscopes, point scanning microscopes and a whole host, a plethora of new technologies in microscopy that have bid on the same most recently. And I think we'll hear a little bit more about some of these as we go today.

The ability to combine these high contrast labeling techniques with this very, very high level of automated control on the microscopy, at the level of the microscope itself and the ability to accumulate that data very rapidly and handle the numeric and computational side of
this problem gives us an enormous facility from imaging techniques. And really I think is opening a new era of experimental biology.

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But there's no such thing as a free lunch. And in this context, we have to take on board that while there are many technologies such as fluorescence resonance energy transfer, which tells us about molecular proximity, fluorescence lifetime measurements, which allow us to make conclusions about the environmental context of the fluorophores necessarily, the disposition of a fluorophore inside the cell or in milieu. Fluorescence recovery after photo bleaching, optogenetics, manipulation of light to manipulate signals inside cells, which we'll hear a little bit more from Klaus later one. Fluorescence correlation spectroscopy, yet another technique that allows us really to get molecular information.

[0:10:01]

Now, despite all these very powerful techniques, there is an overhead and what I would like to do is use the time I have to explain what I think is a very simple rule that I think needs to be repeated ad infinitum when we're looking at new technologies for imaging. And in many ways, optogenetics and fluorescence bleaching, photoactivation, these technologies actually give us some sense of what it is where that first overhead comes.

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And -- I'll just advance. The first overhead or the most important overhead is really about photobleaching. When we look as we see on the dark contrast image on the left-hand side of the screen, we see a bead that is a subresolution fluorescent particle that's been reconstructed on a confocal microscope in the Z-axis. So we're seeing it as a planar image so instead of seeing a bead, we see it stretched out. If we translate that into the inverse and we look into a gel that's carrying fluorescent marker that was distributed homogenously, in the middle image of this series of gray level images, what we see is actually the bleached region, but that same objective. So it's made the image from the sub-resolution bead. The bleached region of that objects you create inside the milieu of the homogeneous fluorescent gel.

To the right-hand side, we simply see a change in objective so that the back plane is not overfilled with the objective by the laser and so the laser is really being fed in all its intensity through the objectives. So, by changing the numerical aperture of the objects, the magnification, we can change these bleaching characteristics. Now
these are extremely powerful, they're useful ways of manipulating light on a microscope, but they do not necessarily all give us good effects.

**Slide 10**

Because photobleaching is something that we understand, but necessarily it's not something that -- we can describe, but we can't necessarily understand what its downstream impacts are. So photobleaching is a process that we can understand in the context of this Jablonski plot here where we see the excitation of a photoelectron from its ground state into its excited state where it then decays and releases light that we can then measure. But there's a shortcut that the excited state may take and that means it's going to a triplet state and from the triplet state, necessarily this excited, high-energy photoelectron can go into non-emission states that can react with the very biology that you're trying to get a handle on.

So necessarily when bleaching occurs, when this is combined with certain reactive species such as molecular oxygen, you can end up with triplet states and necessarily free radicals. This can happen also with macromolecules inside the cell that are sensitive to the excited state of a fluorophore. So, by introducing a fluorophore, we necessarily increase the risk of generating free radicals.

**Slide 11**

Now fortunately, cells, biology, certainly high eukaryotic cells have evolved an enormous number of ways of dealing with free radicals. However, when we start generating them using high power lasers focused into tiny volumes inside the cell, we can overcome some of these mechanisms that are aimed to reduce free radical activities. And so it's necessary always to plan what is the overhead for light-budget is. So, light-budget is how much light can I put into the cell before it starts to feel sick.

So, here are a few examples, for people who want to download the PDF, of work that we believe actually represents probably the most important point of view on photobleaching and that is where photobleaching becomes phototoxicity. Actually, very little is known about the specificity of phototoxicity because it's actually rather heterogeneous, as I'll show in the next slide.

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So, in the case of a standard microscope experiment where we may switch the light on and illuminate a cell, we have to take into
consideration a number of important factors. Many of us know, even with a fixed immunostained sample, we know very well that the intensity of light and the duration that we leave that light on may very well cause photobleaching. Translate that into a living live cell system or into a tissue system then in fact this will necessarily have an impact on the viability status of the tissue that we're investigating.

This table summarizes a number of different other factors you may take into consideration when you're designing your live experiment. Necessarily, the wavelength you choose whether it's a blue wavelength or whether it's a red wavelength can have an enormous impact on the type of phototoxicity and the type of impacts you'll have on the biology. The type of cell that you may work on will also change enormously the type of phototoxic effects that you may see. But if you're working on neutrophils, you have very, very different rules too if you're working on epithelial cells from skin. The type of medium you choose, not just because it's autofluorescent in itself, you have to bear in mind that some medium, the very nutrients inside the medium can enhance the process of phototoxicity or reduce the process of phototoxicity.

So, given these elements, we've been working for some time now on trying to understand phototoxicity and we came up with a hypothesis, which is effectively a no brainer. It's a way of saying well if we could measure phototoxicity, we would see the health of the biology. And so in this sort of hypothetical napkin sketch, what you see in the green region is cells that are very happy and there is no phototoxicity. And of course as we accumulate phototoxic damage, we go into the amber and the red areas where in fact red represents cataclysmic cell death.

But it's not all about cell death because cell death is easy to see and that means we can stop an experiment and redo it with different conditions. What is difficult to see is actually when things are just impacted in a more subtle manner. Here we see embryogenesis in a C. elegans and in the top of at the T0, we see the comparison between on the left-hand side a C. elegans embryo in its first hour or first two hours of division that saw a low level of light from our spinning disk system, and on the right-hand side, it saw much more light. And the net result is that you can see after three hours of imaging that the number of nuclei i.e., the number of divisions that
have occurred has actually been impacted in the condition where we've got 30% or 2x more laser light going into the system.

So, these are ranges of light that we will be using in experiments. So, we've chosen these two ranges because they do make a big impact on the way that experiment is going to turn out. If we were to be measuring the number of nuclei in this embryo, we would have the wrong result when we're looking on the right-hand side because we've caused those effects.

**Slide 15**

So we've seen that effect for some time and with our collaborator Vincent Galy in Paris who's an expert in C. elegans. He kindly provided us with a C. elegans that was expressing a GFP histone binding protein and we set about the process of trying to quantify phototoxicity using the regularity of cell division in C. elegans as a means to actually measure how phototoxic is my microscope.

And our aim was to develop a protocol that would allow us very simply to move from one condition of seeing -- sorry, I've advanced that slide -- one condition of seeing the divisions happening in a 4D time lapse process. And of course 4D time lapse in terms of light-budget is a very expensive way of catching your data, but it's also a very, very high content manner of catching your data. It's very data rich. So, of course, there's lots of information that we want to catch about embryogenesis inside this paradigm.

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Necessarily by fixing the sampling frequency, so the types of images and the stacks of images we were collecting over time, we could then just increase the light power. And by function of increasing the light power, we could repeat this experiment 20 or 30 times. And you can see as in this figure here, that each of these little white dots is representing one experiment that ran for three hours where we counted the number of nuclei that were present after we have exposed the cells to a continuous Z stack acquisition during two hours. And at the end of this experiment, we simply counted the number of cells that were present inside the embryo and we compared this over time as we increased the light dose. And what you see is exactly the reflection of our napkin sketch. There's a period on the left-hand side, you can see there's a low dose of laser light, which causes very little damage and so we see similar values, the number of nuclei that we expect after two hours. And then after
Now, the higher level of laser light, we can see that this number begins to diminish rapidly.

Now, the most dangerous part of the experiment is of course where the value is around the point of inflection on this curve. So necessarily where the cells are not dead and the embryo is still dividing, but we don't really see it, and in fact, it's been slowed down radically by the light exposure.

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Now, what's nice about this approach is that you can then use it to quantify things. So actually reproducing this in two different experimental series, we were able to compare in this example. For example, we were able to compare the exposure time of 100 milliseconds with 500 milliseconds and this showed us immediately the long exposure times are actually less phototoxic than shorter ones. But, of course, we forget something and that is the signal-to-noise ratio so that means necessarily while a low level of light exposure over a longer time gives us much lower phototoxicity. Of course, if we need to get the same signal-to-noise ratio that we get in 100 milliseconds with a higher laser intensity then we need to actually get more light in that. So the quality of the image is not measured here, it's just the phototoxicity.

We can compare microscopes and aside from the parenthetical difficulty of making this work, in principle we can compare a wide-field microscope with a spinning-disk microscope for example and make a conclusion about the phototoxicity impacts that our microscope has. Now, what's important again is to underline that this is not a measure of the quality of the image that you would get out of the system because the quality for spinning-disk image is going to be much higher than your wide-field microscope in terms of confocality and sub-sampling of the cell. But you can see the wide-field microscope was giving you a lower signal-to-noise ratio, it's probably going to have a lower phototoxic impact. So you need to consider these elements when you're making your experiments.

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So, having said that, just a very parenthetical note here to say that an alternative to using fluorescence is of course to use bioluminescence. Bioluminescence uses chemical reactions to product photons from genetically modified enzymes, genetically expressed enzymes. So, we can take a genetically encoded enzyme
such as luciferase from the firefly and we can express that in a target cell or a target animal and necessarily measure light from that. And that's a very powerful way to sidestep this problem of phototoxicity.

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And just for the record, it's worth to note that in fact one of the major things that has happened recently albeit very quietly is the sensitivity of detectors now is such that it is possible to actually measure bioluminescence now rather simply from single cells.

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So that means we can express for example an aequorin protein inside an individual cell and use that then as a means to measure continuously light output representing calcium fluctuations in cells. Now, calcium fluctuation is very difficult to measure if anyone is in that field because once you use a chemical dye for example this can cause a lot of phototoxicity and make it a very difficult measurement to achieve. However, using a luminescence approach that can be done almost indefinitely over many, many hours, in some cases even days.

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We use this kind of approach, therefore, for looking at latency in viral infection so we can measure using bioluminescence from single cells during many, many days. And this way, we can actually see either activated or latent revival of viruses inside natural target cells. So here, we're looking at an HIV virus that's had the Nef gene replaced with luciferase. It's been used to infect natural target cells, lymphocytes, and macrophages isolated from human source and we can actually model the process of latency revival happening over time by using this kind of approach.

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So having said that, I point, you to a sketch made by Gaudenz Danuser, which reminds us of the need to keep in mind when you're changing the quality of the camera that you're using, if you're improving that, if you're changing the probe that you're using, or changing the time that you need to be making your experiment run for, necessarily these things all create overhead in light-budget. And the light-budget is for us at least one of the most important elements to control inside the live-cell imaging.
So, with that, I'd like to finish off by thanking PerkinElmer and Science Journal once again and the FP7 MEMI and all of our funding bodies and, of course, the Imagopole team who've given me an enormous amount of support to put this presentation in front of you. Thank you.

**Slide 25**

Dr. Klaus Hahn: Hello, this is Klaus Hahn from UNC Chapel Hill, I just want to say I really appreciate the opportunity to reach out and I hope that I can help a few people here.

**Slide 26**

This first slide, which I guess you're seeing, of our friend Godzilla symbolizes what I felt when I was asked if I could discuss in 13 minutes imaging living cells and animals and how that impacts disease research; clearly, a gargantuan task. And so I thought about that, I knew right away that I wouldn’t be able to talk about everything and asked myself what could be most valuable to you.

**Slide 27**

And when I thought about the field, I divided it in my mind into these three areas, instrumentation, computation, and molecular tools. And I really know and believe that we've witnessed a revolution over the last 15 years as Sean said in the introduction and it's come because there are independent developments in each of these areas, but more so because of the synergy between them. And you saw a little bit of that. I would mention briefly some examples where instrumentation and computation have merged to produce new capabilities, but focus most of the talk on molecular tools, which is what I understand best and perhaps can be most helpful in discussing today.

So, when you think about it, you know, originally people watched molecules move around by tagging them first with dyes and then with fluorescent proteins, then came really the enhancement of computational tools. So a good example of that is speckle microscopy where people, Clare Waterman and Gaudenz Danuser purposely incorporated two little fluorescent cytoskeletal proteins so that rather than staining an entire living organelle, a whole cytoskeletal fiber, you could visualize the flux of molecules through the fiber. That became really valuable when they developed computational tools to quantify the rates and directions, visualized fields where actin was moving in different directions and different subdomains that contained real biological information.
Then came variance of the fluorescent proteins that could actually be controlled, you can turn them on and off with light. That led again, in combination with computation, to these super-resolution tools, which I guess everyone is familiar with. Where you could see below like the limit of resolution of the traditional light microscope by enabling individual molecules to blink on and off and accumulate their fluorescence using mathematical techniques to specify or to identify the position on and on and on.

So, let me get that into one sub area here, which is how you can manipulate molecules and change them to your own ends and the three topics I list on the bottom are what I thought we could go over today.

First of all there's the most straightforward conceptually approach, which is simply tagging molecules and watching them and you want a bright fluorophore so that you can see and you want one that does not respond to environment so that you're strictly looking at the movement and concentration of your protein. Or, and then secondary I want to discuss, you can attach fluorophores or manipulate them in a way that they actually report back to you on the activity and environment of the protein that you're studying. And then the most recent development, you can go beyond that. While visualizing these movements in dynamics, you can do mechanistic studies by manipulating specific molecules in specific places and times. Because we're talking here about live cell dynamics and mechanisms involving very dynamic activity, so you can, with control of both time and space, manipulate one molecule and look at the effects on another.

So, I thought I would begin by asking the question why are we doing this. So, what are some of the exciting things that have come out of people imaging fluorescent molecules, biosensors, and living cells and there are I'd say four major groupings. First of all, the most obvious dynamic cell behaviors. Some things in the cell are best studied in real time. Cells move, subcellular organelles move for example trafficking of vesicles, the mitotic spindle. Then there are some more subtle things that have come out. I love this example of the plasticity of signaling networks given an individual molecule can have essentially opposite functions. It can induce proliferation or cell death. And the reason that happens is that you have subtle control of the localization and kinetics of activity in a cell only visualized by
studying living cells and not by mashing up the cell and doing biochemistry.

That brings us to the final point on this slide, which is that those individual cells behave as individuals. So, if you average the population as in biochemistry, you may get erroneous results. A great example of that is looking at the tumor necrosis factor alpha induced translocation of NF-kB in the nucleus. When you look at cell populations in fixed cells, you see a movement into the nucleus. You simply assume it's induced by tumor necrosis factor alpha. If you look at the little graph there and each color represents a different cell, you see that the cells are actually oscillating with the NF-kB moving in and out of the nucleus, and each cell oscillates at its own rate and the biology is driven by the oscillation. So, only by looking at individual cells can you understand the biology here.

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The next slide we're going to try to use a movie. I hope -- let's see if you're getting that slide. I hope Sean can show you this movie here. There are a lot of movies in my talk. Okay, there we go. So, the final point I wanted to make is that there is a big difference between protein activation and localization. Here, you see a biosensor for RhoA reflects the activation of this protein and on the left, you see that the RhoA is primarily around the microtubular organelle in the center, that's where it's localized. But if you look where it's actually in the active form, you see that the highest activity is in this very fine line at the edge of the cell. In fact, by looking at the left, you can tell that there is almost no Rho protein there, yet that's where the action is. So, the biosensor lets you see where the important subspecies, the very small concentrations in some cases of active molecule are localized.

Slide 30

Let's see. So, what I thought I would do now is try to categorize the different designs that people have used. A great fun in this field is to treat the proteins like molecular legos and try to turn them into all sorts of different engineered tools that can report to you. And I want to show you a little gallery of those I think that represent the major subtypes and even encourage you to build these on your own. And then at the end, I'll talk a little bit about what technical issues you will encounter if you try to use them on your own.

[0:30:16]

So, the simplest or perhaps the easiest and in many cases the best for that reason are the translocation biosensors. These are really
useful if you have a molecule that cannot be labeled with a fluorophore. For example, PIP3, which is synthesized and degraded whose concentration has to be monitored without direct labeling. In this example, from Devreotes lab – actually, the Devreotes example I'm going to show they used a CRAC domain. But you take various domains that bind to say a lipid, when the lipid accumulates in the cell membrane, the domains translocate there and you see a buildup of the associated GFP. And all you need to do is see the localization to know where and to what level the PIP3 has been produced and that's shown on the next slide here, which I hope you're getting as a movie now.

**Slide 31**

And what you'll see is when the chemoattractant is added to these cells that you'll see an accumulation of this GFP in the membrane, very obvious to the eye. You can certainly use this to look at kinetics. The tricky bit though is quantifying the levels. You have a lot of artifacts in the cell in just examining concentration. That and certain kinds of enhanced sensitivity have led people to use FRET instead.

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And I wanted to now show you from simplest to perhaps the most complex the various types of FRET biosensors. So, perhaps the simplest is to just look at dissociation of FRET, you look at a substrate and you watch it being cleaved and that's been useful and commercially available, many types. There's a subgroup in which people attach fluorogenic dyes to one portion of the substrate peptide and some sort of structural change of the peptide produces a fluorescent change. But the FRET biosensors, of course, have the big advantage that they're genetically encoded.

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The next slide shows a more complex design. There are now many of these and it's a different kind of substrate-based indicator in which the molecule that you're trying to study acts upon the substrate and the substrate reflects the activity with a change in its FRET. Here for example, you have a peptide that is phosphorylated by a kinase and you're studying this kinase. So, you have a peptide linked to a domain that binds only to the phosphorylated peptide. Nothing happens until the kinase phosphorylates the peptide bringing the two fluorescent proteins together and generating FRET.
And the final subtype right here I'm going to focus on in some more detail because I have examples that I can use to illustrate some basic points about biosensors and imaging. I would say this is another large class that you might say is based on affinity reagents, a term that some biosensor people use, but it nicely illustrates the concept I think. So, if you look at the gray protein at the top, it has an inactive and an active confirmation, the round ball and the square. You find yourself some sort of a small proteinacious unit, say a fragment of a downstream, a vector, an antibody fragment, something that binds only to the active confirmation. And you put one fluorescent protein on your target and another on this binding unit, this affinity reagent. So, wherever in the cell the targeted protein takes on the active confirmation, this affinity reagent binds and you generate FRET.

And importantly, I think there's representatives of both two different kinds of designs, one in which the two chains are separate and shown at the top and another in which they're unified as a single chain. And those have important advantages and disadvantages.

I want to point out one thing first. If you look at this top example, you might say to yourself, why bother with the FRET, we have here this blue affinity reagent. Wherever the active protein is found, the affinity reagent binds and will see this translocation like I showed you with the PIP3. That blue thing is essentially the translocation biosensor.

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But in the next slide, you'll see why people do trouble with FRET and what its advantages are. So, here, we see the same study where on the left, we're examining the FRET between the affinity reagent and the target, which happens to be an activated Rac. And on the left, we're simply looking -- I mean on the right, we're simply looking at the fluorescence of the affinity reagent. You can see on the right that if you -- and what we see there as that red line in the left image is a ruffle, which contains a lot of active Rac. So in the FRET, it's very easy to see because as Spencer was saying, it's all about contrast and you see this tremendous contrast between the activated species and the ruffle and the surrounding background. If you look at the right at the translocation sensor, if you know it's there you see the ruffle, but the cell has a really thick part neighboring the ruffle where you have a large accumulation of this unbound translocation biosensor and you're trying to see one color over a background of the same color so nothing is apparent.
So, it's this contrast that provides the enhanced sensitivity of the FRET and it's important to point out that the FRET, although it has a better contrast is substantially dimmer than translocation, and those are two different aspects of signal-to-noise that you have to trade off. And I tried to emphasize these things in the next slide.

Slide 36

I don't want to run out of time here so I'll just do this really quick. This slide, I think, primarily contrasts the different between the single-chain FRET and the dual-chain FRET designs. In the top, the single-chain FRET is on slightly. You'll see FRET even when the protein is in the inactive form because the two fluorophores are literally linked together. In the bottom in the middle picture, the dual-chain FRET, when that biosensor is off, it's truly off. So what you have is greatly enhanced sensitivity by using the dual-chain and cutting the chains apart. The dynamic range, the change from on to off is really a function of the signal-to-noise of your microscope system because you go from nothing to whatever fluorescence you get out. You could define it as essentially infinite, but it's really bracketed by what kind of signals you can detect.

There are big differences though in ease of use. The top one is a single gene and the two fluorophores will distribute evenly at the same concentration at any point in the cell. So, you don't have to do the substantial image processing that's required to correct for these differences in distribution.

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I wanted to end this discussion of biosensors with this last slide. For those who really want to get into this and make their own, I think it's important to point that there are certain caveats and ways to look at these things so that you don't perturb the cell. It's almost like the Heisenberg uncertainty principle of cells, you can't stick one of these biosensors in there without somehow interacting with the cell if you want to sense it and having a biological effect.

So, a good biosensor also if you get them from other people, you can evaluate them in your system to see if they do these things in the particular cell type that you're working with. But a good biosensor will not compete with the binding of upstream regulators because what you're trying to sense here is the activation of the protein so you want those upstream molecules to be able to interact. So, given that you have to interfere with something except in a few rare cases where you have a big protein and you can bind your biosensor
without competing with anything, you probably have to compete with a downstream effector and you want to pick one that's not involved in localization of the target.

And the last point to make is that, the two designs, the single chain and the dual chain in most cases have different effects. If you use the single chain, you have to watch out for dominant-negative effects because you'll bind your biosensor and the effector on it, come in and block interactions with downstream effector. You will have essentially generated the dominant-negative reagent and as with all biosensors, the expression level is everything. So if you can work at a low level where you can sort of sense activity without perturbing too much, you're all right. With the dual chain, a more sensitive sensor, an Achilles heel is that it's easy for the negative endogenous effectors to compete away the second chain of your biosensors so you're more prone to false negatives.

Slide 38

I just wanted to end -- sorry, there's one more slide on biosensors. Expression is everything. People call us and they ask about using biosensors. When they have trouble, it almost always have to do with cell health usually mediated by phototoxicity as you heard in the previous talk and the expression levels effect on biology. And I have here an example where a Rho biosensor was showing no response. And this particular biosensor is normally this molecule that it was sensing is regulated in that the inactive form is held in the cytoplasm by GDI and the active form is brought to membrane where it could be activated by GEFs, so it's a GTPase biosensor.

When we actually looked at the cells that were not responding, we saw that all of the inactive biosensor or the biosensor in the unstimulated cells was in the membrane already. So that clued us in that we had expressed so much that we were overwhelming this GDI that was supposed to hold it in the cytoplasm. And the biosensor was essentially all on right from the beginning. So, by co-expressing more GDI, as shown in the middle panel, or by using lower expression levels, we drove the inactive state into the cytoplasm and we saw normal cell behavior.

With the little time I have left, I guess I have about six minutes, I'm going to hit a second topic and that is this more recent development, which is the ability to actually manipulate activity in cells.

Slide 39
In a very broad overview shown on this slide, sort of a little bit of a historical perspective, this began years ago, maybe 20, 30 years ago with chemical approaches. So almost all of those who used this ortho nitrobenzyl group that you see here caging glutamate in this one example, but the idea is the same everywhere. You have a hetero amino on a peptide or a protein and you have to go through this somewhat arduous process of specifically labeling your protein if you want to do that or perhaps not so difficult to label a small molecule, and you block an essential functionality.

Upon irradiation, this chemical group it comes off and frees the peptide. And the Achilles heels here are that you have to load this chemically derivatized things into your cells often requiring injection or cumbersome techniques like that. And for proteins especially, it’s difficult to do the essential site specific labeling. Also, most of these have to be irradiated at less than 400 nanometers, usually around 350, 360 so it can produce more phototoxicity than other approaches.

An exciting revolution that almost everyone probably is familiar with is optogenetics, the method of the year this year in "Nature Methods," I believe in "Science" as well. And this is specifically directed at channel proteins, which can be used to control the conductance of ions in and out of nerves. It’s been extended all the way out to actually manipulating the behavior of animals by using fiberoptics and shining light on specific regions of the brain.

What I thought I would emphasize in the last few minutes is something that I'm most familiar with so hopefully it can be most helpful to you on and that is the extension of this technique to now control cytosolic proteins, things besides channels. And the two domains that have seen the most use are the Phy domain and the LOV domain and they operate differently and therefore have different advantages and disadvantages.

So, the Phy domain binds to PIF when you shine a specific color of light on it, IR light. And the big advantage here is that they remain stuck together until you irradiate with a different color of light, further red wavelength. And that allows you very precise control of activity.

So for example, the paper that I quote here were Voigt, Lim, and Weiner and their labs worked together to control GTPases by driving them to membrane. So one member of the pair was anchored in the
membrane, the other attached to the GTPase and upon irradiation, you drove the GTPase into the membrane through this association. And they were able to irradiate their cells with two different colors of light so whenever a molecule drifted into a second zone, it would come apart or be brought together. So, they had very precise control of their localized activity.

The other domain, the LOV domain, which is the one that my lab has focused on along with multiple other labs is based on a different principle. Basically, it's a protein that unfolds when you irradiate it. And we have used it as a steric blocker as shown here for Rac1 where you attach it to Rac1 when you irradiate, it unfolds and allows the Rac to interact with its effectors. Its advantages, I'd say, is that it's simple to use and it's found a lot of uses in animals. Basically, when you shine a light on it, it unfolds and the protein is turned on. When you turn the light off, the LOV domain closes again and the protein is off. So, you have a very simple switch that you can use.

Slide 40

We go to the next slide here. So, I'm going to go into this in a little detail and then stop talking here, at least temporarily. So, this shows the structure of the LOV domain and it has a globular portion here, the LOV2 core shown in green and there's a flavin. Another advantage of this for animals for example is that this is a naturally occurring co-factor. So, all you have to do is express this protein and it incorporates the flavins. Irradiation anywhere between 400 and 500 nanometer, which is typically available on many microscope systems will cause an interaction of the globular domain cysteine with the flavin leading to a breakdown of hydrogen bonds that hold this blue J alpha helix in place. So, you can think of this as almost like a yoyo. If you look on the right, here's your target protein. The LOV domain is on there with a short string, the J alpha, irradiate, the string is lengthened relieving the steric block, the target protein can do what it wants. You turn the light off and it snaps close again.

Slide 41

And on the next slide is a movie that I wanted to use to illustrate what this actually looks like to prove to you that it works. So, what you see here, I can't see this, but you should be seeing a laser spot moving around activating photoactivatable Rac and Rac is a protein that induces protrusion and polymerization of actin. And so you see very precise localized control. Because it's reversible, wherever the spot goes, you see a protrusion and as the spot moves somewhere else, that part of the protrusion dies down in that part of the cell.
To make a long story rather short, here's one slide representing our research in this area. So, we tested a hypothesis. Let me see if I can get that out there. No, I wanted to go to a different slide. Try this one. There you go. The hypothesis was, you know, put forth by a lot of modeling studies and other previous work that a gradient of Rac was sufficient to generate cell movement at a specified direction. The cell was polarized and chemotaxis and other phenomena were driven by a gradient of high activity on one side and low on the other.

So, where you see the yellow spot on the left, we activated Rac in the cell and sure enough we got not only protrusion around this spot, but on the opposite side, we saw a retraction. We were wondering whether this was simply a pulling of the front of the cell on the back or whether the gradient was responsible. So, we made a dominant-negative version of the Rac to inhibit Rac and we turned that on with light, that's in the right-hand panel. Now, you see where the yellow spot of radiation is. We have a nearby retraction and then on the opposite side, we have protrusion. So, that's been published, the biological issues there. But perhaps I think one of the most important aspects for future work is shown in the movie on the next slide.

And there you see because Rac controls movement simply by specifying this gradient. If you have a spot of light on one side of the cell, you can get it to move around like a horse chasing a carrot on a stick.

And that led to many interesting studies in animals because people could then control the movements of cells and groups of cells within living tissues and animals. And I have two examples of that.

This was done by our collaborators. Here, you can see first the work from Denise Montell's lab at Hopkins. So, she was studying order cell migration in the ovary of drosophila and found that if she activated one cell in the group of these border cells, that the other cells would follow, that they would move. So, she was able to study this coordinated migration, tried to move the cells into different parts of the embryo, finding forbidden zones, regions where the cells could
move, and looking more closely at the group of cells and understanding how they communicate as one cell in the group is induced to move.

**Slide 46**

The next slide, let's see. The next movie I should say is from Anna Huttenlocher at the University of Wisconsin in Madison. I couldn't resist showing this. This is a cell, neutrophils in zebrafish. Anna studies the trafficking of neutrophils in and out of tumors and in this particular case, you're seeing the cells spell the word Rac for us, which is both fun and illustrates this very precise control that you can achieve.

**Slide 47**

Finally, I just wanted to close with two slides that show you a little bit how you could do this yourself if you're interested, how we actually engineered these things. So, this is the generation of the caged Rac that I've used in these examples. So, we shouldn't have a movie at this point. Hopefully, I'll just have a slide here now. There you go. Okay.

So, the first job you want to do is turn off any upstream regulators. You want to be solely in control of this protein with light. So, you introduce point mutations to knock -- or just use a fragment of your protein, knock out any upstream effectors and this was quite complex for Rac, it interacts with GEFs, GDIs, and GAPs. So, we did point mutations to knock out those interactions. But then what you have to do is attach this LOV domain I've shown in the upper right-hand corner so that it blocks the interaction with effectors, the one interaction that you left in your molecule.

**Slide 48**

And we found the most effective way to do that is shown on the next slide with the sort of mini-screen. And it's been primarily a matter of the linker between the LOV domain and the targeted protein. So, what Yi Wu did, who did essentially all of this work, is he took the linker between the Rac and the LOV and varied its length and also chewed a little bit into the J alpha helix and into the Rac itself, and asked how those affected the ability of PAK, a downstream effector to pull down the modified Rac.

And we were looking for an example, we have mutations, which are published of the LOV domain that leaves it always in the lit or always in the dark form. So, you can use an always closed, dark version of
this test protein and ask when you can effectively inhibit interaction with the effector, when your pulldown goes away. You see there at the bottom the variations in length. I won’t go through this whole slide, but if you look at the very right-hand two lanes, you see the difference between the lit and the dark forms when we found the optimum length.

Slide 49

And I’ll just conclude now by saying thank you first of all to Science and AAAS and the sponsors. And I certainly want to thank Yi Wu, Ollie Pertz, and Lou Hodgson who produced all the data that I showed as examples, the funding agencies behind our work. And in case, you’re really interested in doing these things yourself, I wrote down here the two methods or articles that we published on the biosensors and the other. Thanks a lot for your time.

[0:50:09]

Sean Sanders: Great. Thank you very much, Dr. Hahn, and my sincere apologies for the lack of introduction. I was experiencing some technical issues. Viewers can see Dr. Hahn’s bio by clicking the blue speaker bio widget at the bottom of their viewing console.

Slide 50

So many thanks to both of our speakers for their excellent presentations and we’re now going to move on to the questions submitted by our online viewers. A quick reminder to those watching us live that you can still submit questions by typing them into the ask-a-question box, the text box and clicking the submit button. If you don’t see this box on your screen, just click the red Q&A icon and it should appear.

So, the first question that I’ve received that I’m going to put to both of you may be starting with Dr. Shorte is how important is it for a new core microscopy lab to consider getting a microscope equipped with super-resolution capability and what considerations are important in making that decision?

Dr. Spencer Shorte: Probably, a primary consideration has got to be what is the context that the core facility is... [0:51:11] [audio glitch]

Sean Sanders: Dr. Shorte, we are having a little bit of trouble hearing you. I wonder if you could speak up a little bit or get closer to the microphone?

Dr. Spencer Shorte: Do you hear me better now, is that...?
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<th>Sean Sanders:</th>
<th>That's much better. Thank you.</th>
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<td>Dr. Spencer Shorte:</td>
<td>Okay. Probably, the primary consideration will be to look at what the core facility is already doing. It's sure that super-resolution technologies bring something important to the stage and that I would feel, my feeling is that the super-resolution will be a standard approach in labs in the next few years in the same way a confocal microscope is today. So that's the fundamental change in the way that we do things from day to day, but before that happens, super-resolution technologies have got to be a little bit more accessible and do the same things that a normal confocal does.</td>
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<td>So, we see a lot of utility for super-resolution approaches for both immunofluorescence so that means they're fixed cells or they're not live experimentation and ultimately, we would hope that those same technologies could be applied to live. So, if the idea is to go to live, maybe a core facility is not the right place right now unless there's a specific project to drive it, that would be my general feeling. So, it still seems a little fiddly and there are lot of considerations concerning everything from phototoxicity through to actually whether you win very much resolution as well. A lot of this technology is not applicable to live cell paradigm so people who do live cell are going to be a bit frustrated with the time it takes to get a STORM image or a PALM image so it's not very applicable to live. STED can be very phototoxic so there have to be big considerations there.</td>
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<td>So, this is not routine stuff and if you're running a core facility, you really do need routine to happen. So, I would say that if it's a cherry on the cake, something separate then one could look at the structured illuminations, solutions that are around there I think would be important ones. They seem to have a broad applicability. And ultimately, wait a little bit and see what evolves in the coming months to see whether there's a good commercial solution out there for a core.</td>
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<td>Sean Sanders:</td>
<td>Great. Thank you very much, Dr. Shorte. A question for you, Dr. Hahn, this is related to your presentation. How difficult is it to make these GFP biosensors that you talked about.</td>
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<td>Dr. Klaus Hahn:</td>
<td>That's a really important question and it depends very much on the molecule and I think the whole field is moving towards trying to produce high throughput versions of the technology. A lot of those</td>
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are focused on using screening approaches like using phage display screening for example and having a fixed scaffold. So, you have a scaffold whose binding to a given target is altered, most of it stays constant and a few loops are varied via the screening to find one that binds to the active state of your protein. Because the whole scaffold is constant, you're already set up to build in the fluorescent proteins or the dyes or whatever. And I think right now, you know, it's hard to give an estimate, but I would give it a number of months to make one of these things the old fashioned way. But I think turn-the-crank methods are on the minds of multiple labs now.

Sean Sanders: Excellent. So, another question for both you, with all the multidimensional images, it's very challenging for image data management and analysis. Can you describe some of these challenges for you in particular and how you've addressed them and any developments you might see on the horizon? Maybe we'll start with, Dr. Shorte.

Dr. Spencer Shorte: In short, I would say right now, there are not that many solutions out there. There are certainly very few commercial solutions and there are a few open-sourced solutions. Among these is the open microscopy environment, which is a very powerful means to manage large data problems so running up to a hundred, 200 terabyte of images and you need to share that and store that. This is a good solution.

[0:55:04] PerkinElmer itself provides a solution based on the open microscopy environment and that's very encouraging because seeing commercial organizations actually taking hold of what's out there in the open source means that we get the equilibrium between the open source solution and the reliability of your commercial solutions. So, there are solutions evolving and I think there are big visions underlying that some of these including extending data management from imaging to include data management in second-generation sequencing for example and start to actually data link across these.

Sean Sanders: Dr. Hahn?

Dr. Klaus Hahn: Oh, I think a second aspect of that is sort of where the whole field is evolving now. It was in the past that you would create a new biosensor, a new fluorophore and qualitatively observe what it does and learn a lot and then be somewhat stymied because the behaviors beyond that are too subtle for the human eye. So, I think
the best work now comes from coupling the fluorescent tool with new modes of analysis and most of those seem to be tailored to the individual project. So, when you work with someone who does modeling or who does image analysis, I think you have tremendous potential. That seems to be the next wave.

I was thinking of putting this in my slides, but I should mention that there are now packages of software and labs that are out there whose job is to make those sort of things accessible to you. So for example, the NIH funds the Laboratory for Fluorescence Dynamics, which I make a lot of use of. That said, you just Google LFD and you'll see it and there's a big array of software there and a lot of tools. The ImageJ package is free. You're probably familiar with that, also funded by the NIH. And then there are individual examples of labs doing great work where they apply analysis. I would point to Gaudenz Danuser or Tim Elston. But I think the future lies in quantitative analysis of the imaging for sure.

Dr. Spencer Shorte: Yeah, I'd agree with that absolutely, Klaus, it's a very good point. As I said, Bob Murphy, his name is with that list of people out there doing great computational science as well.

Dr. Klaus Hahn: I regret that I didn't mention him. Probably, there are a lot of people that we could list.

Sean Sanders: Uh-hum.

Dr. Spencer Shorte: Yeah, that's a very good point. So, this question about data management has to do with the legacy of the data and right now we have an enormous attrition in data generations. We pay a lot of money to generate these images and then actually they deteriorate because we can't recuperate the information. And actually, the computational scientists are crying into their handkerchiefs because that's an enormous resource for systems biology if only we could connect the pipelines properly and I think NIH maybe one of the organizations who could do that.

Sean Sanders: Great. So, we are coming to the end of our hour, but I'd like to squeeze in a couple more questions and hopefully our audience can stay with us. I'll put this to you, Dr. Shorte, can you comment on whether you've used 3D image analysis and where you see it might add to our understanding of disease?
Dr. Spencer Shorte: In fact, 3D image analysis is basically our day-to-day now. I think when I began in this game, nobody did 3D image analysis and it seems quite cooky to build a 3D imaging system and a bit of a waste of time. Today, actually of the 25 or 30 microscopes that are running every day in our labs, all of them are doing 3D with almost no exceptions at all. So, I think it's fundamentally for cellular work, tissue, and for fixed cell work. This is a fundamental part of what we do.

For live cell, it adds an overhead as I've mentioned during my presentation, which one has to handle very carefully in order to avoid spinning one's wheels for a very long time and not actually getting anywhere because you have a lot of data. So this can be done by, I think, investing carefully in the types of microscopes that will give you a payback of low phototoxicity combined with 3D, multidimensional data readout.

And that's definitely what we see our future is automating that, making that a smooth easy process. And ideally as Klaus referred earlier, I think actually automating the throughput of this, so getting a high content data with the throughput, so in his case talking about production of probes, in our case, getting the data, I think we'd like to see that as almost a hands-off process that could be really automated.

Sean Sanders: Excellent. So, just a final question to both of you, and we can start with Dr. Hahn on this one, how important do you think translational imaging approaches are for the future of disease research?

Dr. Klaus Hahn: Oh, I like to think very important. I'm a basic scientist, but obviously the end goal is to really have impact on human health and on our world and there are more and more examples of that. You know, really an odd side issue is that the big revolution has been the genetically encoded fluorophores and that's precisely what you can't use in humans. So, I wonder if we won't have a renaissance in the dye-based approaches eventually and I see that happening at some meetings and in labs as well. But I think certainly there is a bright future there.

[1:00:08]
Sean Sanders: Dr. Shorte?

Dr. Spencer Shorte: Yeah, that's a very, very good point. I think that, you know, there are very few fluorophores that are approved for use in humans, two I believe. And that's where things are going to change. I think the
chemist, there is a renaissance in chemistry happening right now. A lot of great chemists are turning eyes on to the problem of dealing with how can we generate fluorophores that can be used in vivo. And that's a critically important question, I think both for the fundamental biology and also for what might come from that downstream.

With regards to translational research, our public health mission at Pasteur obviously defines that, but we see that as being something that's driven by the fundamental research that's the priority. There needs to be liberty within the fundamental research, which drives what can then be harvested subsequently. And the process of harvesting is obviously translational research. So, actually, one of our groups is dedicated to the process of translational research in both senses, bench to bedside and bedside to bench so.

Sean Sanders: Fantastic. Well, unfortunately, we have run out of time for this webinar so I want to thank our speakers for providing such interesting talks and discussion, Dr. Spencer Shorte from the Pasteur Institute and Dr. Klaus Hahn from the University of North Carolina at Chapel Hill.

Dr. Spencer Shorte: Thank you very much.

Dr. Klaus Hahn: Thanks very much.

Sean Sanders: And many thanks to our online audience for the questions you submitted. I'm very sorry that we didn't manage to get to all of them. But please go to the URL at the bottom of your slide viewer now to learn more about resources related to today's webinar. I'm going to put that slide up for your right now.

Slide 51

We will be having an additional two webinars related to this imaging series and they will be up on our Science website, www.sciencemag.org/webinar. This webinar will be made available to view again as an on-demand presentation within approximately 48 hours from now.

We'd love to hear what you thought of the webinar, just send us an email at the address now up in your slide viewer; webinar@aaas.org.

Again, thank you to our panel and to PerkinElmer for their sponsorship of today's educational seminar.
Goodbye.

[1:02:24] End of Audio