“Predicting Immunotherapy Response with a New Test" (BostonGene) [#75]

Adrienne Nugent and Brad Power
November 1, 2023

“When we speak about multiplex immunofluorescence, we're mostly focused on the tumor microenvironment because we would like to provide a prediction of therapy response to immuno-oncology.” – Katerina Postovalova

“The tissue assay, the blood assay, the MxIF – we're trying to go deep to decode the cancer in unique cases to really understand what's driving response, or lack thereof.” – Michael Hensley

Meeting Summary

Advanced cancer patients need advanced testing to personalize and guide their treatment decisions. Beyond the increasingly common DNA sequencing, there are emerging RNA sequencing and other tests which show your "tumor mutational burden", “microsatellite instability”, and mutational and immune landscapes. These state-of-the art tests are performed by certified labs. They then apply sophisticated analytics and bioinformatics to the test data to generate comprehensive personalized reports on the unique molecular portrait of your cancer and your treatment options. These tests can tell you whether you will be a likely responder to a therapy. For example, understanding the spatial relationships of where the various immune cells are within the tumor microenvironment is important if you are considering an immunotherapy.

Katerina Postovalova is uniquely qualified to explain these novel tests. She is Head of Digital Pathology at BostonGene, a diagnostics company. She can describe, for example, how BostonGene's "multiplex immunofluorescence" platform, in which many (30-50) labeled antibodies can be applied to your tumor tissue sample to provide a comprehensive overview of your tumor cells and tumor microenvironment. Michael Hensley is a Senior Strategic Account Manager at BostonGene, managing their relationship with MD Anderson.

Why should you care about newer tests of the tumor microenvironment?

Understanding your tumor microenvironment – the spatial distribution of the cell types in your tumor tissue – can aid in predicting your response to immunotherapy and guide your treatment decisions. A three-dimensional picture of your tumor microenvironment – the types of cells that are present in and around your tumor tissue, such as whether your immune cells are located primarily within important tumor niches or are isolated – can show whether an immunotherapy has a good or bad chance of killing your cancer cells. For example, if your tumor has a high level of infiltration of immune cells, your tumor may be more likely to respond to immunotherapy.

What can multiplex immunofluorescence tell you that is new and different?

Multiplex immunofluorescence is a complement to the more common tumor tests (an “oncopanel”, which looks at 100-300 driver genes), whole exome sequencing (about 20,000 genes/DNA which code proteins), and RNA (transcriptome) sequencing. The sequencing tests

tell you about mutations in your tumor cells which are different from normal cells. Multiplex immunofluorescence is a technology that can stain up to 40 markers on a single slide of tissue, enabling a deeper analysis of the tumor microenvironment than traditional staining of tumor tissue.

Multiplex immunofluorescence can tell you the following about your tumor microenvironment:

- The presence or absence of different biomarkers, e.g., PD-L1
- The different subtypes of immune cells
- The location of immune cells
- The distances between different cells
- The contacts between different cells

What input do you need to provide?

You probably had a biopsy of your cancer at your diagnosis (the primary site), or maybe you have a more recent biopsy of a metastatic site. This will have been typically stored in a formalin-fixed paraffin-embedded (FFPE) block. Slides can be made from this tissue. Primary or metastatic tissue can be analyzed. While tissue that has been stored for a long time can be used, samples older than five years may be less practical. Factors such as tumor purity and necrotic (dead) tissue can affect the ability to use tissue samples. Frozen tissue can also be used as it can then be embedded in paraffin. Importantly, tumor tissue must be stored in a block so that fresh-cut slides can be prepared. Tumor tissue stored as slides will not work for this test.

Tissue slides are a very precious resource, so an algorithm is used to predict the number of slides needed for tumor analysis. This ensures that each slide can be put to maximum use. While tissue samples from multiple time points can be assessed to provide valuable insights into tumor response and evolution, the most informative sample to test would be the most recently available tissue.

Does this test work for all cancer types?

Multiplex immunofluorescence works well for all cancer types. The only issue arises when there are unique preservation methods required for certain types of tissue. For example, if a metastatic site is in the bone, then the lab uses a specific solution to prepare the sample for multiplex immunofluorescence.

Can the same tissue sample be used for typical tumor sequencing and multiplex immunofluorescence?

Each test requires a separate tissue sample for a separate workflow. Depending on the amount of tissue provided, the lab will use an algorithm to determine if there is enough tissue for both multiplex immunofluorescence and their standard tumor sequencing, which includes whole exome (DNA) and transcriptome (RNA) sequencing.

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The lab can also do immunoprofiling of blood samples. This is a comprehensive flow cytometry test (a laser-based technique used to detect and analyze the chemical and physical characteristics of cells or particles) used to describe immune fitness and can guide immunotherapy-based decisions.

To maximize your knowledge and inform your treatment decisions, you could get three tests:

1. Tumor Portrait: Whole exome and transcriptome (bulk RNA) sequencing to understand genomic and RNA expression alterations within the tumor.
2. Multiplex immunofluorescence - MxIF: spatial proteomics to characterize tumor tissue architecture and cell-to-cell interactions in the tumor microenvironment.
3. Immunoprofiling: blood cell composition characterization to determine the immune cell types present in blood.

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Meeting Notes

SUMMARY KEYWORDS

tumor, tissue, immune cells, samples, work, tumor microenvironment, analyze, pdl, slides, patients, analysis, cells, stromal, clinical utility, immune, boston, sequencing, rna, gene, tissue sample

SPEAKERS

Katerina Postovalova (40%), Michael Hensley (18%), Rick Stanton (10%), Brad Power (9%), Allen Morris (8%), Brian McCloskey (7%), Gitte Pedersen (6%), David Plunkett (1%), Kaumudi Bhawe (1%)

OUTLINE

1. Immunotherapy analysis and treatment options for cancer patients. (0:00)
2. Analyzing multiplexed immunofluorescence images for cancer research. (6:07)
3. Analyzing audio transcripts for tumor microenvironment insights. (12:44)
4. Using spatial analysis for cancer treatment. (19:35)
5. Cancer biomarkers and tissue analysis. (23:55)
6. Cancer treatment and immune microenvironment. (34:00)
7. Tissue analysis and immune profiling for cancer diagnosis. (36:00)
8. Tumor profiling and immune profiling in cancer treatment. (39:16)
9. Leveraging tissue samples for cancer treatment decisions. (41:41)
10. Immunotherapy treatment and tumor evolution. (45:39)
11. Validating biomarkers for cancer treatment using a platform. (48:52)
12. Validating biomarkers for cancer immunotherapy. (50:21)
13. Prostate cancer diagnosis and immune response. (54:46)
14. Using technology to analyze RNA data for cancer research. (1:01:43)
15. Cancer genomics and personalized medicine. (1:03:59)

SUMMARY

- **Immunotherapy analysis and treatment options for cancer patients.** 0:00
    - BostonGene scientist Katerina Postovalova discusses cancer research with patients and caregivers.
    - BostonGene: Leading immunotherapy analysis for prostate cancer treatment decisions.
    - Multiplex fluorescence data analysis for tumor diagnosis.
- **Analyzing multiplexed immunofluorescence images for cancer research.** 6:07
    - Researcher uses Acquire Boston's pseudo-service provider for acquire to analyze tissue samples for immune cell subtypes and heterogeneity in tumor tissue.
    - Researchers develop AI-based approach to analyze multiplexed fluorescence images of tissue samples to identify cell types and their distribution within the tumor.

    - Analyze cell types in tumor microenvironment using 20-30 antibody panels.
- **Analyzing audio transcripts for tumor microenvironment insights.** 12:44
    - Katerina Postovalova highlights the limitations of bulk RNA sequencing data in understanding the spatial organization of tumors, citing examples from prostate cancer research.
    - Katerina Postovalova emphasizes the importance of supplementing tumor positive polls with spatial analysis to predict therapy response to immune oncology, using multiplexing or fluorescence techniques to analyze the tumor microenvironment.
    - Katerina Postovalova analyzes the distribution of stromal component and tumor cells in the tissue, noting less immune alterations in the malignant area.
    - Katerina Postovalova presents a graph-based analysis of cellular niches and cell-to-cell interaction, highlighting the presence of different subtypes of immune cells in various segments of the tissue.
    - Katerina Postovalova presents analysis of tumor imaging report, focusing on malignant cells and tumor microenvironment.
- **Using spatial analysis for cancer treatment.** 19:35
    - Rick Stanton praises Katarina's spatial analysis, hoping it will lead to directed therapies for prostate cancer patients.
    - Rick Stanton discusses using 40-plex immunophenotyping to predict cancer response to treatment.
- **Cancer biomarkers and tissue analysis.** 23:55
    - Michael Hensley discusses using MX if to better understand why some tumors respond to immunotherapy while others do not.
    - Kate's research focuses on comparing multiplex fluorescence data with bulk sequencing data to identify the biology of the tumor and why some tumor hubs do not exhaust, even with a lot of immune cells in the non-malignant component around the tumor.
    - David Plunkett asks about the durability of tissue samples for analysis, with Katerina Postovalova explaining that samples older than 5 years may not be practical to use.
    - Katerina Postovalova also notes that there can be significant differences between primary tumors and metastases in patients with available samples, and that some cancers are difficult to analyze due to their nature.
    - Katerina Postovalova discusses the challenges of analyzing bulk RNA sequencing data from metastatic and primary tumors, including differences in tumor purity and the need for proper sample preparation.
    - Katerina Postovalova also highlights the importance of considering necrotic tissue in NGS data analysis and multiplex fluorescence staining, and how it can affect the results.
- **Cancer treatment and immune microenvironment.** 34:00
    - Michael Hensley discusses testing metastatic lesions to understand cancer behavior changes.
- **Tissue analysis and immune profiling for cancer diagnosis.** 36:00
    - Brian McCloskey asks about including his 7-year-old prostatectomy tissue for immune profiling, and if running both spatial and standard tests on the same tissue is possible.
    - Developed algorithm to predict number of slides needed for tumor analysis, prioritizing multiplex immunofluorescence.
- **Tumor profiling and immune profiling in cancer treatment.** 39:16

    - Hensley discusses challenges with RNA extraction in old tissue, but successful DNA extraction can provide a comprehensive report.
- **Leveraging tissue samples for cancer treatment decisions.** 41:41
    - Brian McCloskey seeks advice on prioritizing tissue samples for diagnostics, given limited availability.
    - Brian McCloskey and Katerina Postovalova discuss how to leverage tissue samples for personalized cancer treatment decisions, with a focus on analyzing all three time points to see the full history of tumor response.
- **Immunotherapy treatment and tumor evolution.** 45:39
    - Michael Hensley discusses the clinical utility of immuno profiling tests, which can provide valuable insights into a patient's immune system before and after treatment.
    - Brian McCloskey is considering using immuno profiling tests during his cancer treatment, and Michael Hensley offers to change the test if needed.
- **Validating biomarkers for cancer treatment using a platform.** 48:52
    - Gitte Pedersen expresses interest in collaborating with the speaker's group, validating findings using I'd see, and inquires about validation of PDL one and PR two biomarkers.
- **Validating biomarkers for cancer immunotherapy.** 50:21
    - Katerina Postovalova validates PDL1 IHC slides by staining in-house and sending to external vendors for validation.
    - Collaboration proposed to validate biomarkers for immunotherapy in FFPE tumor samples.
- **Prostate cancer diagnosis and immune response.** 54:46
    - Allen Morris hypothesizes that Gleason pattern 3 tumors are never inflamed, based on 40 years of light microscopic HIV staining.
    - Allen Morris hypothesizes that immune desert in prostate cancer is actually high, backed by Provenge data.
    - Katerina Postovalova mentions that their algorithm can identify immune cells in different areas of the tumor and distinguish between malignant and non-malignant cells.
    - Katerina Postovalova also mentions that they have not looked at Gleason pattern 3 and stromal changes, despite Allen Morris's suggestion that this could be an important area of research.
- **Using technology to analyze RNA data for cancer research.** 1:01:43
    - Rick Stanton expresses frustration with lack of access to advanced bioinformatics tools for cancer research.
- **Cancer genomics and personalized medicine.** 1:03:59
    - Michael Hensley emphasizes the importance of the Boston GTuner portrait test for Brian's cancer treatment.
    - BostonGene's sequencing pipeline provides unique insights into tumor biology, leading to clinically actionable therapy choices.

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TRANSCRIPT

Brad Power 0:03
I'm the Co-Founder and President of the Cancer Patient Lab. This is our weekly webinar series where we talk either to patients about their cases or to innovative companies, experts at the cutting edge, and then the patients do a “question and answer” to learn about whatever that area is.

Today, we're honored to have Katerina Postovalova, a scientist who's working at BostonGene.

We connected initially with BostonGene through Sumit Subhudi, one of our very honored and esteemed gurus that we really listen to closely. He's an expert in immunotherapies in prostate cancer, and he described how at MD Anderson, he and others are working with BostonGene to guide their treatment decisions with patients. And so we said, “We need to make BostonGene available to the patients in our community.

I want to credit Rick Stanton with educating all of us about immunotherapies as a possible best path of treatment options because it's fighting the disease, a cancer system, with the immune system. All things being equal among your treatment options – targeted therapies, chemotherapy or radiation – if immunotherapy works, then you have a much greater chance of a durable response. Anything that would help guide us towards immunotherapies is valuable. Rick was early in pointing us to a couple of companies: Akoya and NanoString, that are hardware manufacturers that provide some of the tools that allow spatial analysis, that can help look at the tumor microenvironment and figure out whether an immunotherapy is a likely response. We talked to them, but they don't talk directly to patients, they only talk through labs, and it seemed that BostonGene was at the cutting edge of using that technology to help guide patients, particularly with their tumor profile and their immunotherapy guidance, and whether immunotherapy would be a likely therapy option for them. We've been very interested in this.

In our initial conversations with Michael Hensley about BostonGene, when Sumit Subhudi put us in contact with BostonGene, Rick was very interested in getting behind the curtain to see the Wizard. What's behind the curtain? What's going on inside that lab? What are you doing? So that we can understand in more detail what happens inside a lab that's doing the kinds of analysis to help us patients guide our path forward.

We already had a session with Michael and with some of his colleagues to get introduced to BostonGene. This session is with Katerina to go deeper into the technology and pipelines and processes of the lab that enable BostonGene’s reports.

We have a number of patients who have taken advantage of the BostonGene services, including Brian McCloskey and Bob Gurmankin. They will have some questions that come from that perspective.

Katerina Postovalova 5:01

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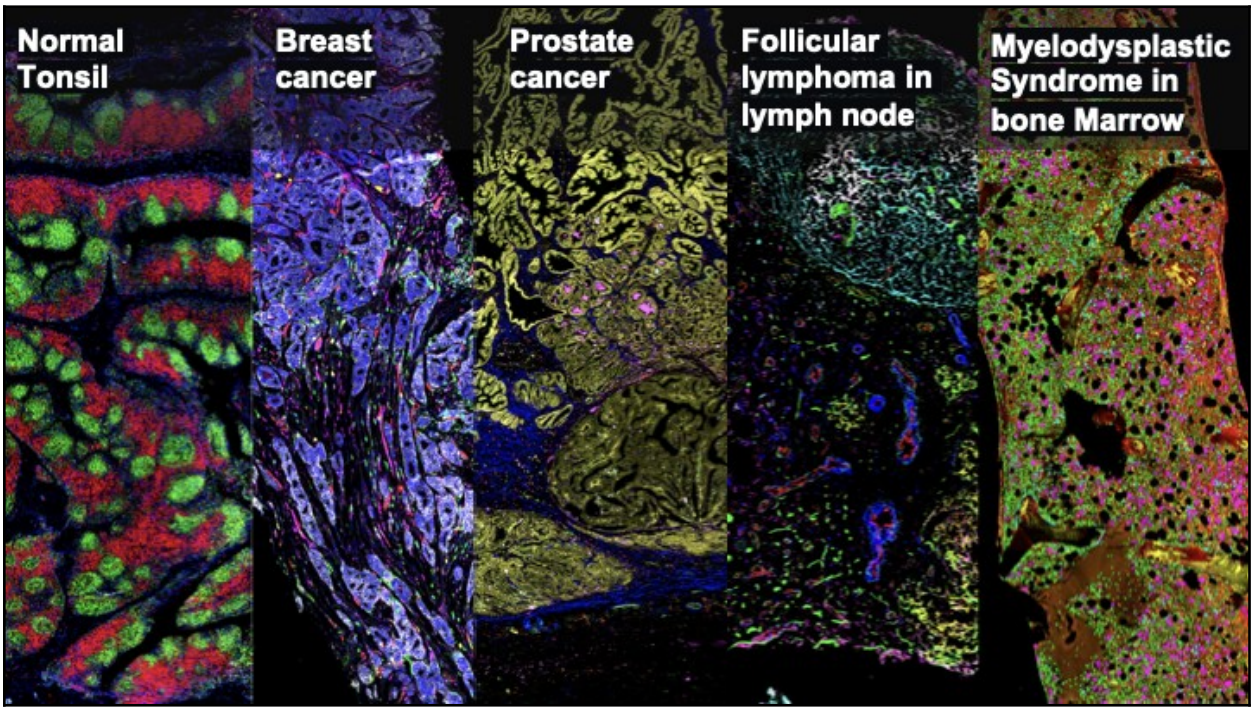
I've been with BostonGene for about four years. I started as an analyst, analyzing the MxIF (multiplex immunofluorescence, a new imaging technology which can look at a large number of cellular and other markers on a single tissue slice) data. I am happy to present those results and our product to you.



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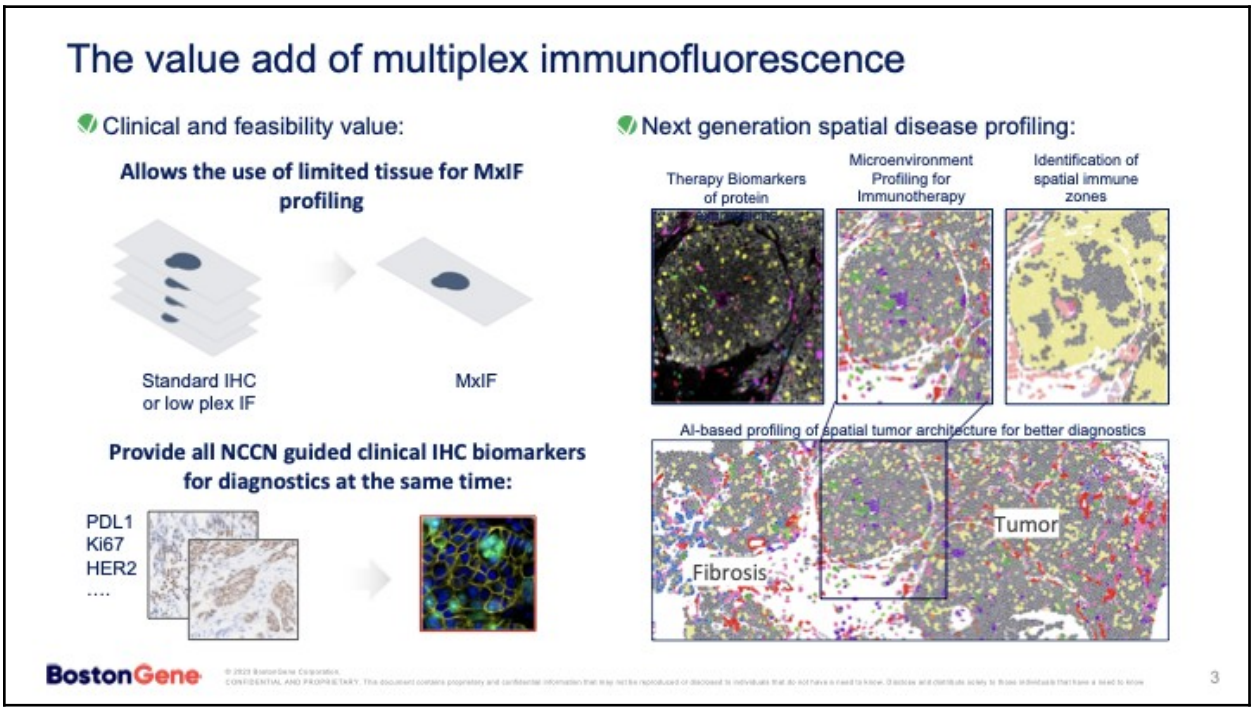
This is multiplex immunofluorescence data. We can work with non-malignant tissue. We can work with malignant tissue. We can work with solid malignancies. We can work with hematological malignancies. Or if your tumor is located in the lymph node or in bone marrow samples. Here's just an example of images we can receive and analyze.



We offer multiplex immunofluorescence (MxIF), in addition to, or instead of, standard IHC. For standard IHC we usually utilize one marker per slide. For multiplex immunofluorescence we can stain up to 40 markers. This can be increased in the near future and analyze many more biomarkers on a single slide. Multiplex immunofluorescence gives us some capabilities to study not only the presence or absence of different markers and calculate the scores, but also identify the different subtypes of immune cells and identify the localization of those immune cells and some complex metrics like contacts between different cells, distances between different cells, etc.

I will provide more examples later in this presentation.

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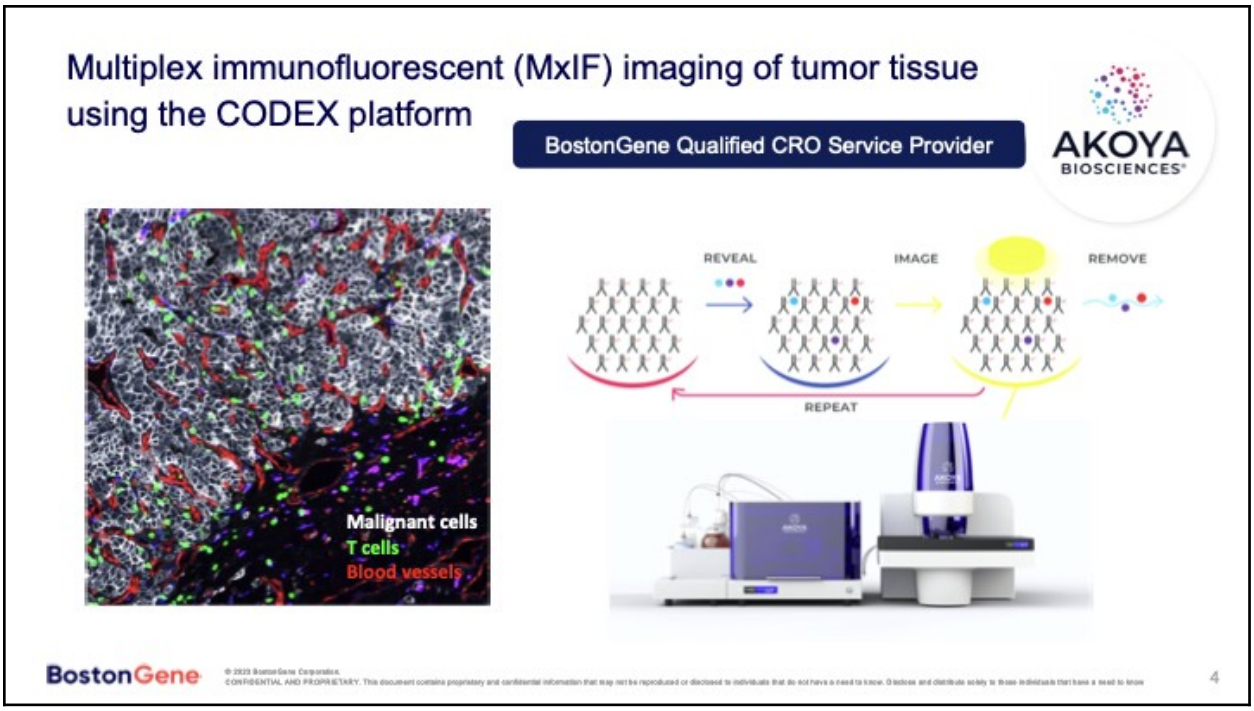

In house we have the PhenoCycler™-Fusion machine. (This machine is from Akoya that makes three-dimensional pictures of cells at single-cell resolution from a slide of a tissue sample through automated repeated stain washes.) BostonGene is a qualified CRO service provider for Akoya.

How this technology works: The first step is to cover the whole tissue with all of the antibodies. Then we put on three reporters, visualize them, remove the free reporters from the tissue, and repeat the cycle until all the antibodies are covered. If we decide to select the panel of 40 markers, we just repeat the cycle until all our markers like CD3, CD4, PD-L1, and some epithelial markers will be covered and will be imaged for us for the analysis.

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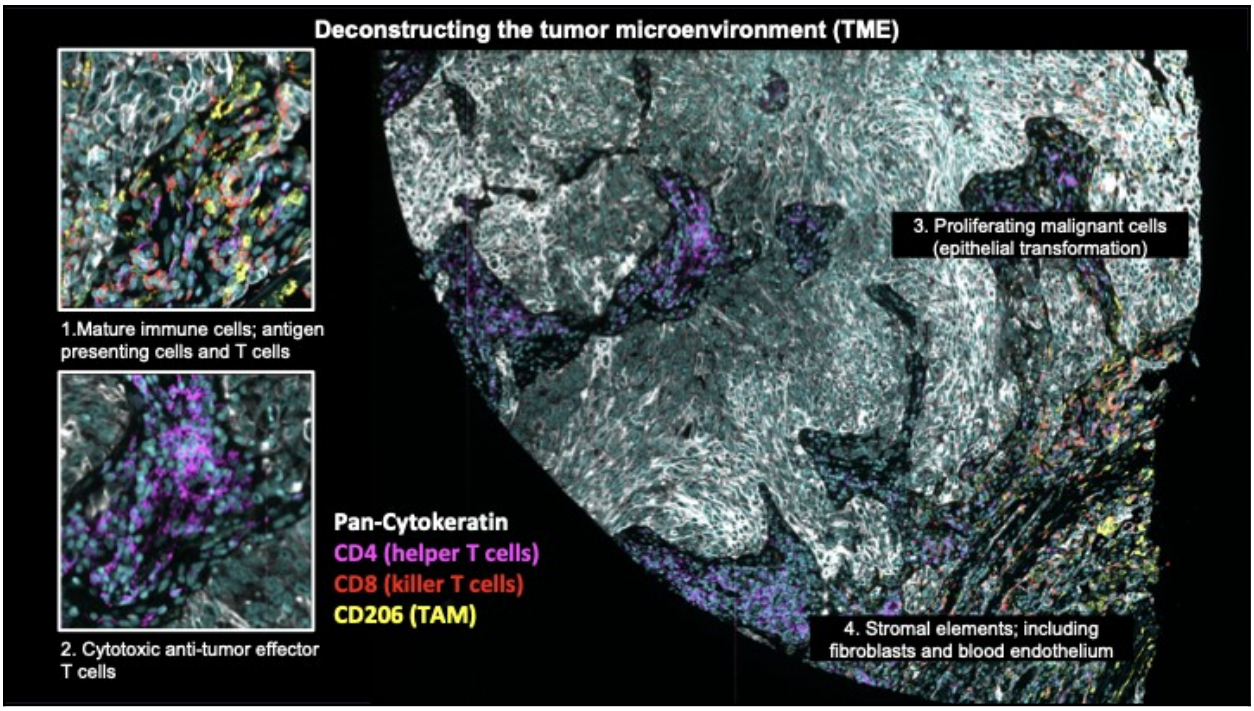

The result of this processing is some raw images, like in this example. We see the morphological structures of the tissue. We can see the tumor enriched area is sun and a gray color on the image. We see some colored dots in purple, which are different subtypes of immune cells. We also see some colored dots here, red and yellow, which represent different subtypes of immune cells. On this step, even without any analysis, and any comprehensive bioinformatics support, we see the heterogeneity of the tumor tissue. We see the tumor area. We see some stromal components. We see increased immune infiltration in the stromal component. We also see that some immune cells are present in this part of the tissue and some of the immune cells are present on this part of the tissue. Next, we need to investigate the cell types. For example, if red dots here are CD8 T-cells, which are cytotoxic T-cells, are killers, and those cells can potentially affect the tumor. We need to investigate how far away those cells are from the tumor cells within the malignant tissue sample.

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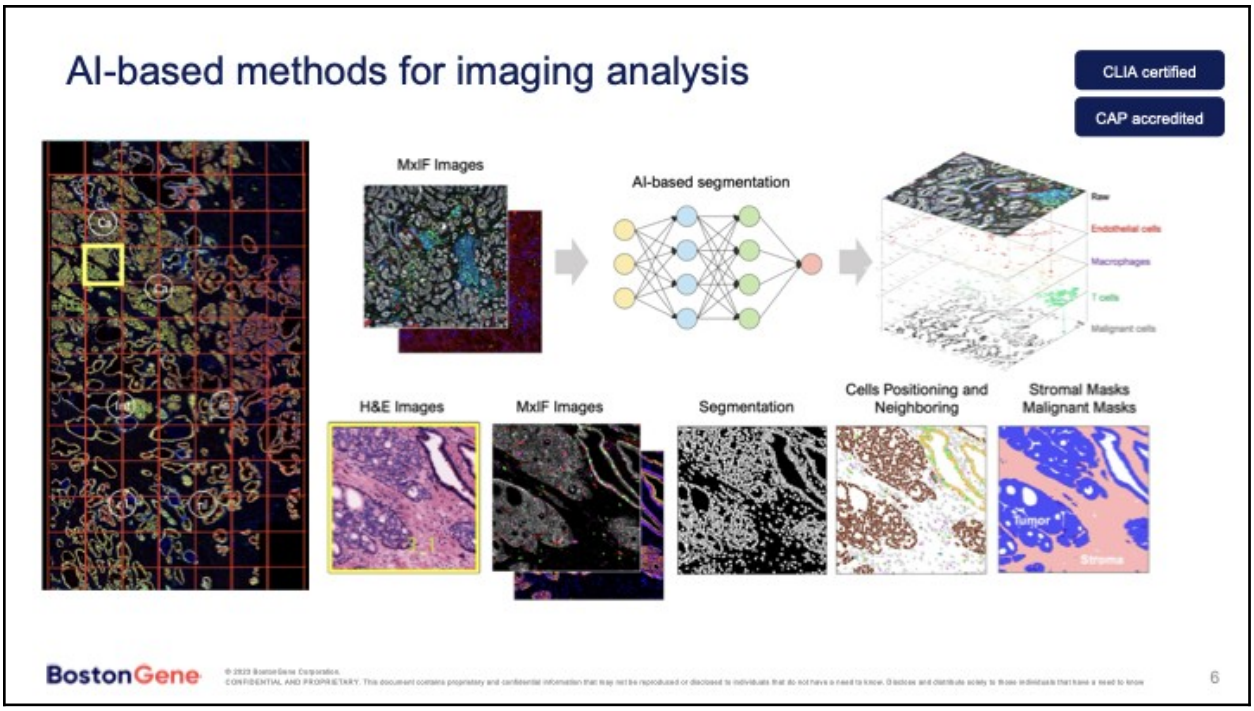
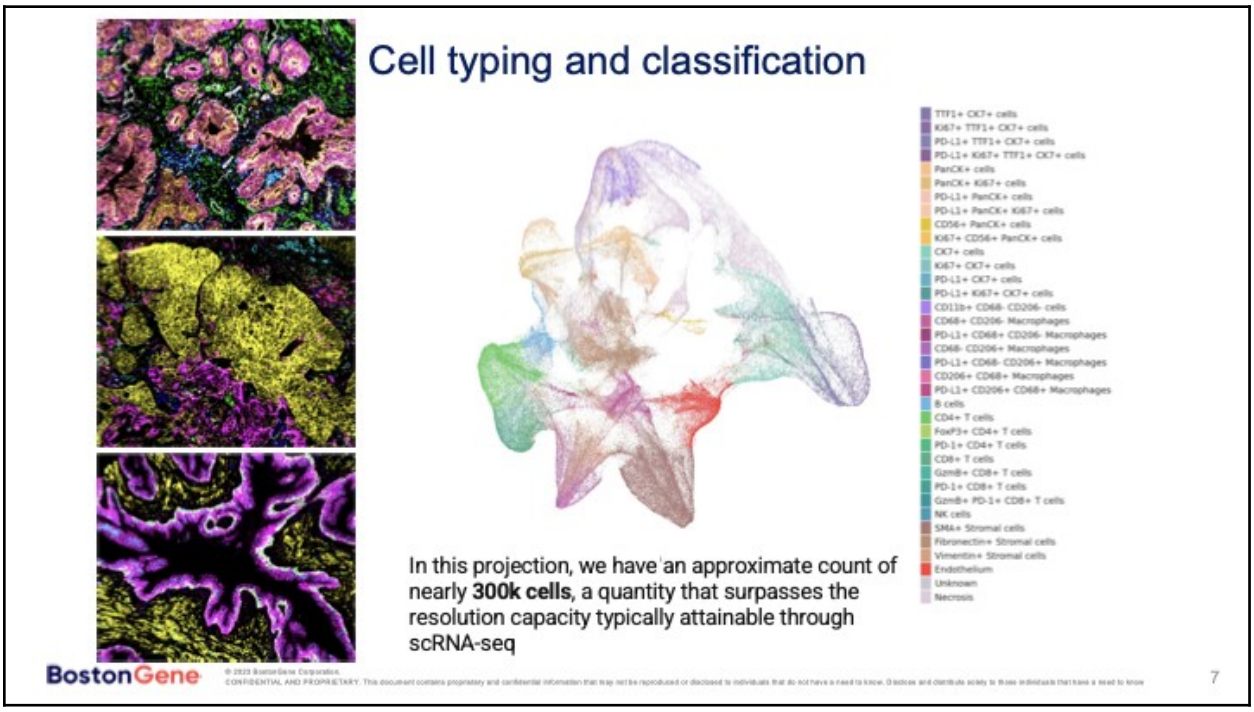


For that purpose, we have developed our own artificial intelligence-based approach to analyze these multiplex immunofluorescence images. We always start with raw MxIF images. We can work with the whole tissue section, for example, if this is reduction material. We can work with core needle biopsies. We can work with tissue microarrays, for example, if we would like to analyze multiple samples like multiregional analysis or several different time points. All of this can be analyzed in stains.

The first step is an AI-based **cell segmentation**. We have developed our own approach for segmentation and as a result of this step we receive a binary mask with multiple counters. Each counter here represents a specific cell type on the slide. If you compare those three images between each other, this is like a standard H&E image which a medical doctor pathologist analyzed. For example, looking at this small region of interest, you can immediately see malignant components, normal components, and non-malignant components. We can see the same on the multiplex immunofluorescence image, and we can see the same presence of different cells on the cell segmentation. That is the segmentation step.

Next we need to assign each specific cell segment to the specific cell type it belongs to. This step is called “**cell typing**”. You can see it here. On the cell segmentation, we just received the information about all of the cells without specification of the cells. For the next step, we see all the cells on the slide, and now we know the specific cell type of each cell. For example (pointing to the image labeled “Cells Positioning and Neighboring”), the brown color represents the tumor cells. The gray color represents the stromal component. Some colored dots represent different types of immune cells. The yellow color represents the non-malignant epithelial cells, and what was included in our pipeline matrix, not only based on the cell segments, like counts, different cells, calculating the distance between different cells, calculating the direct contacts between

those cells, but we also take into account the different approach like Stromal Mask-based approach to analyze morphological structures which cannot be described in the format over the cell segmentation, like stromal components, blood vessels, sometimes macrophages, because they have a very complex shape.



Katerina Postovalova 11:42
After the cell segmentation step is completed, we do the cell typing analysis, and using the 20 or 30 or 40 antibody panels, we can identify many different cell subtypes. Here's an example of images we can stain and receive after the wet lab processing. Here (pointing to the image in the middle of the slide) is an example of cells which we can identify from the single slide, and each dot on this image projection could correspond with different cell types. Using our standard panels, we can analyze different subtypes of epithelial cells, immune cells, like CD8 T-cells, CD4 T-cells, both of them PD-1 positive, PD-1 negative. We can really focus on the tumor microenvironment.

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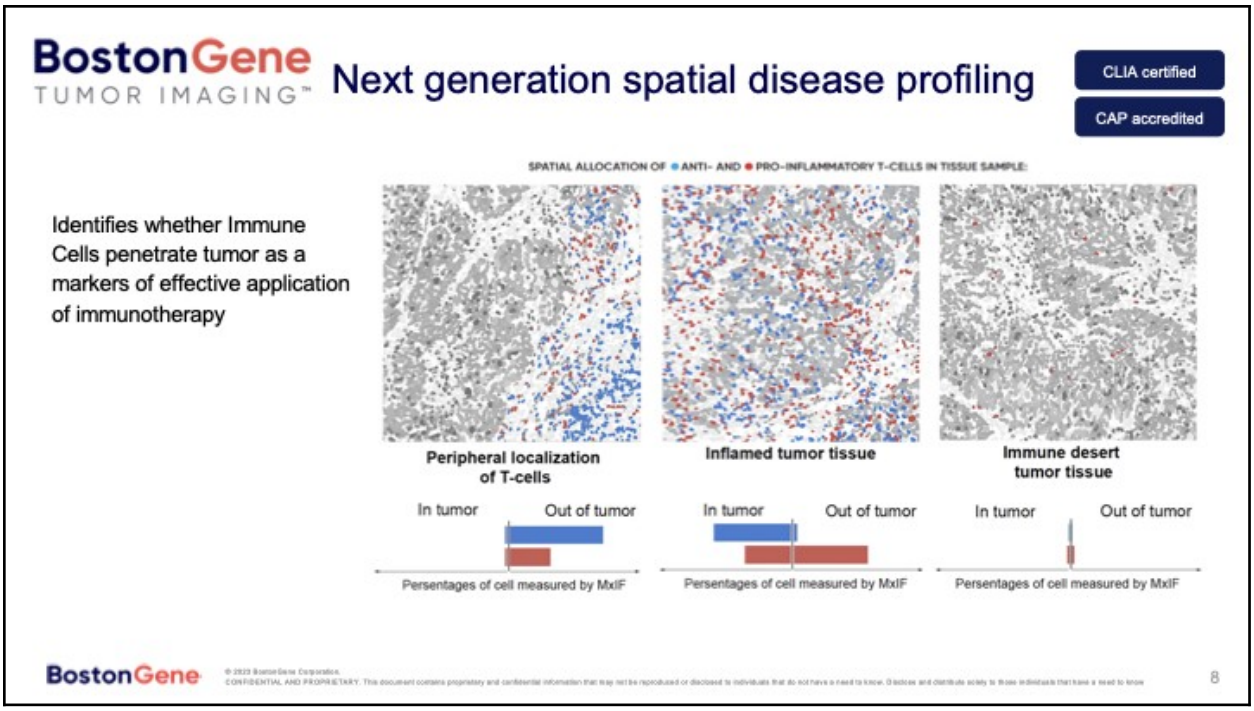

Why do we need to have this? I learned that you heard a talk about our Tumor Portrait Report. (Referring to Cancer Patient Lab meeting #72.) In our Tumor Portrait Report we focus on the tumor microenvironment. We utilize several approaches to study the tumor microenvironment based on the bulk RNA sequencing data. In one approach, we analyze or predict the percentages of different subtypes of immune cells within the bulk of the tissue. Second, we analyze which components within the tumor are present or absent using the gene sequencing-based approach. Both approaches completely lack information about the spatial organization of the tissue.

Here on this slide it shows why this is important. I put three different examples. For example, example one and example two show us almost the same amount of immune cells based on the bulk RNA sequencing results, when we see the percentages of immune cells in the tissue. But these cases can be different from the spatial organization point of view. For example, in the first case (pointing at Peripheral Localization of T-cells), you can see the gray color here, which represents the malignant compartment. We see blue and red colored dots here, which represent the different types of immune cells. We see that almost all of the immune cells are located on the tumor stromal border and the tumor itself.

We see the opposite picture in the second image (Inflamed Tumor Tissue), where we see a lot of color blue and red dots, which have penetrated the tumor cell. This will be a real tumor sample. We can see those differences on imaging data on the multiplex immunofluorescence stain. This is the main reason why we would like to supplement our Tumor Portrait Report and the bulk RNA sequencing data with spatial analysis.

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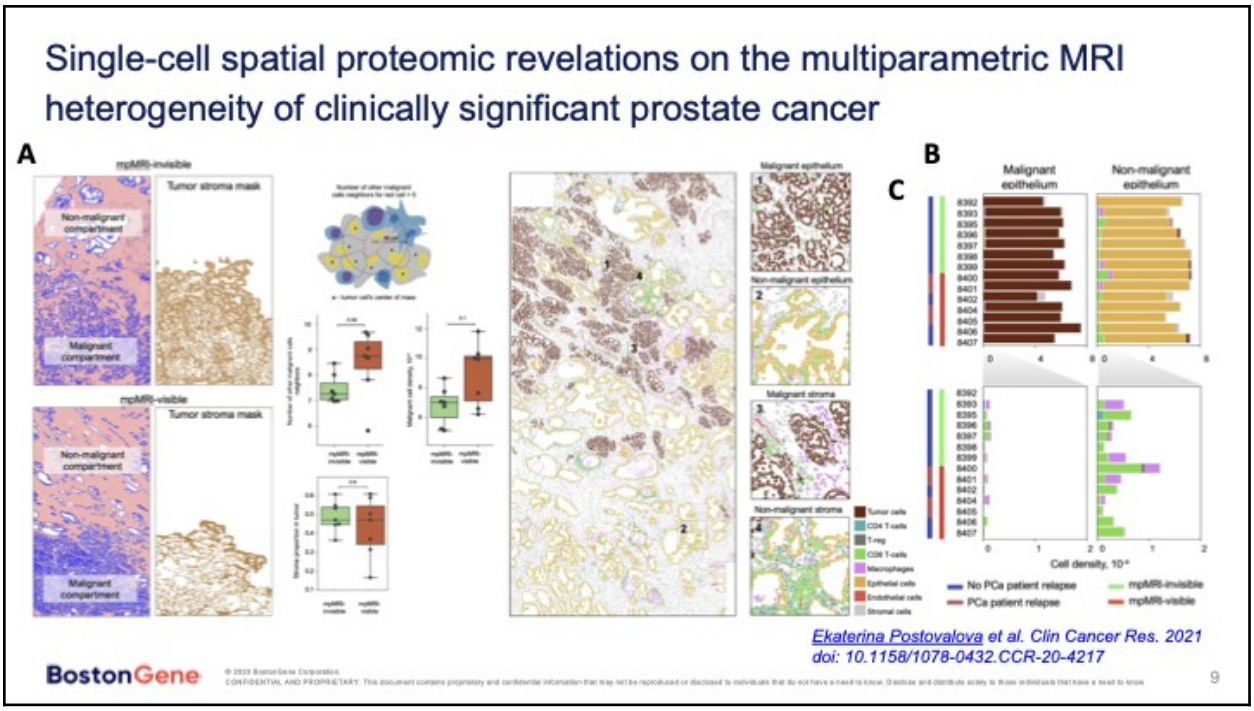

Here are some examples from one study we did in prostate cancer. I would like to highlight that, of course, when we speak about multiplex immunofluorescence, we're mostly focused on the tumor microenvironment because we would like to provide a prediction of therapy response to immune oncology. But we also can analyze the different metrics and morphological structures and find their associations with, for example, MRI points, patients with better or worse prognosis. In this work, we analyzed the distribution of the stromal component, the density of tumor cells, the tumor cells itself, and we analyzed the presence of immune infiltration in different compartments on the tissue. The color coding here (pointing at B) is the same: the brown color represents the tumor-enriched area of malignant cells, and we can see the tumor here (pointing at the brown area). The yellow color represents non-malignant components, or the non-malignant compartment of the tissue, and color dots green and purple represent different subtypes of immune cells. Green dots represent T-cells, and purple dots represent macrophages. If we analyze the tumor microenvironment deeper, we see that in the malignant tumor cells-enriched area, we see less immune alterations and a non-malignant capacity. We can expect, for example, that most of the immune cells we see on the bulk RNA sequencing probably came from the non-malignant component.

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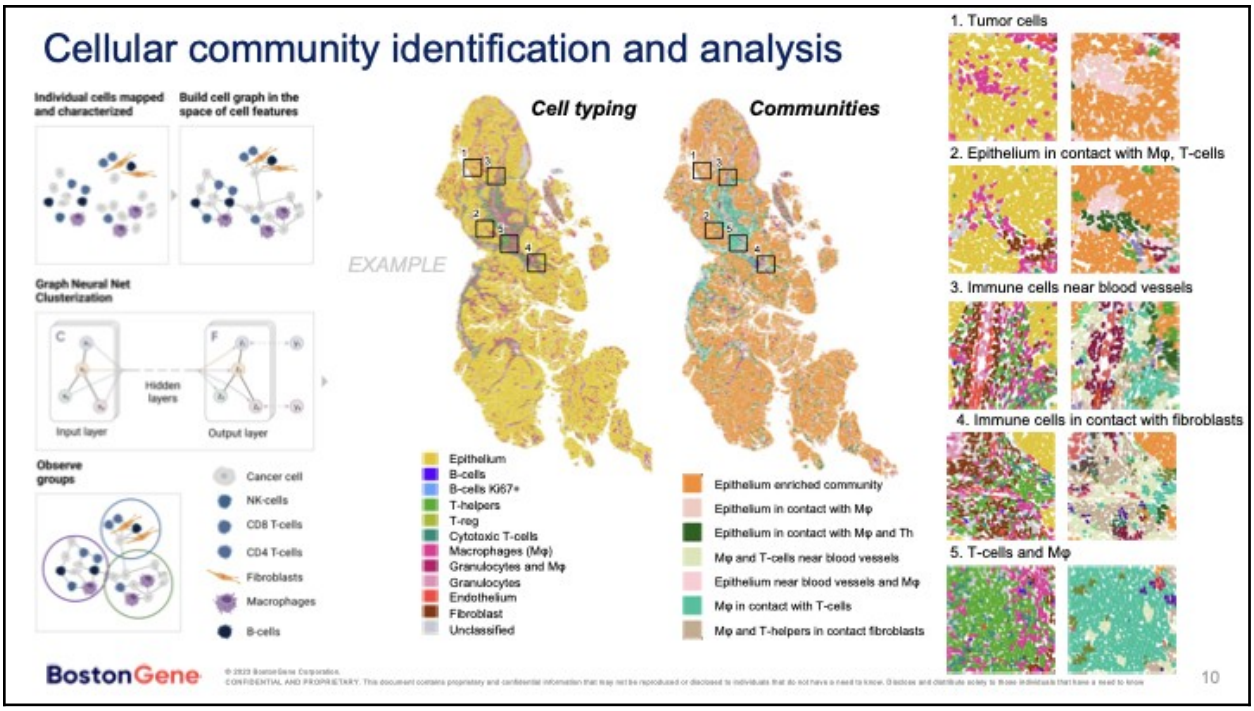

Using another approach, we can calculate the percentages of immune cells. We can calculate the absolute amount of immune cells. We can calculate the distribution of these immune cells – those allocated in the tumor stromal border, in the stromal component near the blood vessels.

You also can do some comprehensive analytics. Here's an example of this type of analysis – the analysis of cellular niches or cell-to-cell interaction analysis. This is how it works. This is a graph-based analysis. On the left picture (pointing at Cell Typing), you can see this is actually a cell segment analysis, colored by different cell types: yellow color zones are epithelial cells, and the colored dots are the same, representing different subtypes of immune cells. On this image (pointing to the image labeled “Communities”), we have the same segments but annotated in the cellular niches. Now, for example, when we see the orange color here, we mean, not like epithelial cells, but we mean, for example, high density of epithelial cells. There was almost zero presence of immune cells in this type of community. For example, the green color here, we mean that this is a stromal component with an increased proportion of immune cells within this community. We have more spatial analysis and spatial meaning and color coding and analysis.

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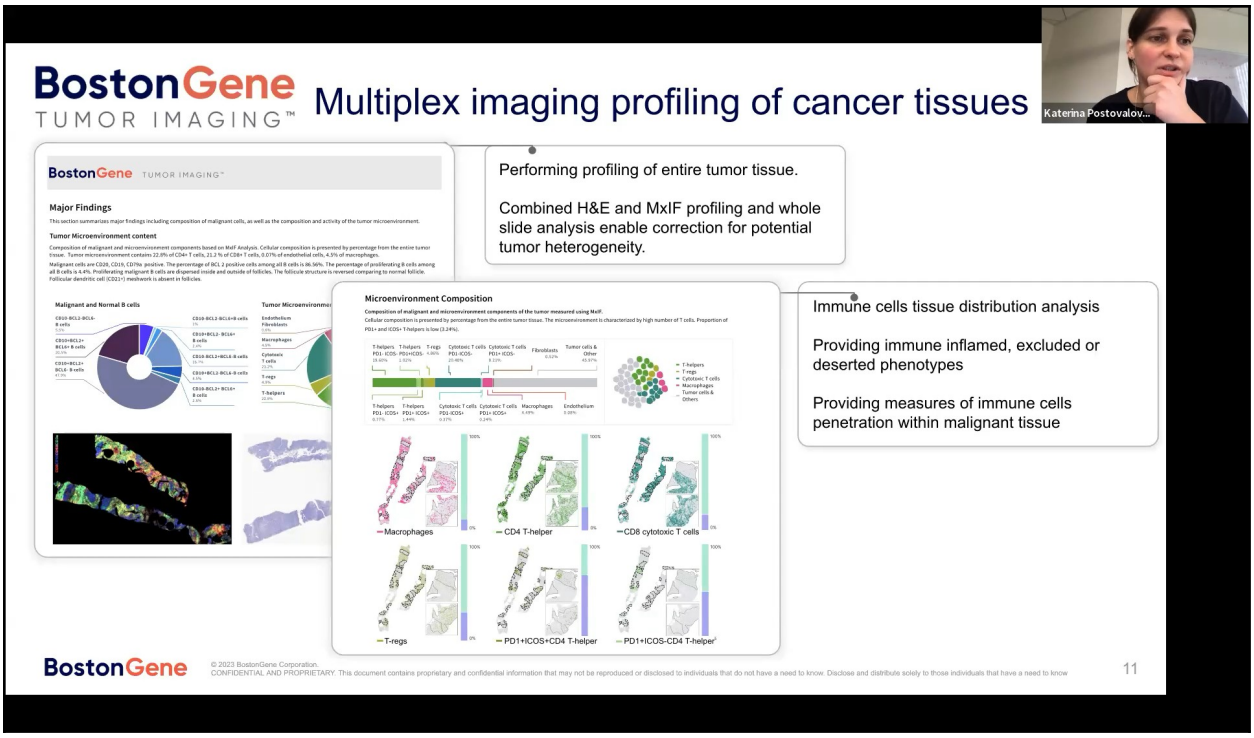

We also have prepared the tumor imaging report. I mentioned that this analysis and this information can be provided as a supplement to our Tumor Portrait report. We focus on the malignant cells and on the tumor microenvironment. We, of course, try to simplify the analysis we provide not to push all of this complete, comprehensive analytics and data visualization. We just calculate the percentages, trying to compare current patients with the patients with the same diagnosis, like we do in our Tumor Portrait Report, trying to match with widely-used biomarkers and clinical practice.

Rick Stanton 19:47
Emphasizing why we care is probably coming up. For example, Phil just responded to Pluvicto. That's awesome. We were commenting that not many people in our little lab responded to Pluvicto. Would your spatial analysis have predicted a response? One would hope so, rather than the bulk RNA seq, which is like, take all the tissue and you throw it in a blender, and you get the components. It’s like you are going to get a smoothie, and you said, “There's so many blueberries and such.” But now with your spatial analysis, which I'm loving, we can get the nuances. Of course, we're hoping to go beyond just PD-L1 since we, especially as prostate cancer patients, are mostly cold tumors. This type of glimpse inside what's happening in the tumor microenvironment is fantastic. I hope someday it will lead to directed therapies. It probably didn't matter in Phil's case, but I would suspect we would have seen an infiltration of T-cells. Because if Pluvicto was going to work on PD-L1, you have got to have some T-cells. You have to have the right players in the microenvironment.

Next up, a prediction of response is something that this would be able to provide, far in excess of the standard nice stains. When we think about the 40-plex, you're going 40 dimensional, you

have a lot more information. You can nuance beyond PD-L1. You could look at B7H3, like the clinical trial I'm on, or how to reverse the desert. Reversing the desert, to enable your immune system to work. It's something that I've been looking into, and we're all interested in it, of course. You now have 40-plex, and you can guide those 40 markers to help take us to the next step in the revolution against cancer. The only trouble is, we need tissue. We have this tissue. We see where it was and make a prediction. Now we intervene in some way. What happened? That requires more tissue to find out what happened.

Are you offering RNA seq on circulating tumor cells? Is that something inside your portfolio that would maybe be of interest?

Katerina Postovalova 23:47
Not yet. So right now we're developing these technologies in our lab.

Michael Hensley 23:55
I came from a conversation with Brian. [Todd Issue?] is leading this effort in our lab with this technology. It is in development. RNA within this level is extremely fragile. A lot of it has to do with blood collection and everything that happens until it gets to our lab. That's one of the most difficult aspects.

Going back to MxIF (multiplex immunofluorescence), the question is clinical utility. In a lot of the projects that we're doing with MD Anderson, the broad projects are doing the Tumor Portrait testing, and then gauging response to whether it's immunotherapy or whatnot. But then what we've done is in some of these high responders or some of these non-responders, we are going back to use MxIF to better understand. One thing that Kate shared, and is very exciting to us, is to be able to identify the different cells that have penetrated the tumor versus those that are outside the tumor. The question is “Why?” Why is that happening or not happening? Stand alone, it's not there. But to offer greater insight, it's definitely there. That's where the MxIF is being used from the research side. Kate may have something to add because I'm focused only on MD Anderson. But when we talk about this – the tissue assay, the blood assay, the MxIF – we're trying to go so deep to decode the cancer in unique cases to really understand what's driving response, or lack thereof.

Katerina Postovalova 25:50
Right now we're working in both directions. The first is just described by Michael: when we provide a tumor imaging report, in addition to our Tumor Portrait report, for example, in cases when we see highly inflamed keys to just confirm where those immune cells are located. We also do a lot of research work. We compare multiplex immunofluorescence data with the bulk RNA sequencing data. For example, if we see that a lot of immune cells that came to the tumor, but we see almost all of these immune cells are on the tumor stromal border. Next, we use the bulk RNA sequencing to see the cytokines and chemokines, to see what happens in the tumor while the immune cells are stacked on the tumor stromal border and do not penetrate the tumor itself. This is more likely research work right now. But we're working in this direction as well, trying to identify the biology of the tumor itself and why some tumors are exhausted or not

exhausted – the phenotype if we see a lot of immune cells in the non-malignant component around the tumor.

David Plunkett 27:21
What's your experience with the durability or fragility of the tissue samples? Is there an age after which it's not really practical to do an analysis, then you need a fresh tissue sample? For example, my biopsy was four-and-a-half years ago. I don't know if it would actually be useful to try to do any analysis on those samples at this time, even if I had access to them.

Katerina Postovalova 27:53
We have known for years that it's not that old biopsies age. For our Tumor Portrait Report, we can utilize this sample and for multiplex immunofluorescence it also can be utilized. For our assay, we need an FFPE (the Formalin-Fixed Paraffin-Embedded preservation method) tissue sample. If you have a fresh-frozen tissue sample, it doesn't matter, we can embed this in paraffin and proceed with the staining and with the following analysis. If it is already embedded tissue, we can cut one slide and then do multiplex immunofluorescence to see the morphology of the tissue.

Michael Hensley 28:41
The vast majority of tissue samples – over 98% that we receive – are preserved in formalin blocks.

Katerina Postovalova 28:57
If the FFPE block is younger than five years, we can work with the block. It will be better to have a block instead of just slides. For example, if it is five-year-old slides, it looks like a sample will not work well from this type of the material because we need a fresh cut at the tops.

David Plunkett 29:22
Letting a patient know in advance what the limitations or requirements are is important because if I can avoid going through another biopsy, I'd certainly prefer that.

Katerina Postovalova 29:37
It's true on the one hand, and on the other hand, the tumor can change within these four years significantly. So if you would like to have the actual information about the current status, it would be better to have the fresh stuff.

Kaumudi Bhawe 30:02
Do you see significant differences between the primary tumor and metastases in patients where you're able to obtain that sample? I know it sounds limiting.

Katerina Postovalova 30:33
From the bulk RNA sequencing point of view: we do not have any batch effects between those two types of samples (primary site and metastatic site). For example, when we receive some solid malignancies from a metastatic site, we will still be able to compare this sample with our

internal cohorts, because we do not see any batch effect with primary sites for multiplex immunofluorescence. We just do not have that many cohort samples. We have some multi-regional samples. Those samples can differ from each other mostly because of the presence of tumor or immune cells itself. It's not that much of a difference, like this is the primary site, this is metastatic. It's mostly because of the tumor sample itself, for example, one site will have more tumor cells, another site sample will have less tumor cells.

Kaumudi Bhawe 31:36
Are there any cancers that are just so notoriously difficult to read that you just avoid them upfront?

Katerina Postovalova 31:41
Speaking about multiplex immunofluorescence: we don't have any issues with different cancer types. We have only one issue, which is with the preservation method. For example, if it will be a metastatic site in bone, we will need to do declassification. To be able to perform bulk RNA sequencing, a whole exome sequence, or multiplex immunofluorescence sequencing, or multiplex immunofluorescence staining, these bone marrow samples should be declassified with EDTA (ethylenediamine tetraacetic acid) solution. Usually in the pathology lab for declassification, they use [Achis?]. But for NGS and for multiplex immunofluorescence, this solution will not work. We require a different type of solution for bone for declassification of the sample.

Kaumudi Bhawe 32:34
When there's highly necrotic (dead) tissue in the tumor, how does that work?

Katerina Postovalova 32:44
For example, if we're speaking about NGS (next generation sequencing), and we see necrotic (dead) tissue, we analyze the presence of the necrotic tissue in the sample. For example, if it is not broad necrosis, we can just perform the macrodissection to remove the necrotic tissue and still be able to proceed with NGS data generation and analysis. If we're speaking about multiplex immunofluorescence, necrotic tissue doesn't affect us at all. From the research perspective, it is also really interesting to analyze this type of morphological structure within the tissue because sometimes it is full of macrophages and such. For NGS, we're trying to remove this as well, like blood on the slides. From the multiplex immunofluorescence point of view, we don't see any issues here and can proceed with the staining. We will just decide whether we want to mask this area and analyze this as a separate morphological structure, or we can perform the cell segmentation, and then we will see a difference in the cell typing step.

Michael Hensley 34:00
In various departments at MD Anderson, and for some cancer types, it's Uber important to test the most recent metastatic lesion to see how the behavior of the cancer has changed because we know that a lot of therapies, like endocrine therapies in breast cancer can change the histology. But there are some cancers, like in a project we're doing with Dr. Subhudi, where

we're going back to the 2011 prostatectomy for the tumor microenvironment. It’s a great area of interest.

We've discussed on our prior calls how you make an immune microenvironment hot, turn cold to hot. What makes a hot tumor turn cold. In many of our cases, we are being able to do longitudinal testing. If we have a treatment in the middle, what we do at BostonGene is we will do each individual test, and deliver the Tumor Portrait Report. Then our team will add a tumor evolution report. We will show differences in key biomarkers. We'll show differences in TMB (tumor mutational burden) and PD-L1 and so forth. But we are also a CAP-CLIA certified lab, so we can show individual RNA expression. We can show changes in RNA expression levels between the two different tissue samples. This is mostly hypothesis generating, because we don't know yet, but it's interesting how some therapies can really affect these key biomarkers.

Brian McCloskey 36:00
I'm just in the process of going through your pipeline. As a matter of fact, I was just sending an authorization to UCSD to release my tissue from 2016, which is from my prostatectomy, as well as my most recent surgery, which was in November 2022. There are a few things that you mentioned that I want to drill down on. You said that beyond five years, the tissue starts to degrade, and you'd rather have a block of tissue. My prostatectomy was seven years ago. Should we include that? This is getting into some of the details. We're going to do the immune profiling. I don't know if we can do spatial profiling. Michael, you still may be working on that.

If a patient has access to do both spatial and your standard immune profiling work, and you have limited tissue, can you run both tests on the same tissue? Or do you have to prioritize one or the other? Tissue is a precious thing.

Katerina Postovalova 37:40
We should prioritize the Tumor Portrait test. For our Tumor Portrait test right now, we request ten unstained slides, but we have developed the ART algorithm, which can predict the number of slides which we will need to take into the extraction. We can request ten unstained slides, but when we see the tissue, if we see that the similarity of the tumor is great and the area is enough, we can utilize the fewer slides than we requested for the Tumor Portrait analysis, and then we can make a decision to run the additional test, like one slide for the multiplex immunofluorescence assay. So it definitely should be prioritized because we cannot run two tests in parallel. So we can run the Tumor Portrait test, which is bulk RNA sequencing and whole exome, in parallel from the one set of slides from the one step of the extraction, but multiplex immunofluorescence should go further, if we only have enough materials for one slide to perform this test.

Speaking about the old biopsy, we have experience working with a seven-year-old biopsy for the Tumor Portrait test, so we can process this for bulk RNA sequencing and whole exome sequencing. You just need to decide if it is crucial to know the information from the seven-year-old biopsy.

Michael Hensley 39:16
I see a lot of older tissues that are requested for Tumor Portrait testing. Obviously, the older the tissue, the higher the difficulty of mainly RNA extraction. RNA can degrade. But if there's really strong tumor content, like Kate was explaining, that we can go and analyze and microdissect, that enhances our chances. Obviously, if it's very small tumor content, the failure rate goes very high. But in many of the cases of some of our projects that were requesting old tissue from the primary site, what I've seen is that we're usually pretty successful with DNA extraction. It's RNA that really becomes difficult. A lot of the BostonGene magic is with the tumor microenvironment profiling, and all that stuff comes from RNA sequencing. But we can deliver a DNA-only report, which basically shows everything we found in whole exome sequencing.

Part of your case, Brian, is to get that primary lesion – fingers crossed we are successful. Then do your most recent November 2022 surgery. I’m confident with that, because you said that there was pretty significant tumor content there. Fingers crossed, we will be able to deliver both. Then we can deliver the tumor evolution report and really understand your transition. It'd be nice to add it to your amazing portrait of your whole timeline. It'd be really exciting.

Katerina Postovalova 41:00
You also mentioned immunoprofiling. We do immunoprofiling from blood samples. This is our comprehensive flow cytometry approach. The team has developed an immune fitness score. We know about the presence of different subtypes of immune cells in the blood, from healthy donors and from cancer patients. The team can predict the therapy response to immuno-oncology using the blood samples as well. Immunoprofiling, or the blood test, is a really, really powerful tool in the immuno-oncology point of view too.

Brian McCloskey 41:40
This is a real life case, which will hopefully be useful for everyone because to a certain extent we are defining maybe a little bit of a new path in terms of what to do with longitudinal information about me, tissue specifically.

Rick just brought up a good point, which is to say, I had a midpoint resection in 2020. I have three tissue samples, (1) my prostate in 2016, (2) a met resection in 2020, and (3) another met resection in 2022. So, does it make sense to take all of it? Or would we be better off taking the 2020 resection? Again, there's only so much tissue that a patient has. It's precious, precious currency. Understanding how to maximize the utility of that tissue is critical before a patient actually goes through this process.

Please help me understand what is the best way for me to prioritize my tissue for diagnostics.

Katerina Postovalova 43:15
If you take all three time points, and you receive treatment, you can definitely see how the tumor changed the tumor microenvironment subtypes. For example, “Was it cold?” Or, “Was it hot?” “Was it more hemo subtype?” “How did the tumor change when you took the drugs?” We definitely see more stories when we have three time points.

Brian McCloskey 43:45
Ultimately, I love data as much as anybody on this call. But really, it's about how to translate that data into treatments.

I've got a meeting that Michael set up with Mike Goldberg (Director, Immunology and Immunoprofiling at BostonGene) coming up on Friday. I'm going to be bringing, hopefully, if he can make it, Saul Priceman. He's the brains behind his lab, which drives CAR-T development for the City of Hope.

One thought is, “How do we use all this great insight to develop some type of immunotherapy, whether it's CAR-T or something else, that can attack my cancer?” In the context of this menu of tissue and corresponding data, you're going to have three resections, three tissue samples.

What is the best way for us to leverage that tissue?
Please give me insight into my treatment decisions.

Katerina Postovalova 45:07
For the real treatment decision, the most relevant will be this latest tissue sample. If you would like to see how your tumor reacted to the previous treatments, it will be better to analyze all three to see the full history. For example, you received one drug, and next check to see, “How's the tissue?” How did the tumor respond to this drug? You try another drug, and see how the tissue responded to this other drug.

For your real prospective treatment decision, the most important one is the most recent tumor sample.

Michael Hensley 45:39
I would agree with Kate: the greatest clinical utility is the most recent surgery. Going back to the original surgery is more thought-provoking where you started. If Dr. McKay or Mike Goldberg think that we should go to the midpoint because you're so far gone from that original diagnosis, then we could do that. But we've had quite a few private-pay patients that have done five or six BostonGene Tumor Portraits, not only evolutionary, looking at different time points. If they have multiple anatomic lesions taken at the same time, at different metastatic sites, then we've done local analysis.

Outside of Brian's unique case where we're doing this partnership, the elephant in the room is insurance reimbursement because that's very tough. For commercial patients, we're often limited on how many tests we can run. We believe that there's a huge profound opportunity for serial testing before and after treatments, but the real gatekeepers are insurance companies and reimbursement.

As regards immunoprofiling: in our research setting this is being used serially, like while patients are on immunotherapy treatments. We have a metastatic trial where the immunotherapy is

being treated like once every month. Before each treatment, the patient does a blood test. So they're adding immunoprofiling to their normal blood tests.

In Brian's case, I don't think that we've identified the clinical utility for our immunoprofiling tests, because it's based on pre-treatment, and then during treatment and post-treatment. That was the topic for his meeting with our chief immunologist, Mike Goldberg, because Mike Goldberg has developed this test, and he is the expert on this test. It will be very interesting to see where that conversation goes. You want to have clinical utility behind the test, even though it's exciting to get insight, and it'd be thought-provoking. But Brian, if in your discussions, you take this feedback to your oncologist, we've promised to test and then to do the tumor evolution. If you decide you want to go to that midpoint, we can change it. It's no problem at all. So just let me know.

Gitte Pedersen 48:52
We do bulk RNA sequencing, and we translate it to treatment options using a platform.

We are super interested in potentially collaborating in some form. We do not do anything on the spatial side. We have a project with the NCI where we are interested in validating some of our findings using IHC.

Because typically we do one biomarker on one slide, and we talked about the availability of tissue is always the issue, my question is: given the other biomarkers you said you have – I know not all of them are FDA-approved, but PD-L1 is – if you have done any validation against the existing IHC methods for PD-L1, androgen receptor, ER, PR, HER-2, in particular?

Katerina Postovalova 50:21
This is exactly what we're doing. This is a separate flow for us to provide the clinical utility of bulk RNA sequencing data. We'll do the correspondence analysis between IHC and bulk RNA sequencing. We stain the same samples with IHC and do bulk RNA sequencing and provide the cutoffs – which is low, medium, and high – on bulk RNA sequencing through analyzing the IHC data, so we have already performed this analysis for PD-L1, ER, PR, HER-2, TROP, [Palat ?] receptors. So right now, the work you're asking for is done.

Gitte Pedersen 51:04
I understand that you are also validating your bulk RNA seq. My question was more for PD-L1: have you also sent your slides out for third party staining? Has your internal staining been validated?

Katerina Postovalova 51:27
During the LDT (Laboratory Developed Tests) validation process, we sent samples outside. We stained a sample in house and also sent it to external CRO (contract research organization) vendors to provide a proof of validation. Now, in our everyday practice, we launched our own PD-L1 [22 C three?] clone, so we don't need to send the samples externally. We can stain all of them in-house. But during the LDT validation, actually [UNVC?] validation for our multiplex

immunofluorescence markers as well. We stain MxIf markers. We stain multiple slides with the individual IHC markers in-house, and we sent those unstained slides to the external CRO to stain IHC externally. It was a huge process.

Gitte Pedersen 52:19
If it's validated, maybe there's a way we can collaborate, because obviously, there's a plurality of biomarkers that we would like to validate in this particular project. Back to what we all know is the limited amount of tissue in those FFPE (fresh frozen, paraffin embedded) samples: If you have validated the biomarkers, we can potentially send you a slide, and we can see what we find there and consider that as an LDT-validated PD-L1, or androgen receptor, ER, PR, or HER-2. We don't know yet exactly what the list is going to end up being. But those are the most common ones I see. Our focus is really also cold tumors and tumors with low mutation frequency, which don't, at this point, benefit that frequently from immunotherapy.

Katerina Postovalova 53:31
We'll be happy to cooperate.

Brad Power 53:35
You're both in Boston.

Gitte Pedersen 53:37
We can meet at any time and also on Zoom to figure this out. As long as we can claim that the markers that you have are validated, we do know that most of the findings will not have any IHC biomarker that is validated. That's the other problem that we have: it's not FDA-approved or used in the clinic. So this is best on mets on validated space out there. I like what you're doing with the multiplex a lot.

Allen Morris 54:46
I saw that you do spatial analysis. It looks to me like you're trying to separate tumor tissue into inflamed states and then two separate cold states, one immune-excluded in an immune desert. Is that correct?

Has your group ever looked at segregating the Gleason scores patterns? For example, do you have an idea of what percent of Gleason score 3 is inflamed?

Katerina Postovalova 55:38
I do not know because we don't have that many samples to receive an equal group of samples with each Gleason score.

Allen Morris 55:50
I’ve looked at prostate cancer for 40 years. I'm going to give you my hypothesis. I certainly have not done it in any kind of sophisticated fashion, where I'm looking at CD8, CD4, tumor associated macrophages, or stromal cells. I look at it the way we've looked at it from 1850 on: by light, microscopic H&E (Hematoxylin and eosin) stain. I've been looking at Gleason pattern 3

for 40 years, and it's never inflamed. I shouldn't say there are tumor infiltrating lymphocytes because here's another problem I saw with your three things. That is, prostate cancer naturally has prostatitis. So I would hope that your algorithm subtracts out background nonneoplastic prostatitis because, for example, you have an immune desert as having absolutely no inflammatory cells. I can tell you, that is not the reality of prostate cancer. In other words, as far as my hypothesis goes, my view of the immune desert is that the cancer cell has not evoked a natural adaptive immune response to the tumor. Your immune desert picture was defined as something that had no inflammation anywhere, and I can tell you, that's not the reality. The reality is you have background prostatitis, and you frequently do not have the immune desert state. I'm giving you my hypothesis. The immune desert state in prostate cancer is actually incredibly high. It's backed up by the Provenge data, which shows that the patient is immune naive before they even get Provenge. I guess that was really compound. It behooves you to get a pathologist to look at patterns 3, 4, and 5, and I'm going to give you my hypothesis: 3 isn't an immune desert; 4, incredibly infrequently will you have an immune reaction to it. It's only the 5s. the really poorly differentiated ones, that have high tumor mutational burden, that are going to evoke an inflammatory spot response, especially those that are undergoing comedonecrosis (a duct filled with dead and dying cells). You're probably not a pathologist. So you don't know that the comedonecrosis pattern is a Gleason 5 pattern that is particularly inflamed. I'm sorry, I'm sure I overwhelmed you. But you guys probably don't have a pathologist that's really honing down on this stuff, I suspect.

Katerina Postovalova 58:47
We have some medical doctor pathologists who help us analyze the samples and help us to prepare the quality control of the tumor samples prior to sequencing. But, “Yes,” I understood, and, “Yes,” we see a lot of immune infiltration and non-malignant components because it comes from the inflammation around the tumor. Yes, we see this as well.

Allen Morris 59:12
Your AI algorithm obviously is very complicated. It subtracts out the background prostatitis, is that correct?

Katerina Postovalova 59:23
We can split malignant tissue malignant cells from non-malignant cells, and we can differentiate prostatitis.

Allen Morris 59:34
Then your pictogram of immune desert should be modified to include prostatitis in the background as potentially immune desert state. Is that right?

Katerina Postovalova 59:47
We do not have enough samples. This can be so from the data analysis standpoint. We can identify the immune cells in different morphological areas. We can split the tissue. Is it a real cold tumor or is it an inflamed tumor? Is it a cold tumor with the presence of immune cells? In an

area of inflammation we can split all of these morphologically different conditions based on the data.

Allen Morris 1:00:19
Have you looked at Gleason 3 pattern to see whether or not there's a stromal change?

Katerina Postovalova 1:00:51
We do not have that many samples to make a conclusion.

Allen Morris 1:00:55
This is incredibly important. If you go down that route, since you're a theoretical company, you will make a huge breakthrough in the prostate cancer exam. I've been asking around, and none of the academics are on to this. A Gleason 3 has no stromal reaction. That's why there's no hard digital rectal exam by virtue of palpitating it.

Rick Stanton 1:01:43
I have some technical bioinformatics questions. How do you normalize your RNA seq? How do you pick your 40-plex membership?

But the real question here for the last is, “What are we going to do with Brian?” I've seen Akoya’s presentations, and NanoString’s presentations. I've tried to get them, but it always falls short. Especially spatial. It looks like spatial is falling short here as well. We're talking about all this advanced stuff that we really can't access.

This is the nature of this question. What can we access? How do we break through? With your amazing analysis, if we can't access it, it's just like, just researching stuff that is pie in the sky, and it's almost a waste of time for us. We need to access your amazing technology. As for your RNA seq, I have no idea what you do. It's probably based on immune deconvolution in R. There's no magic sauce there to me. But if you're going to do that, that's great. You probably have a pipeline. Awesome. But that's really the nature of my question. What can we do with Brian? Open this up so that BostonGene and Cancer Patient Lab start working together? Can we run it up the flagpole? “Hey, look, BostonGene did this for us.” Rather than, “We talked about it. It's unaffordable. Yeah, we can't do that.” I'm so frustrated with NanoString, Akoya, and the other interpretations on codex. Can we actually move something forward here? Like maybe a low cost pilot, one off?

Michael Hensley 1:03:59
As we spoke about that last time with Michelle, the elephant in the room with MxIF is the labor and the technologies are very expensive. We do offer it in a private pay situation. I think that the best way – and this is where I was going to go – is if we could find some type of efforts to fund the Cancer Patient Lab project with you guys, where – and this is what we do with many of our cases – we offer a discounted rate to be able to collaborate together. But I think that the most important part is, with Brian's case, is it's really going to be the most recent tissue, doing the BostonGene Tumor Portrait test on that, and understanding the behavior, the personality of his

cancer at this moment in time, and that is going to be the clinical utility. Everything else that we do as far as MxIF and stuff is just offering insight and maybe getting higher confidence for future treatment selection. But the clinical utility for some of these tests is not there yet. Now, that's the promise – to develop the clinical utility. And how does it change treatment decisions? We're not there yet. Right now we are there with the BostonGene Tumor Portrait. And so all these other exciting technologies just help us to decode the cancer, so to speak, to continue to understand the biology before and after treatment. And unfortunately, that's just where we're at. But I believe that we are moving the needle as fast as possible. From Brian's case, the Tumor Portrait is going to be the most important. Everything else is just value-add to bring this story to life – knowing what Brian's been on before and giving greater insight to why he responded or progressed on previous therapies.

Rick Stanton 1:06:08
Is the Tumor Portrait test based on RNA seq?

Michael Hensley 1:06:12
It's whole exome and whole transcriptome. It's DNA and RNA. Most of your action comes from the DNA component because that's where you're finding all your biomarkers that are mostly actionable. On the RNA side, we're looking at fusions, individual gene expression, as well as we use our deconvolution tool to create the tumor microenvironment. In the microenvironment, composition, and so forth. So the RNA is kind of a lot of thought-provoking insight. DNA is still where most of your clinical action comes from.

Rick Stanton 1:06:49
So you don't have any secret sauce, really. You don't have anything. You have a pipeline.

Michael Hensley 1:06:54
I think we have a lot of secret sauce. hat's in our depth of coverage, our ability to call not only fusions, but expressions, and tumor microenvironment profiling is unique to BostonGene. We're seeing a lot of clinically actionable therapy choices based on that. There's a lot of talent just in the sequencing. Sequencing is not a commodity.

Rick Stanton 1:07:29
I don't see anything novel. But let's push it forward and prove me wrong. You have a pipeline.