

Untangling the Tumor Microenvironment: Illuminating the complex interactions and functions of immune cells Webinar 10 December 2014

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Sean Sanders: Hello everyone and a very warm welcome to this *Science*/AAAS webinar, "Untangling the Tumor Microenvironment: Illuminating the Complex Interactions & Functions of Immune Cells". My name is Sean Sanders and I'm editor for custom publishing at *Science*.

It has been found in recent years that certain antibody techniques have demonstrated the potential for directing a patient's own immune system against humans.

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Further advances in this area will depend upon a detailed understanding of the tumor microenvironment and characterization of the location and status of immune cells and their interaction with tumor cells.

This requires methods that provide phenotyping of immune and cancer cells combined with information about the spatial relationship in tumor regions. Additionally, a deeper understanding of the signaling cascades active in immune recognition of cancers is crucial.

During today's webinar, we will be discussing the bringing together of multiplex fluorescent immunohistochemistry, advanced microscopy techniques, and bioinformatics. We will look at how these are enabling new insights into cancer biology and immunology.

It gives me great pleasure to introduce our speakers to you for today's event. They are Dr. Scott Rodig from Brigham & Women's Hospital and the Dana-Farber Cancer Institute in Boston, Massachusetts, and Dr. Ed Stack from PerkinElmer in Hopkinton, Massachusetts. Thank you both very much for being with us on the line today.

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Before we get started, I'd like to share some 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 a "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.

After speaker presentations, we will have a short Q&A session, during which the speakers will address some of the questions submitted by our live online viewers. So if you are joining us live, start thinking about some questions now and submit them at any time by clicking the "Ask a Question" button below the slide window, typing the question into the message box, and then clicking "OK".

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 icon at the top of the screen. For tweets, you can add the hashtag #sciencewebinar.

Finally, thank you to PerkinElmer for sponsoring today's webinar. Now, I'd like to introduce our first speaker, Dr. Scott Rodig.

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Dr. Rodig joined the faculty at Harvard Medical School in 2004. He is currently an associate professor in the Pathology Department at HMS and an associate pathologist at Brigham and Women's Hospital. Dr. Rodig is also a consultant pathologist at the Dana-Farber Cancer Institute.

His interests are in translational and clinical research in hematopathology and molecular pathology with an emphasis on the development, validation, and implementation of new biomarkers to improve diagnostic practice. A very warm welcome to you, Dr. Rodig.

Dr. Scott Rodig:

Thank you and good day. I'd like to thank the organizers for giving me the opportunity to discuss our work using multiplex immunofluorescence staining and image analysis to unlock the complex biological information that is encoded in fixed tissue biopsy samples from human patients.

A detailed and quantitative assessment of the tumor microenvironment, we believe, will better guide targeted therapies for the treatment of common cancers.

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Here are my disclosures.

Slide 5

I am primarily a diagnostic surgical pathologist and as such, I use a range of tools both simple and complex to analyze primary tissue samples and to diagnose disease.

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Today, I will focus on some novel techniques that allow us to better examine tumor cells microscopically within their natural microenvironment. These are at heart an extension of basic histomorphology and chromogenic immunohistochemistry.

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Over 90% of tissue biopsy samples taken by a surgeon are placed into formalin which cross-links cellular proteins and stabilizes tissue structure. Tissue is then processed for dehydration and further fixation, embedded in paraffin wax, and sectioned very thinly and placed on glass slides.

Application of chemical stains to the tissue section such as hematoxylin and eosin allows us to visualize the cellular structure of the tissue under white light microscopy.

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Thus, histomorphological evaluation is an efficient means to define the cellular composition, cellular morphology, and tissue architecture.

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Despite its technical simplicity, the visual information on an FFPE tissue section stained with an H&E is information-rich and essential for essentially all diagnoses in surgical oncology.

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An important ancillary test for analyzing primary tissue samples is immunohistochemistry. By this method, a select antibody is applied to specially prepared, formalin-fixed, paraffin-embedded tissue sections. If the antibody recognizes the protein of interest, it remains bound during a series of washing steps and is then detected with secondary reagents that include chromogenic dyes.

In this case, a B-cell lymphoma, the cells stain positively as indicated by the brown color for the B-cell lineage marker PAX5. The surrounding non-malignant T-cells in this case do not stain for PAX5 and are blue with hematoxylin counter stain.

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Thus, simple histomorphology under light microscopy allows us to identify specific cell types and tissue architecture in a qualitative manner.

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This is often enough to diagnose cancer in general and even specific cancer types such as lung adenocarcinoma.

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But judicious use of monoclonal antibodies amenable to IHC allows us to further subtype cancers, most commonly according to their cell or tissue of origin.

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Oncologists can use this information to select two more specific chemotherapies for their patients.

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But of course, cancer is a complicated biological system that is driven by multiple genetic, epigenetic, biochemical, and microenvironmental mechanism.

With the accumulated insights from oncological research, the pharmaceutical industry has developed many agents that effectively

target one or more of the signaling pathways that can be dysregulated and contribute to oncogenesis, but every cancer is different and any one cancer is unlikely to respond to an individual targeted therapy.

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Therefore, we need to guide targeted therapy by identifying the dysregulated signaling networks in the tumor microenvironment. Some signaling pathways are so intrinsic such as activation of the B-cell receptor in B-cells. This can occur in normal B-cells as it would occur in an immune response or can occur abnormally in a B-cell lymphoma.

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Other signaling pathways are extrinsic to the tumor cells and such signaling networks include those that govern anti-tumor immunity.

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An understanding of the multiple signaling pathways allows us to select among multiple agents in a rational manner for our individual patients.

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Capturing this network of complex biochemical and cellular interactions requires us to identify multiple proteins and multiple cell types simultaneously and in a quantitative manner.

Multiplex immunofluorescence coupled with digital image analysis fulfills these needs. This approach allows us to perform co-localization studies, visualize signaling networks, quantify protein expression, and quantify cellular and architectural features.

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In the first example using this approach, I will discuss detecting B-cell receptor signaling in a common and aggressive non-Hodgkin lymphoma termed Diffuse Large B-Cell Lymphoma.

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In vitro analyses first performed by Margaret Shipp at the Dana-Farber and Dr. Lou Staudt at the NIH revealed that many large B-cell lymphoma cell lines have constitutive signaling through their cell surface antigen

receptors. At least some cases, this activation is independent of antigen stimulation. Those cell lines that exhibit this quality are dependent upon B-cell receptor signaling for growth and for survival.

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It was unknown whether the signaling cascade active in tissue culture cell lines also occurs in tumors in patients, yet several companies had developed pharmacological inhibitors of B-cell receptor signaling. And so, these drugs were tested on patients with relapsed and refractory Diffuse Large B-cell Lymphoma.

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A substantial subset of these patients showed a response, yet it was less than 50% of all patients.

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We therefore ask the following questions. First, can we detect constitutive B-cell receptor signaling in tissue biopsy samples from patients with Diffuse Large B-cell Lymphoma?

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Second, how common is BCR activation in Diffuse Large B-cell Lymphoma?

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And third, does B-cell receptor signaling correlate with the cell-of-origin of the Diffuse Large B-cell Lymphoma as defined by gene expression profile?

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To address this question, we use primary antibodies in a multiplex immunofluorescence assay. These antibodies recognize three critical signaling molecules in the proximal B-cell receptor signaling cascade, pLYN, pSYK, and pBTK. The antibodies recognize phosphoepitopes in these protein kinases only when they are biologically active.

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After co-staining the tissues with these antibodies and a lineage-specific marker to identify the tumor cells, the slides were digitally imaged and subject to a variety of image analysis algorithms and allowed us to identify individual cells including their nuclei and cytoplasm, and to quantify the expression of the biomarker of interest in each compartment.

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We validated the protocol on formalin-fixed, paraffin-embedded cell lines that are either unstimulated or antigen-receptor activated. These cell lines are shown here and subdivided into those that are either sensitive, the top three cell lines, or insensitive, the bottom line, Toledo, to inhibitors of the BCL signaling molecule pSYK.

In each panel, blue indicates as the DAPI stain and indicates the cell nuclei. The colors represent positive staining of the cell cytoplasm for either pLYN, pSYK, or pBTK either with untreated or cross-linking.

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Post-imaging data analysis using TissueFAXS software allowed us to convert the visual data into a more familiar and quantitative FACS plot. Each dot in these plots represents an individual cell and positive stainings indicated by a shift of the dots from left to right on the X-axis.

We were able to detect proximal BCR signaling at baseline which increased with surface BCR stimulation and the cell line LY7, DHL4, and LY3. These are all sensitive to the pSYK inhibitor. The cell line, Toledo, which is insensitive to pSYK inhibition showed no evidence of signaling for any of the phosphomarkers with or without surface cross-linking IgM.

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We then applied this technique to a series of 60 primary biopsy samples of Diffuse Large B-cell Lymphoma and here is the summary of the results. Each dot indicates a case of Diffuse Large B-cell Lymphoma. The yellow dots indicate cases in which there is quantitative immunofluorescent evidence of concordant activation of all three signaling proteins, pLYN, pSYK, and pBTK together.

By this criteria overall, 45% of cases showed unequivocal evidence of B-cell receptor signaling in situ. This level of signaling surprisingly was

comparable to the level of same cell lines after B-cell receptor cross-linking with anti-IgM antibody.

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We observed the same phenomenon in an independent cohort of 94 DLBCLs. In this case, 47% of all cases showed evidence of concordant activation of pLYN, pSYK, and pBTK. We did not observe a correlation between cases that showed high-level B-cell receptor signaling and the subtype of Diffuse Large B-cell Lymphoma by the cell-of-origin classification system using the Hans classifier.

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Together, we found that one-half of primary Diffuse Large B-cell Lymphoma shows evidence of active B-cell receptor signaling. The intensity of B-cell receptor signaling in-situ is often comparable to that with anti-IgM cross-linked cell lines if they have no correlation with DLBCL subtype.

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We proposed that this may be an adequate and excellent assay and selection criteria for B-cell receptor in the inhibitor trials.

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Next, I will discuss the detection of signaling within the tumor microenvironment. An increasingly recognized signaling network critical for tumor progression is that between cancer cells and the immune cells.

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In this example, I will discuss Classical Hodgkin Lymphoma. This is an aggressive lymphoma of B-cell lineage that is unusual in that the malignant cells, the Reed-Sternberg cells, are a minority within the tumor microenvironment.

The majority of the cells in the tumor microenvironment are non-malignant immune cells that include numerous T-cells, macrophages, plasma cells, and granulocytes, but of course, the immune response is ineffective at stopping the lymphoma.

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Several years ago, the Shipp laboratory described a genetic amplification of chromosome 9p24.1 in Hodgkin Lymphoma cell lines using high density SNP arrays. This locus is notable for containing among other things the genes encoding the programmed cell death ligands PD-L1 and PD-L2.

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They went on to show by flow analysis that PD-L1 and PD-L2 proteins are overexpressed in these cell lines. Importantly, the levels of PD-L1 and PD-L2 protein expression by each cell line correlated with 9p24.1 copy gain.

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We've worked with members of the Shipp laboratory who went on to dissect Reed-Sternberg cells from cases of primary Hodgkin Lymphoma. By qPCR analysis, approximately 40% of cases had 9p24.1 copy gain.

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Cases of Hodgkin with copy gain at 9p24.1 were exclusive of tumors with EBV infection. We subsequently described how EBV can upregulate PD-Ligands by independent mechanisms. In addition, we and others have found that JAK-STAT signaling can promote PD-Ligand expression in numerous cell types.

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It is now obvious why this is such a critical observation. PD-1 and the PD-Ligands are well-known to immunologists as a signaling network that dampens the immune response. This may be helpful to prevent immune-mediated organ damage during chronic viral infection and to suppress autoimmunity. The PD-Ligands are often expressed by macrophages or dendritic cells, which then bind to PD-1 on T-cells to inhibit T-cell activation. This leads to so-called T-cell exhaustion.

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Critically however, this phenotype is reversible with PD-1 blockade.

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In collaboration with Gordon Freeman at the DFCI, we screened a large number of anti-PD-L1 antibodies to develop an effective IHC test to detect the protein in FFPE tissue sections. When tested against the panel of Classical Hodgkin Lymphomas, we've found that approximately 80% have expansive PD-L1 expression on the Hodgkin Reed-Sternberg cells.

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This number exceeds the expectation on genetic studies alone, but maybe explained by the addition of EBV and JAK-STAT signaling. Based upon the genetic and phenotypic data, anti-PD-1 therapeutics were tested as single agents in patients with relapse and refractory Classical Hodgkin. The overall response rates were very high, over 86% per one drug and over 50% for seconds. These are higher than the results observed for solid tumors.

We believe that PD-1 blockade is likely to be part of the standard treatment of Classical Hodgkins in the very near future. However, despite these dramatic results, a minority of patients developed progressive disease. They are analyzing the tissue from these patients now to try to figure out why.

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As part of the original trial however, we did have tissues from a subset of patients. All of the patients have responded to the drug. We performed two sets of double chromogenic immunohistochemical stains to show that in each of these cases, the Reed-Sternberg cells were positive for PD-L1, PD-L2, and had evidence of JAK-STAT signaling as evidenced by nuclear staining for pSTAT3. There were also genetic alterations of either chromosome 9 or the 9p24.1 locus in all cases.

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But we also noticed and reported that PD-L1 in the tumor microenvironment is not restricted to the tumor cells in Classical Hodgkin, but it's also expressed by additional non-malignant cells. Morphologically, these appear to be macrophages and dendritic cells. This is also seen in solid tumors.

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Indeed, as the way you've purported, in most cases of Classical Hodgkins, the majority of the PD-L1 in the tumor microenvironment is expressed by non-malignant cells.

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This raises the question, "Does PD-Ligand expression on the malignant cells, the inflammatory cells or both, predict response to PD-1 blockade in Classical Hodgkins?"

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We're taking the approach of multiplex image IF in digital image analysis to address this question. In this effort, we're using multispectral imaging platform that Dr. Stack will talk about momentarily.

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Here are examples of a data we have generated. In this case, CD30 highlights the malignant Hodgkin RS cells and tissue sections.

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Here, we have added on anti-PD-L1 stain. We see that most of the CD30 positive cells have co-expression of PD-L1, but there are also areas with PD-L1 staining, but without CD30.

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Here, we've added pSTAT3, which indicates active JAK-STAT signaling in the Hodgkin Reed-Sternberg cells and to a lesser degree, in non-malignant cells within the microenvironment.

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Here in purple is CD68, which shows that PD-L1 that is not already expressed in the RS cells is mostly localized within CD68 positive macrophages.

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Here, we have added in CD163, which captures a subtype of macrophage known as M2 macrophages. And in this case, or at least in this section, it is a source for much of the PD-L1.

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We can do the same procedure with T-cells to identify T-cells and T-cell subsets. Here, we start with CD30 for the tumor cells.

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Here, we see CD4 and CD8 to identify T-helper and cytotoxic T-cells.

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Here's the combination with also FOXP3 added in to identify regulatory T-cells in the microenvironment.

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After imaging with these multiplex immunofluorescence stains, we used image analysis. In this example, we used a combination of positive staining, tumor-specific markers, and shape to identify individual cells.

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Once we identify the cells of interest, we can quantify the biomarker of interest.

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This is the same procedure applied to macrophages in the same tissue section --

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-- and this is the quantified result.

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Here's a summary of the results of at least a small subset of cases that we have run indicating the co-expression of PD-L1, the Hodgkin RS cells indicated in blue -- these are CD30 positive -- and in macrophages in the red and green bars post-stained by CD68 and CD163.

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Here, we see that in some cases, there is a small amount of PD-L1 in the RS cells and tumor-associated macrophages.

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In a larger proportion of cases, there's extensive co-expression of PD-L1 and the Reed-Sternberg cells and in the macrophages. They are currently applying this approach to the cases of Classical Hodgkin Lymphoma treated with PD-1 inhibitors in clinical trials.

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Thus, multiplex immunofluorescence and image analysis allows us to simultaneously characterize and quantify many parameters in the tumor microenvironment including better subtyping cells and enumerating them.

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It also allows us to localize and quantify critical immunoregulatory proteins.

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Finally, it allows us to start establishing the geometric relationships between the actors in this play, between immune surveillance and tumor escape.

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It is a combination of quantifying both cell intrinsic and extrinsic features that works that are dysregulated in individual tumors that will allow us to tailor combinations of targeted therapies to individual patients.

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I would like to thank you for your time and acknowledge many people who have helped us on this study.

Sean Sanders: Great! Thank you so much, Dr. Rodig. We're going to move right along to our second speaker, and that is Dr. Ed Stack.

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Dr. Stack was an associate director of the Dana-Farber/Brigham & Women's Center for Molecular Oncologic Pathology, and an instructor in the Pathology Department of Brigham and Women's and the Harvard Medical School. Dr. Stack was also a member of the Dana-Farber/Harvard Cancer Center in both the gastrointestinal malignancies and prostate cancer research programs.

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Dr. Stack is currently a pathology scientist at PerkinElmer where he is interested in developing cancer immunology-focused, tissue-based assays, which can provide deeper insights into cancer-host interactions and assist in clinical management of cancer.

A very warm welcome to you, Dr. Stack.

Dr. Ed Stack: Thank you very much and good afternoon, everyone. I'd like to thank the organizers for the opportunity to share with you today some of our work to improve multiplex IHC approaches to enable refine microenvironment assessment in tissues and how this is driving our understanding of the immune system interactions in cancer. I'd also like to thank Dr. Rodig for a very insightful and enjoyable presentation.

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To begin, I think it's important that we recognize the biological complexity of the cancer immunology landscape. As we improve our ability to develop immune checkpoint inhibitors and with the great promise this class of compounds hold for cancer treatment especially with the recent publication from Ansell and colleagues in the New England Journal of Medicine this week showing very impressive response rates in a cohort of refractory Hodgkin Lymphoma patients.

We must endeavor to more precisely define the interaction of the immune system within the tumor microenvironment. This requires an understanding of the multiple cell types which may be present such as dendritic cells, tissue macrophages, and various subsets of T-cells, and finally the tumor, yet phenotyping cell types alone is not sufficient as we

must also understand the functional state of these cells through assessment of specific co-factors such as TIM-3, or PD-L1.

The great complexity of the immune system in the tumor microenvironment requires significant multiplexing in order to improve our understanding of the biology, which may one day soon help to better guide clinical assessment of cancer immunology.

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Yet when we consider the challenges of multiplexing, we must take note of some of the significant impediments which often precludes successful multiplex IHC beyond two or three markers.

The primary difficulty is encountered when we consider antibody species interference. This is circumvented often through the use of different primary species, yet consistently finding different primary species for previously optimized protocols as difficult as it's finding enough different species to enable significant multiplexing depth.

Assuming that antibody species interference can be avoided, we will then encounter signal intensity issues such as weak or imbalanced signals. While weak signals may result from low target abundance, they often require increased exposure times for any image capture. This can compound any imbalance in multiplex signals, which then can reduce imaging effectiveness.

An added complication of using fluorescence dyes and any multiplex IHC assay is the tendency for any fluorophore to quench with time, exposure, or a combination of each, an issue pathologists loath.

Lastly, should one overcome all obstacles above, there is the issue of endemic autofluorescence associated with FFPE tissues. This has the potential to obscure signal in some cases and more problematic is the inability to distinguish autofluorescence from signal contributing to unwanted bias. All of these make multiplexing hard, but I'd like to tell you about our solution.

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In terms of staining, the Opal Multiplex protocol can overcome several of these complications including antibody species interference, weak

signals, and signal imbalances. This can be achieved using Opal's iterative IHC approach.

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In this instance, imagine three targets of interest and the need to interrogate each using Rabbit primary antibodies. With Opal, this can be done. Basically, the first target can be interrogated as follows.

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A Rabbit primary to Target X can be applied to detect the target exactly as we've done in classical immunohistochemistry. This primary can then be interrogated by specie-specific secondary.

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This secondary possesses an HRP conjugate, which is used to facilitate the deposition of a TSA detection reagent.

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In the following example, a fluorescein-linked TSA reagent is added. TSA is a substrate for the HRP and the interaction causes the TSA to covalently bind tyrosine residues within the epitope.

Importantly, because the TSA and its fluorophore are covalently bound to the epitope, it can withstand certain protocols for the primary and secondary antibodies, which themselves are linked to their respective epitopes by non-covalent interactions.

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So when the antibody stripping is performed, which is represented in this instance by heat, the antibody complexes are removed while the TSA reporter for Target X remains intact, as is represented here.

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Importantly, because the primary and secondary antibodies are completely removed, the assay is now set up for the second round of immunohistochemistry. And as before, a new Rabbit primary to Target Y can be applied to detect the target.

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As before, the new Rabbit primary can be interrogated by the same anti-Rabbit secondary.

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Once again, the secondary HRP can be used to facilitate the deposition of a different TSA detection reagent, so in the following example, the new detection reagent is a Cy3-linked TSA.

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Again, with the TSA serving as a substrate for HRP, the interaction causes the TSA to covalently bind tyrosine residues within the epitope and as before, the TSA and its fluorophore can withstand the stripping of the primary and secondary antibodies supporting higher degrees of multiplexing.

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Again, the stripping can remove the antibody complexes for the second Target Y, yet the second TSA reporter for Target Y remains intact.

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And now, we see Targets X and Y labeled with detection reagents represented here. As well, the assay is now set up for additional rounds of immunohistochemistry. And as before, a new Rabbit primary for Target Z can be applied to detect the third and last target in this example. This new Rabbit primary for Target Z can also be interrogated by the same anti-Rabbit secondary.

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With this last HRP secondary, the deposition of a third and final TSA detection reagent can be achieved with a new Cy5-linked TSA --

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-- which results in covalent deposition of the last TSA reporter molecule.

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And when antibody stripping is performed for the final time --

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-- the result is a multiplex that has avoided any specie's cross-reactivity and is thus well positioned to advance our understanding of the tumor microenvironment.

Importantly, this represents a significant advance beyond the common multiplexing difficulties outlined earlier, which has the potential to fundamentally change the way in which multiplex IHC can be seamlessly employed in the research setting. As well, such multiplex IHC can provide a deeper view into the biology of the interaction between a tumor and a dissociated immunological milieu.

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A perfect example of our multiplexing IHC can be seen here. This is a breast cancer sample stained with CD4 to identify T-helper cells, CD8 for the detection of T-cytotoxic cells, and CD20 for the detection of B-cells. In addition, the sample was stained with cytokeratin, which is used to identify the epithelial breast tumor from other stromal or non-tumor tissues.

I think the benefit of this kind of multiplexing can be seen from the staining that we can achieve, but this is only the first step in our data acquisition, which is a goal of any biological assay and is certainly our ultimate goal, yet to achieve this, we must also be looking toward a robust imaging modality and we have focused our efforts on multispectral imaging.

A main reason for this in addition to reliable signal separation of each of the markers in the image you see here is that multispectral imaging also helps us to overcome the confounding effects of autofluorescence.

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This is the last major impediment to reliable quantification that can support robust multiplexing and this is how this can be solved.

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Here is an example of a simple experiment to detect EGFR in breast cancer. In the top panel, the image is acquired using conventional fluorescence microscopy where a monochrome bandpass image is acquired.

In order to detect true signal from general autofluorescence, measurements are obtained from the membraneous component where EGFR is expected and a nuclear compartment where no EGFR expression is expected.

While this normally allows for proper balancing a signaling background, what can be seen is that due to autofluorescence, the signal to background ratio is very poor, which would confound any meaningful interpretation of EGFR expression in this example.

In contrast, as seen in the lower panel, the image can be acquired using multispectral microscopy. Unlike standard fluorescence microscopy, multispectral imaging collects images at discrete bandpass integrals of 10 to 20 nanometers. This creates a multispectral cue where both the EGFR spectra and the autofluorescence factor can be separated so that the intensity of EGFR alone can be measured.

In this instance, it is clear that multispectral imaging can significantly improve the signal to background ratio, which supports a more specific EGFR assessment in breast cancer.

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Using both the Opal Multiplex IHC staining method and multispectral imaging, I would like to now describe for you how we are using these tools to advance our knowledge of the tumor-infiltrating lymphocytes in breast cancer.

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In this study, we first performed an Opal Multiplexing proof of concept using tonsil, a tissue that is rich in lymphocytes, and this proved very useful for optimization efforts. Once we had each marker perfected in the Opal Multiplex assay, we moved into the breast cancer tissues.

As can be seen in Panel A, we successfully multiplex CD4, CD8, CD20, cytokeratin and DAPI in breast tissue, and perform multispectral imaging

where we prepared unmixed image samples for further downstream phenotyping studies.

An example of this process can be seen in Panel B where each specific cell type, CD4 in green, CD8 in purple, CD20 in red, or cytokeratin in yellow, is identified. Importantly, the position of each cell is locked within a Cartesian coordinate system that's seen in the phenotype map in Panel C. This coordinate system is then amenable to a wide variety of analytical approaches that can support robust biological and pathology-based assessment.

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To demonstrate the simplicity of our imaging approach, the system will automatically capture all of the tissue and images at a 4x resolution. From this step, areas identified by pathology assessment for further analysis are then selected. The system then automatically collects all the pathology-defined 20x fields for data acquisition and data output as can be seen in Panel A.

In this workflow, the data acquisition supports tissue identification. So for our purposes, we train the system to recognize and denote all tumor tissues in each selected field. In this instance, as shown in Panel B, the tumor is segmented by a red mask while all non-tumor or stromal tissue is denoted by a green mask.

From this step, the phenotyping can identify all CD4, CD8, and CD20 cells in either the tumor compartment as indicated in Panel C or the stromal compartment as in Panel D. With the tumor stromal border defined by the tissue segmentation and the cell phenotype defined by the Opal Multiplex immunohistochemistry, we can now ask questions of spatial orientation of cells to morphological entities such as the tumor stromal border or from compartmental cell then to the measurements.

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Our Opal Multiplex assay and the multispectral analysis provided us with the ability to assess the average distance of every CD8 positive cell from the tumor stromal border probing both the intraepithelial and intrastromal compartments.

For either of the tumor or the stroma, ANOVA of the average CD8 distances did not distinguish any differences between the various breast

cancer subtypes of estrogen receptor positive or HER2 positive or triple negative indicated by green, blue, or red respectively.

Similarly, when assessing the average CD4 cell density in the tumor with stromal compartment, ANOVA failed to demonstrate any significant difference between any of the breast cancer subtypes.

In contrast, assessing the average CD8 cell density in the tumor compartment revealed differences approaching significance in the tumor density of each subtype while the effect was less robust when assessing stromal CD8 density. While this pilot population is small, we feel confident that the assay, when properly powered, will yield valuable cancer biology.

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Indeed, Loi et al. reported a prognostic role for tumor-associated lymphocyte in triple negative breast cancer in the large Breast International Group Clinical Trial back in 2013.

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This may lead one to think that this subject with the increased lymphocytic infiltrate as was described by Loi et al. may show a reduced relapse rate and/or improved survival.

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So to summarize, while the case study was relatively small, there were still some very interesting observations. Importantly, we believe that their study demonstrates a new capability for interrogating the intricacies of the tumor microenvironment and the cancer immune response for research.

As shown, it appeared that estrogen receptor positive tumors have a lower immune response in terms of the intraepithelial and stromal fields when compared against the HER2 and triple negative breast cancers while the HER2 tumor showed a large range of immune response, possibly dichotomous, so the significance of this remains unclear.

In contrast, the triple negative breast cancers had generally much higher immune response and this was particularly so for the CD8 cell density in the tumor compartment, which revealed differences approaching significance between the ER-positive and HER2 subtypes.

Based on these results, we've begun repeating this assay on a larger cohort while also expanding the multiplexing to add both PD-L1 and FOXP3 to assess the contribution of checkpoint blockade and tumor survival.

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And so, I would like to finally share with you some of the more recent data we've now generated using an expanded Opal Multiplex assay, which includes CD4, CD8, CD20, along with FOXP3, PD-L1, cytokeratin, and DAPI done on a larger cohort of breast cancer cases. With this data, we've also advanced our phenotyping tools and analytical assessment to help further explore and characterize the tumor infiltrating lymphocyte profile seen in the various subtypes of breast cancer.

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As an example, here we have a breast cancer where the Opal Multiplex assay is identifying B-cells, T-helper cells, cytotoxic T-cells, T-regulatory cells, and PD-L1 positive cells, all shown in a multispectral composite. From this data, we can locate examples of each phenotype and demonstrate each to the system such that when sufficient examples of each phenotype are defined, the system can then manually define the phenotype of every other cell in the image.

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With this phenotype information, we can now determine the specific numbers of each phenotype for each case. You may recall that while using this system, the images generated also retain their Cartesian coordinate structure. So with the structure mat and the phenotype information, we are able to expand our analytical approach to dive deeper into the data.

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Here is what the deeper dive looks like. Here, we performed the nearest neighbor analysis and we have asked the question, "What is the nearest cytotoxic T-cell neighbor from the point of view of every tumor cell?" The phenotype map is expressing this distance by black lines connecting every tumor cell indicated in yellow to the closest cytotoxic T-cell, which is indicated in blue.

This provides a very detailed view of the nearest neighbor assessment or perhaps more interesting is using the actual distance measurements represented by those black lines to show that in this case, 33% of the tumor cells have a cytotoxic T-cell within 25 microns.

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Here, we have another example of a breast cancer shown in the multispectral composite and I think it can be seen right away especially when compared to the previous example that this case will display a markedly different immunological profile.

Slide 104

Indeed, the phenotype information shows a very different phenotypic profile and by using that embedded Cartesian coordinate structure, the deeper dive into the data reveals a markedly different result.

Slide 105

Here is what the markedly different deeper dive looks like. Again, we performed the nearest neighbor analysis. As can be seen, the distance lines here are remarkably longer when compared to the previous case such that when the actual distance measurements are examined, they show that in this case, only 3% of the tumor cells have a cytotoxic T-cell within 25 microns.

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In summary, I think that pairing Opal Multiplex immunohistochemistry with multispectral imaging will enable more comprehensive measurements of multiple immune-related analytes within the tumor microenvironment.

And by combining phenotypic as well as functional co-factor markers, as well as adding more advanced data metrics such as our nearest neighbor analysis, we can increase our knowledge of underlying biological mechanisms that could be leveraged to optimize the efficacy of immunotherapy protocols.

I hope that I have shown you the potential of this approach to successfully assess and distill the tumor microenvironment in cancer.

[0:45:09]

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In closing, I would like to acknowledge the invaluable collaborations and contributions of my PerkinElmer colleagues, Clifford Hoyt and Kent Johnson, along with Kristin Roman and Chi Wang, as well as Mike Feldman at the University of Pennsylvania and Beth Mittendorf at the MD Anderson Cancer Center, and with that, I thank you.

Sean Sanders: Great! Thank you so much, Dr. Stack, many thanks to both of our speakers for the fantastic presentations.

Slide 108

We're going to move right along to the questions submitted by our online viewers.

Just a quick reminder to those watching live, you can still submit your questions by clicking the "Ask a Question" button below the slide window and typing the question into the message box, and then clicking "OK".

The first question I'm going to put I think to both of these speakers -- let's start with you, Dr. Rodig -- is if you could talk a little bit about pre-treatment of the slide for antigen retrieval, how this might affect the results, and also if you have any recommendations specifically for slide preparation for the techniques that you used.

Dr. Scott Rodig: Sure. We prepare our slides by a variety of mechanisms, and a lot of it ends up being empiric. We love doing everything by multiplex immunofluorescence, but we do optimize staining for individual biomarkers with individual antibodies by traditional chromogenic staining, and then move whatever is the optimal method over to immunofluorescence.

And for antigen retrieval, we've done all types of methods including microwave retrieval as discussed today, but also using heated baths, steamers, and pressure cookers. I think the folks out there who do immunohistochemistry realized different antibodies require different retrieval mechanisms for optimization.

Dr. Ed Stack: I think that's a great summary, Scott. I would add, in fact with our work here, we have noted while classical chromogenic immunohistochemistry does often require optimization of the antigen retrieval, I think given the great efficiency of the TSA reagents, I'm surprised by how many of what I

would consider to be more challenging stains, including nuclear markers where a higher pH stringent antigen retrieval will be required, we find that even with citrate buffer, we're achieving results that certainly surpass what I would typically expect.

Sean Sanders: There's a follow-up question to this, and a number of viewers have asked them in a variety of different ways. How can you be sure that the microwave treatment, Dr. Stack, that you described to strip the antibodies away completely removes both primary and secondary antibodies?

Dr. Ed Stack: Well, there's been data that's been published with this methodology from Toth and Mezey's group where they have shown in fact that they can perform antibody stripping. And through manipulating the secondaries, to use the same secondary, but going with different TSA molecules, they will not see after antibody stripping and the reintroduction of a rather primary any HRP-mediated deposition of a novel TSA after the stripping protocols.

And so, I think there's a good deal of evidence to show that this is quite an effective way in which to not only potentially inactivate the HRP, but really remove any of the confounding species' cross-reactivity. I would encourage all of our listeners to consult that study by Toth and Mezey in I believe 2007.

Dr. Scott Rodig: I can second in that, too, in that we've done extensive work using known antibodies which when we've started multiplexing them, we know that they're in different compartments such as PAX5 as I showed, we know to be a nuclear antigen in B-cells, and you can do something such as CD68, which is known only to go to macrophages.

And when you start multiplexing these, it becomes quite clear whether or not there's any cross-reactivity during the sequential staining so you can cross-check very quickly.

Sean Sanders: Let me stay with you, Dr. Rodig. Have you found a limited number of times that you can strip and re-stain the slide for multiplexing?

Dr. Scott Rodig: No, we've not. We've maxed out with the maximum number of dyes that we've been able to get our hands on. At least as far as what we've done -- and I think probably the most we've done is seven stains -- we have not noticed any difficulties. For us, it's a matter of literal bandwidth. That's the limitation.

[0:50:07]

Sean Sanders: And Dr. Stack, have you reached the limit yet? Is there a limit or is it just based on the number of dyes currently available?

Dr. Ed Stack: Well, unlike in some of the previous multiplex efforts such as the simple method where in fact it was pointed out that four or five rounds of antigen retrieval were sufficient to begin to start to see tissue lifts from the slides, in our hands, we have been able to do nine rounds of microwaving with no detectable tissue loss or tissue lifting in the sample.

I have had it reported to me in fact that people have gone to higher lengths in that with the same kinds of results. This seems to be I think given the fact that the citrate buffers with their low pH are the least stringent of many of the antigen retrieval strategies. I think that may be contributing to why we still see very good tissue morphology after multiple rounds in the retrieval.

Sean Sanders: And Dr. Stack, I'll stay with you for a second. Have you validated this methodology by dye comparison between single and multiplex IHC?

Dr. Ed Stack: Yes. In fact, we recently published a paper where we did exactly that within a five-panel breast cancer including ER, PR, HER2, T67, along with cytokeratin and DAPI. What we were able to show in that study was we could reliably separate the HER2 signal from a multiplex and take a sister section and stain for HER2, and do quantitation within the singleplex HER2 versus the multiplex analysis of the HER2 section.

We were achieving concordance rates that were as high as 0.9, so there's very good concordance between the singleplex and multiplex especially when you consider that when you move from one level of section to another, you're often looking at a slightly different morphology.

Sean Sanders: Let me actually ask both of you this question. Let's start with you, Dr. Stack. This viewer asks if this is possible to do on cryo sections rather than FFPE.

Dr. Ed Stack: That's a very interesting perspective because one of the things that formalin fixation does is adhere our tissue sections to the slide, whereas frozen sections are simply dried on to the slide and they're not really adhered to any great degree. So I have every faith that a single round of immunohistochemistry could be done there. I'm not sure as it's never been done by us whether or not multiple rounds of immunohistochemistry could be done on frozen section.

I think there probably could be some strategies that could assist with that as opposed to a post-sectioning, formalin fixation dip, but as far as I'm aware, no one has tried that. I don't know, Scott, if you have anything to add there.

Dr. Scott Rodig: Right. I think maybe a post-fixation might be a wise thing to try to do, but we have never tested that.

Sean Sanders: Excellent! I'm going to get on to some more general questions now.

Dr. Rodig, this was a question that came in during our presentation. You said that 22% of patients responded to BCR signaling inhibitors while 45% of tumor cells seem to have an active BCR. Can you explain this discrepancy?

Dr. Scott Rodig: Yes. Well, the way to answer it, of course, is to actually analyze tissue from the actual clinical trials, which we're working very hard to get our hands on.

I don't have a direct answer for what this reason is. We don't know at this point. Our data would suggest that perhaps even a subset of patients that do have evidence of active B-cell receptor signaling, perhaps they do not respond very well to some of the drugs on the market, but until we've done the actual primary experiment, it's hard to say why.

Sean Sanders: I'll send another question your way. In what ways do you think this work can help find better immune therapies for cancer patients better than the current chemotherapy techniques? Any thoughts?

Dr. Scott Rodig: Yes. I think we've seen tremendous success through even the limited trials that have come through with human antibodies against CTLA-4, for instance, and PD-1 and PD-L1, but as most of the folks in immunology know, there's a whole host of additional immunoregulatory proteins out there. And not surprisingly, there are antibodies that are either activators or antagonists against many, if not all, of these additional immunoregulatory proteins.

[0:55:04]

We know that many of these can be expressed by tumor cells and can be expressed in the tumor microenvironment. So very soon, there's going to be an overwhelming number of therapeutic options coming on to the market.

The challenge, of course, will be to figure out which patient would benefit best from which therapeutic choice based on the nature of their tumor and the nature of their immune response. So we believe that this is the tip of the iceberg in terms of characterizing the microenvironment and using that to tailor the most effective therapy.

Sean Sanders: Dr. Stack, a question for you about digital imaging. This viewer asks us to address the issue of digital image acquisition analysis, especially concerns around throughput and area of acquisition. Any thoughts on that?

Dr. Ed Stack: In terms of throughput, there are automated strategies one can use to help deliver the slides to these instruments and I think that can actually really improve slide throughput. The reason of interest is a very interesting one because while we're doing multiplexing, we're capturing a great deal of data and we're doing so at 20x objectives in order to be able to see what we need to see.

I think there are some pathologists who would argue that you need to get into 40x or maybe even 100x to be able to see various cellular components. One thing to take away from this though is that the strategies that we've used while we tend to sample individual regions of interest, there is nothing that prevents us from taking the entire tissue section and doing the multispectral analysis at 20x level.

There is obviously a data component associated with that and you have to ask the question, "Where is the rate limiting step for the amount of valuable information you're pulling out versus the amount of information you're actually acquiring?"

I certainly don't have the answer to that. I think that differs from individual disease contexts, the different cancer types or specific tissue presentation, all of which I think need to be addressed when the analysis is taking place.

This is why I think pathology can very much utilize this technology and guide that because the pathologists have the greatest understanding of how in which to interpret the tissue sections that they analyzed.

Sean Sanders: I think we have time for just one more question for you, Dr. Rodig. Have you seen any evidence for the role of tumor stromal cells in inducing chemo resistance?

Dr. Scott Rodig: I can say that we have not looked at that in any detail. In terms of standard chemotherapy, there's a number of hypothesis out there. I can't say we've looked at that. And even in terms of immunotherapy, to my knowledge, it's not been looked at too much of a degree.

Certainly being able to identify the malignant cells, the immune cells, and the stromal cells within the microenvironment there allows us to answer that question more concretely going forward, but we've not looked at it.

Sean Sanders: Excellent! Well, I wanted to say many thanks to both of our speakers for their fantastic presentations. Unfortunately, we are out of time now for this webinar, so on behalf of myself and our viewing audience, thank you very much for being with us today, Dr. Scott Rodig from Brigham & Women's Hospital and Dana-Farber Cancer Institute, and Dr. Ed Stack from PerkinElmer.

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

[0:59:26]

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