

## **“Single Cell and Cancer Heterogeneity Analysis” (Wendy Fantl, PhD) [#17]**

Brian McCloskey and Brad Power

July 13, 2022

*“We’ve had DNA and RNA seq. Those are all great proxies for proteins. I don’t want to monkey around anymore. I want to get a good definition of what’s happening.” – Brian McCloskey*

*“You need somebody that’s really, really committed to understanding how new technologies can work. You need really good physician scientists.” – Wendy Fantl, PhD*

### **Meeting Summary**

Wendy Fantl, PhD, Assistant Professor, Urology, Stanford Medicine, led a discussion on "Single cell and cancer heterogeneity analysis". She gave a basic introduction to two single cell technologies she works with: "Cytometry by time of flight" ("CyTOF") and "CODEX" (Co-detection by indexing) multiplex imaging. They provide important information at a single cell level about protein expression, using antibodies to identify cells based on the types of antigens or markers on the surface of the cells.

*Why are these single cell technologies important?*

Tumors are a mass of heterogeneous cell types. Single cell proteomic technologies allow you to unravel tumor heterogeneity, to peer inside and identify key cell types in the tumor, their numbers, and how they are arranged spatially into neighborhoods. They can help characterize immune function and cancers, and understand cells' response to therapy. You can identify minority cell populations that are the bad guys, that have key roles in tumor initiation, maintenance, cell migration, and drug resistance.

For example, Wendy shared research that looked at blood collected from men with metastatic prostate cancer and found natural killer cells that didn't kill anymore. With CyTOF, you could measure populations of these distinctive natural killer cells to monitor disease progression.

Since CyTOF looks at cells in suspension (usually blood), you lose information about which cell is next to which cell. That's important, and that brings up seeing cellular neighborhoods using CODEX multiplex imaging. For example, you can see one neighborhood that has tumor cells, or another that has immune cells. A "hot tumor", which is more likely to respond to immunotherapies, has immune cell infiltration into the tumor cells in a neighborhood, while a "cold tumor" does not.

Brian McCloskey asked how he could have a conversation with his doctor about using these technologies in his treatment decision-making process. “We've had DNA and RNA seq. Those are all great proxies for proteins. I don't want to monkey around anymore. I want to get a good definition of what's happening.”

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Wendy responded: “You need somebody that's really, really committed to understanding how new technologies can work. You need really good physician scientists. They need to really get it. Sadly, a lot of physicians don't have time. I am a former patient. I had ovarian cancer. I fired so many doctors you don't even want to know. I told them: ‘I am the patient from hell. I am going to ask you questions, I am the boss of me. I am a scientist and a cancer biologist, and I am not a dummy.’ I did find somebody who was amazing. She's one of my best friends, and we are collaborating. You need somebody who's really going to engage and get what it is, and a lot of them they just don't want to.”

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### Meeting Transcript


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### SUMMARY KEYWORDS

cell, tumor, antibodies, proteins, ovarian cancer, question, samples, patient, technology, tumor cell, imaging, nk cells, tissue, important, papers, oligo, immune cells, cancer, neighborhood, assays

### SPEAKERS

Wendy Fantl, Anonymous Caregiver, Dr. John Laird, MD, Brian McCloskey, Brad Power, Rick Stanton.

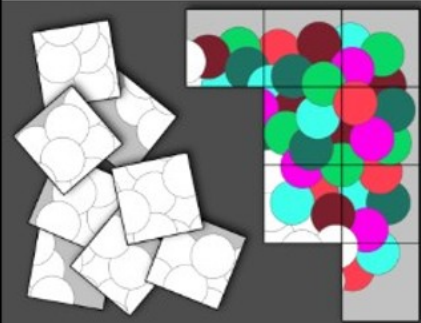


Applying Single Cell Proteomic Technologies to Expedite Patient Benefit

Wendy J. Fantl Ph.D

Department of Urology

July 13th, 2022



Wendy Fantl 00:03

## “Single Cell and Cancer Heterogeneity Analysis” (Wendy Fantl, PhD) [#17]

My lab is focused on applying single cell proteomic technologies to expedite patient benefit. We're all about translation. The focus areas of my lab are ovarian cancer and kidney cancer. But all this work can be applied to any malignancy.

### Outline

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- Background
  - Motivation for developing multiplex single cell proteomic technologies
    - Mass cytometry/ CyTOF
    - CODEX multiplex imaging
  - Clinical samples

There are two multiplex single cell proteomic technologies up and running in my lab: mass cytometry or cytometry by time of flight (“CyTOF”), and CODEX multiplex imaging. (Mass cytometry labels antibodies with isotopes of rare-earth metals, resulting in a theoretical capacity to distinguish up to 100 unique characteristics of cells simultaneously. CODEX is a high-parameter imaging technology that relies on DNA-conjugated antibodies and the cyclic addition and removal of complementary fluorescently-labeled DNA probes, enabling up to 60 markers to be simultaneously visualized.)

I'm also going to talk about the quality of clinical samples.

## Multiplex Single-Cell Technologies –Motivation

- Deep phenotyping of single intact cells unravels tumor heterogeneity
  - Identifies minority cell populations with key roles in initiation, maintenance, EMT, and drug resistance
- Mass cytometry ( Cytometry by TimeOf-Flight (CyTOF)) characterizes single intact cells in suspension based on protein co-expression patterns (60 parameters/cell)
  - Possible to include RNA
  - Proximity ligation assays to measure Pr:Pr interactions
- Multiplex imaging technologies (CODEX) use tissue sections and maintain information about tissue architecture
  - Cellular neighborhoods – different cell types and their spatial relationships

Wendy Fantl 02:04

*What is the motivation for developing multiplex single cell technologies?*

Tumors are a mass of heterogeneous cell types. These multiplex single cell proteomic technologies allow you to deeply phenotype what kind of cell it is. Is it a tumor cell? Is it a blood cell? What is it? They allow you to unravel tumor heterogeneity. And why is that important? If you took a tumor and you processed it, just mashed it up, you get sort of average values of transcripts and proteins. But because you're analyzing or measuring what's going on in single, intact cells, you can identify minority cell populations and those are the populations that you're interested in. They have key roles in tumor initiation, maintenance, epithelial-mesenchymal transition (a process by which epithelial cells – cells that come from surfaces of your body, such as your skin, blood vessels, urinary tract, or organs – lose their cell polarity and cell-cell adhesion, and gain migratory and invasive properties to become mesenchymal stem cells – multipotent stem cells found in bone marrow that are important for making and repairing skeletal tissues, such as cartilage, bone and the fat found in bone marrow), and drug resistance. Those are the bad guys. Those are the guys we need to understand.

To address this need, about over a decade ago mass cytometry was developed. It characterizes single, intact cells in suspension, and it's based on protein co-expression patterns. I'm not going to talk about transcripts or DNA. This is all about proteins, which really are the functional convergence point from DNA, to RNA, and to protein. You can include RNA in the assays, and

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you can look at protein-to-protein interactions. But CyTOF, or mass cytometry, depends on cells in suspension, and so therefore, you lose any structural information. That was the motivation for the development of multiplex imaging technologies, and several are available. Code detection by indexing, or CODEX, is the technology I have in my lab. There are other technologies, such as Multiplexed Ion Beam Imaging (MIBI) and Cyclic Immunofluorescence (CyCIF) imaging, a whole slew of them. But essentially, they're all doing the same thing. They're good at providing information about tissue architecture and allowing you to identify cellular neighborhoods. Samples of the highest quality are essential.

### Clinical Samples and Processing

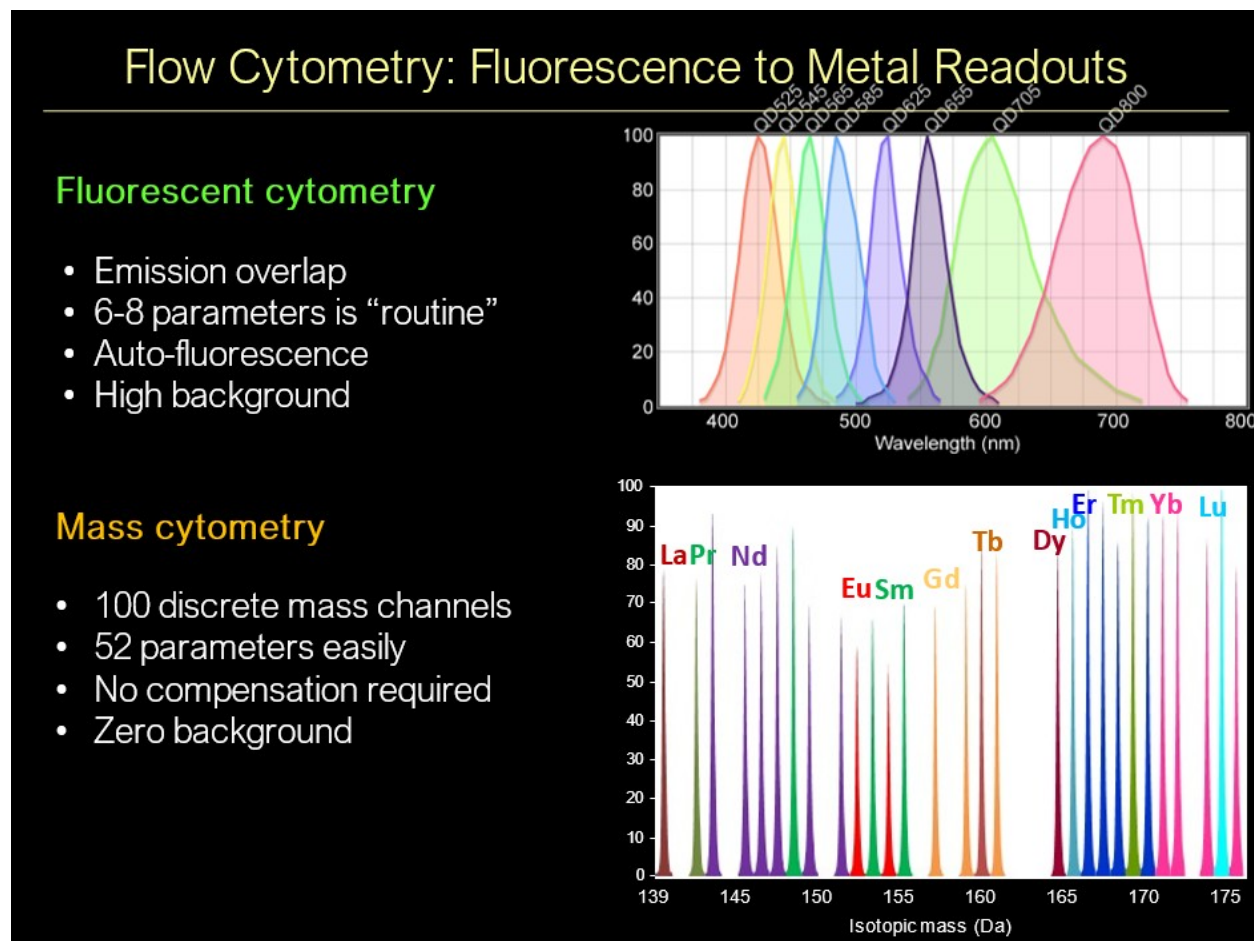
- Samples of the highest quality essential
  - Stating the obvious!
  - **Many variables to consider**
- CyTOF- cells in suspension
  - Blood processed with Smart tube proteomic stabilizer
  - PBMCs
  - Dissociation of solid tumors
- CODEX multiplex imaging of tissue sections
  - FF
  - FFPE

Gaudilliere et al. STM 2014  
Gonzalez et al. Cell Reports 2018  
Hickey et al Nat. Methods 2021  
Gonzalez et al. Methods in Mol Biol. 2022  
Phillips et al. Nat Comm. 2021

There are many variables to consider when collecting samples, and the devil is in the details. CyTOF cells in suspension means that blood is the perfect medium. People generate peripheral blood mononuclear cells from whole blood. But what I'm using is a buffer that allows you at the clinic, at the site, not to do any of the peripheral blood mononuclear side preparation, just add this buffer to incubate and freeze it. That's it. And what's really cool about that is that with peripheral blood mononuclear cells, you don't have any granulocytes. Granulocytes are also important in cancer. Smart2 buffer allows you to preserve granulocytes. For other CyTOF analysis, blood is perfect, but it also works with solid tumors. I think my lab was one of the first to look at solid image ovarian tumors and to work out in excruciating detail all the protocols and details of dissociation into high quality single cells. CODEX doesn't use cells in suspension, but it uses tissue slices. These can be fresh-frozen or formalin-fixed paraffin embedded (FFPE).

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And again, the devil is in the details. Everybody is very sort of "hoo ha" about fresh-frozen paraffin embedded samples because they're available worldwide, so it shouldn't be a problem. But I can tell you, there is such variation in the preparation. Here are some of the papers and some of them are from my lab and others. They go into the details about producing high quality samples. I think this is very important for this group. Maybe we could have a separate meeting about how to request samples and the quality control that we need to have working with the collaborators and the people providing the samples.



Wendy Fantl 06:53

With flow cytometry, which is reused routinely, the technology is based on fluorescence and looking at fluorescence emission spectra. What you can see here from this diagram is that there's tremendous overlap. That makes it very difficult to measure if you're a routine practitioner. There are six to eight experts in the field, but they're few and far between and they're swamped.

Rick Stanton 07:28

Wendy, I don't think everyone knows how to associate these wavelengths with any problem, or what are these wavelengths?

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Rick Stanton 07:43

I mean, I know flow cytometry, but I don't think other people here do.

Wendy Fantl 07:48

You basically take antibodies that recognize specific proteins on cells. The antibodies are labeled with fluorescence, fluorochrome. The fluorochromes, when they get excited, emit light in these wavelengths. That's the signal you will measure. What you can see from this graph is tremendous overlap. It's difficult to pull out specific signals because of this overlap. You must apply all these algorithms to do that. Does that clear it up?

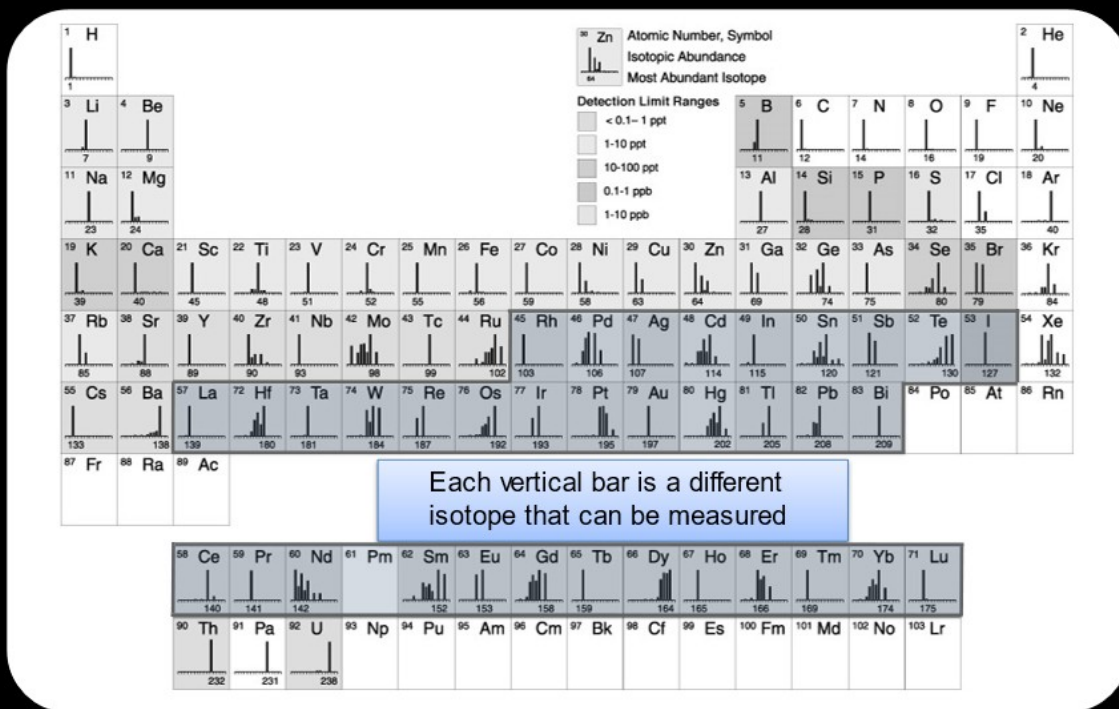
Rick Stanton 08:30

Fluorophores with these emission spectra, like the red and the green on the right, show there's overlap. If something was emitting, it would be between 600 and 100. You don't really know whether it was a red or a green, and that red and the green are bound selectively to different antibodies. Without some kind of deconvolution algorithm which is trying to optimize red from green, you don't know. That's the meaning of this overlap - there's an ambiguity of whether I am looking at a fluorophore that's tagged to this antibody, or that antibody. That is going to make a difference in the cell type in your conclusions because you're querying with these antibodies tagged to these fluorophores. If you don't know where they're coming from, you lose your ability to interpret.

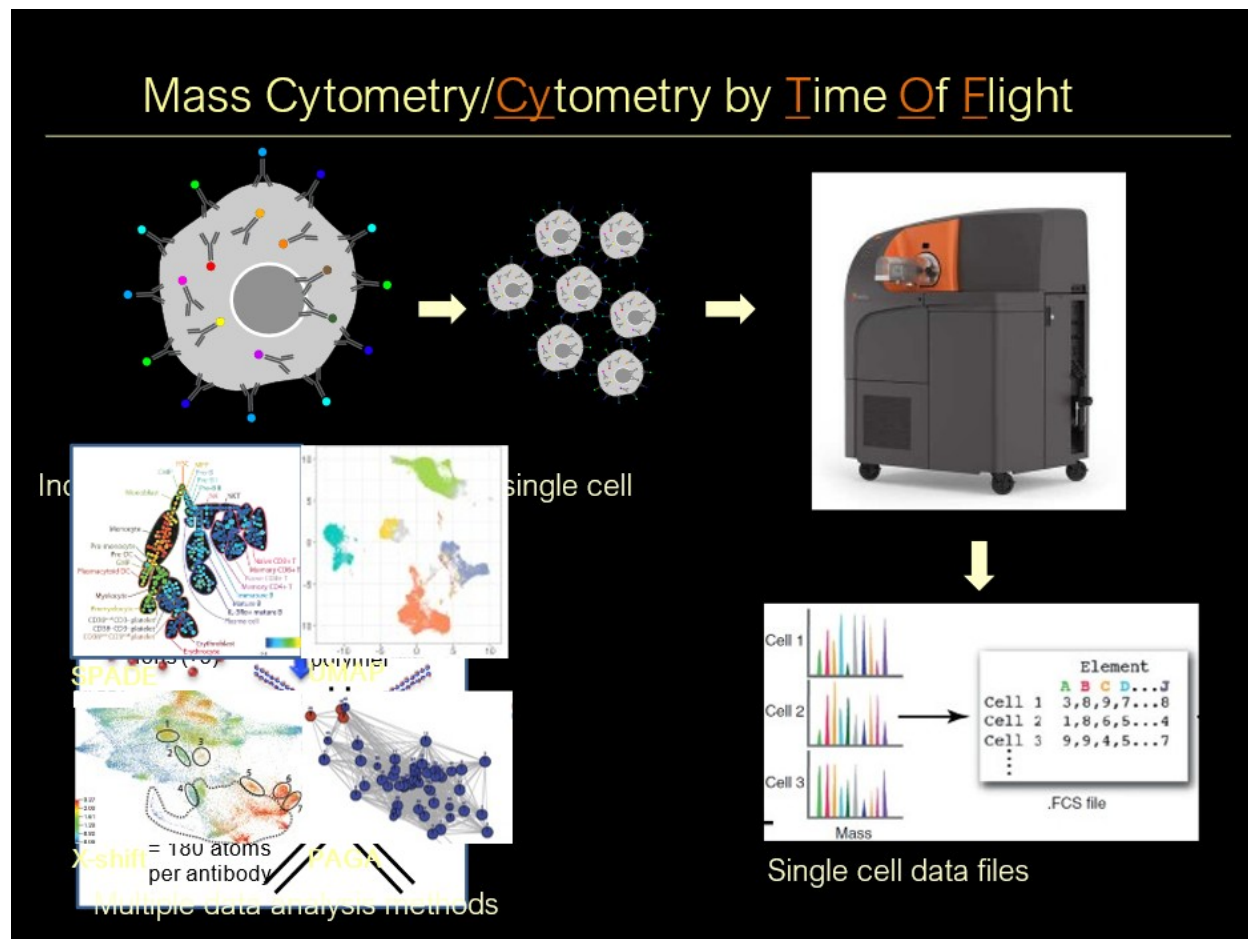
Wendy Fantl 09:35

Absolutely. But I would say the algorithms are actually very good at untangling that. In any case and to address the problem, enter Scott Turner. Scott Turner was a professor at the University of Toronto who is no longer there, but he reasoned that he would label antibodies with rare metals. Those metals are rare or absent in biology and they're at the bottom of the periodic table.

## How Do You Get 100 Channels From 35 Elements?

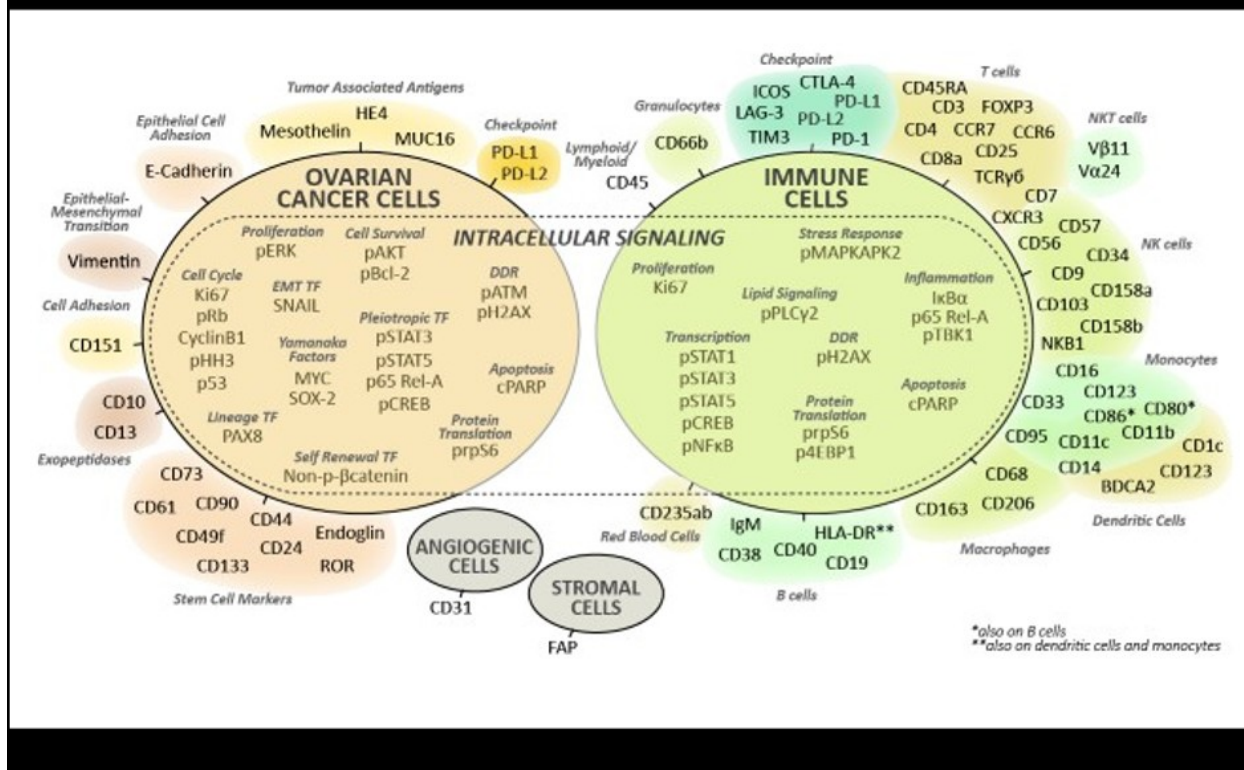


Here they are - the lanthanides. They're rare or absent in biology and what's cool is they are composed on the planet when they mine stable metal isotopes. There are ways to purify these stable metal isotopes. Now you can label antibodies with these metals, and you can get rid of the overlap that you've just so beautifully explained. The number of parameters per single cell is just dependent on what Earth provides us, but maybe we'll go to Mars and get more, but I don't know. For example, you see that samarium, or Sm, has 6, a reasonable abundance. We label our antibodies with an isotopically-enriched lanthanide.



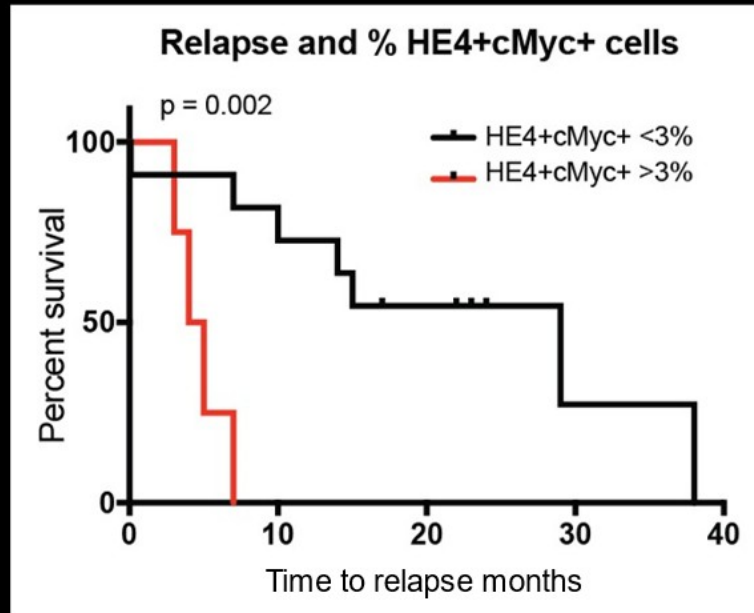
Right now, we're up to about 60 parameters per single cell. We label ourselves with antibodies against proteins that delineate surface markers. If it was a T cell, the tumor cell, the cell type, and antibodies, we permeabilized the cells, and we could go inside the cell and look at what's inside the cell. It's incredibly powerful. We've got the samples labeled, and this is a picture of their CyTOF instrument. So now you're introducing into the CyTOF hundreds of 1000s, if not millions of cells. For each cell, you get a readout of the amount of protein. This has really spawned the computational folks to develop all sorts of algorithms that take a lot of the intuition from RNA expression. These are just some of them. You don't need to know the specifics. It's just the power of this that is just fantastic.

## Tumor and Immune CyTOF Antibody Panels



Because this is publicly available, these are the panels that we made in my lab, and there are plenty, plenty more that we made ourselves – the conjugations. They're commercially available, and there are antibodies against immune cells. It'd be like the checkpoint proteins that you're familiar with: T cells, NK cells, natural killer cells, macrophages, etc. against tumor cells. If you need anything else in the panel, and it's not available commercially, but there's a good antibody, we can conjugate it and include it in the CODEX panel. With prostate cancer, if there aren't any CyTOF antibodies metal conjugated, then they can certainly be made. Here are a few examples of some results from our publications.

## Minority Cell Type That Correlates with Poor Prognosis



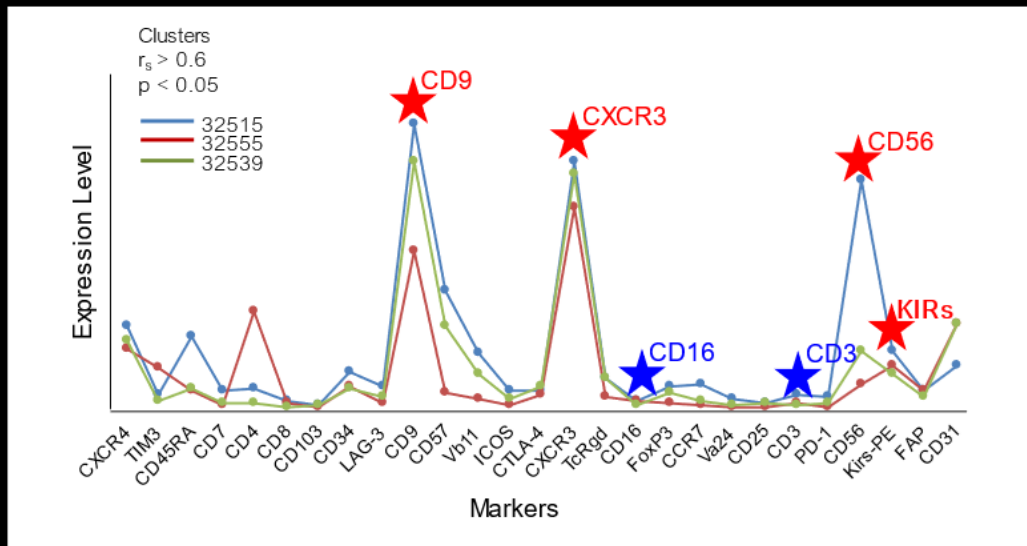
Metastatic features

Gonzalez et al. Cell Report 2018

This is data from untreated ovarian tumors. We identified a minority cell type with metastatic features. The more you have, the worse time to relapse. It's much, much shorter. We would have never found this any other way.

## Intra-tumoral NK Cells With Decidual-Like Phenotype

### Correlated with ovarian tumor mass



There can be no stronger connection in biology than a mother tolerating her fetus

And then another example, we were analyzing the intra-tumoral immune cells and found this cell type. This cell type turns out to be decidual-like. (Decidual cells are a distinctive cell population observed in the mammalian endometrium – the mucous membrane lining the uterus – during pregnancy.) Why is that? Because this CD9 protein is a hallmark of decidual NK cells. These NK cells, natural killer cells, are found in about 70% of lymphocytes in the first trimester of pregnancy. And what do they do? They prevent the mother rejecting her fetus. We found that in ovarian cancer, even though they have exhausted T-cells, ovarian cancer patients have had very poor response to checkpoint immune blockade. Our working hypothesis is that these decidual-like NK cells could be overriding any effect from the immune checkpoint blockade. There can be no stronger connection in biology than a mother tolerating her fetus, and the cancer has taken this over. These cells are found in other humans. It's not just ovarian cancer. Adriana Albini, who I had the great pleasure of meeting, has done a lot more and looked at a lot more cancers than I have. One of the cancers is prostate cancer. That brings me on to this paper here.

## Decidual-like NK Cells: Relevance to PC

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- Prostate Cancer Peripheral Blood NK Cells Show Enhanced CD9, CD49a, CXCR4, CXCL8, MMP-9 Production and Secrete Monocyte-Recruiting and Polarizing Factors. Matteo et al. *Frontiers in Immunology*, 2021

If you don't have access to the library, and you want papers and things, I'm more than happy to send them to you. But this is a very interesting paper. (See [here](#).)

Brad Power 15:22

The word "decidual" is a new one for me. Can you define it, please?

15:26

Decidual relates to how the uterus gets a decidual lining before fetal implantation. It makes it kind of cozy for the fetus to develop. The key here is that the immune cells set up this maternal environment so that the mother doesn't reject the fetus. One of the most prevalent cell types is decidual NK cells. We found them through a CyTOF analysis. This paper was very, very interesting because her group found that there were these natural killer cells that had decidual-like features. Decidual-like features are CD9, CD49A, CXCR4. It doesn't matter what they are, but they are hallmarks. They endow the NK cells. These natural killer cells do what its name says, it immediately sees something like a virus, or a bacterially infected cell, or a tumor cell in early stages, and it kills it. That's what it does. But we showed, and she showed, that once they get certainly CD9, those are natural killer cells, and they don't kill anymore. They have looked at the blood collected from men with metastatic prostate cancer and found these cells

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intratumorally. One of the ideas is that in blood, we know how to measure all this with CyTOF. You could monitor populations of these natural killer cells. A lot more work needs to be done, but it could be used to monitor progression. I think another application of CyTOF for patients with cancer generally, is ex vivo looking at drug responses with blood. I've done this a lot. There's a lot of publications on it. It's really kind of a subject for another seminar, but I just wanted to mention that. I'll give you one example. We published a paper from my lab looking at leukemia patients, taking blood, and treating them ex vivo with daunorubicin. We showed that in looking into cellular features, whether a patient will respond or not respond. That's a very powerful application. It's a blood sample. The key here is getting samples that are easily attainable. I don't think anybody in this audience is going to object to a blood sample.

Brad Power 18:34

Wendy, can I be a "Wendy for Dummies", and I'll be the dummy? Let me play it back to you. What I hear is, you have some magic technology that allows you to get insights down to a cellular level, or molecular level, or tumor microenvironment. From that you can then find signatures that are characteristic of ovarian cancer or prostate cancer, or some other cancer, and then you can see that this is distinctive, it's got a signature, it's unique. And then you're going to use it in two ways. One is you're going to say, "Why is that? Can I model it? What does that have to do with the disease and how it progresses?" The other is that I can monitor if this is a unique signature. For example, I was talking to a friend of mine, Ricardo Salgado, who has lung cancer. He wants to use Bob Gatenby-like evolutionary treatments, adaptive therapy, but he doesn't have a biomarker. The beauty of prostate cancers is we have PSA. You can try a drug and see how the patient responds because their PSA goes down or it doesn't. So you're uncovering potential biomarkers. It would allow you to do it after you get a blood draw and determine if my disease is progressing or regressing. Is my understanding correct?

Wendy Fantl 20:09

Absolutely right on, or as we'd say in the UK "on the nail". Very good summary.

Brian McCloskey 20:21

Just a follow up. With respect to the blood assay to test drug response ex vivo. You mentioned leukemia. What about solid tumors such as the prostate? Ovarian?

Wendy Fantl 20:32

Yes, great question. There are a couple of things you could do. I assume with prostate, getting tumor tissue is difficult. With ovarian cancer, sometimes you're removing masses, so you'd get enough tumor tissue. You could get a lot of information from blood cells, peripheral blood. Think of it this way. The immune system is a system. There is communication between immune cells that are in tissues and in the periphery. They're not independently operating. Our working hypothesis is that as a system, you could pick something up in the periphery that would be a proxy, or a surrogate for response to what's going on in the tumor. Yes, you could get tumor tissue and dissociate it. A very good friend and collaborator in New York, [Derek Tan](#), has developed a medium where you can culture the tumor cells, dissociate tumors, ovarian tumors, and culture them and do drug assays that way, which is very exciting. You probably get a lot

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more tumor tissue than you do for prostate cancer. But it's a very exciting area because blood is easy to get. So, a lot of testing to do, but a very exciting area.

Anonymous Caregiver 22:12

I have a question about the tissue. What are your parameters for what type of treatments a tissue is allowed to have undergone? Are you able to still receive signals? For instance, radiation of certain types? Do you need fresh untreated tissue? Even with metastatic sites.

Wendy Fantl 22:36

We all say fresh, untreated would be best. And that certainly is very good. The best scenario, just logically is, if you have longitudinal samples, before treatment, after treatment that is of course the gold standard, but that's just fraught with practical problems. I'm of the opinion that any sample, so long as it's high quality, would be acceptable.

Anonymous Caregiver 23:29

Can you define high quality just a little bit?

Wendy Fantl 23:31

When we look at our samples, the cells have to be intact. Right at the get go, we do something called a trypan blue stain. If your cell took a bashing and it has holes in it because it's necrotic or it hasn't been handled correctly, the blue dye will enter the cells and you can look at it under the microscope. Then you can see white because they didn't get the dye, and blue because they did get the dye. So trypan blue is important. With fresh blood, it's so fresh you can add the buffer immediately. So you don't really need to do that. But what we typically do in all our assays, is add an antibody that actually lets you know whether the cell is about to die. You wouldn't have picked that up before. But that's also so we have cells that are dead. We can measure that all by mass cytometry. The first assay with the blue dye is very, very easy. You take some of your sample, you add a drop of the dye. I've got it all written up in published protocols, because I know that even if it sounds simple, the devil is in the details. I intend our work to be for everybody. I want them to benefit from everything. It's not just me sitting here, working on how many papers we can get and forget it. No, the work we do is for patients. I feel very, very strongly about that. I'm always willing to help and train and all the rest of it. Does that answer your question?

Anonymous Caregiver 24:11

That was helpful. I think really the primary gate, maybe from a patient point of view, is when there are parameters that they already know that they would fail, where it's not worth going through the risk or the effort to get a biopsy versus when is it worth the risk, and then your stain will tell whether you can use the sample, and it's high quality enough?

Dr. John Laird, MD 25:55

When you're getting a spectrum from the cells, spectral readout, these are proteins?

Wendy Fantl 26:12

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Yes. Proteins.

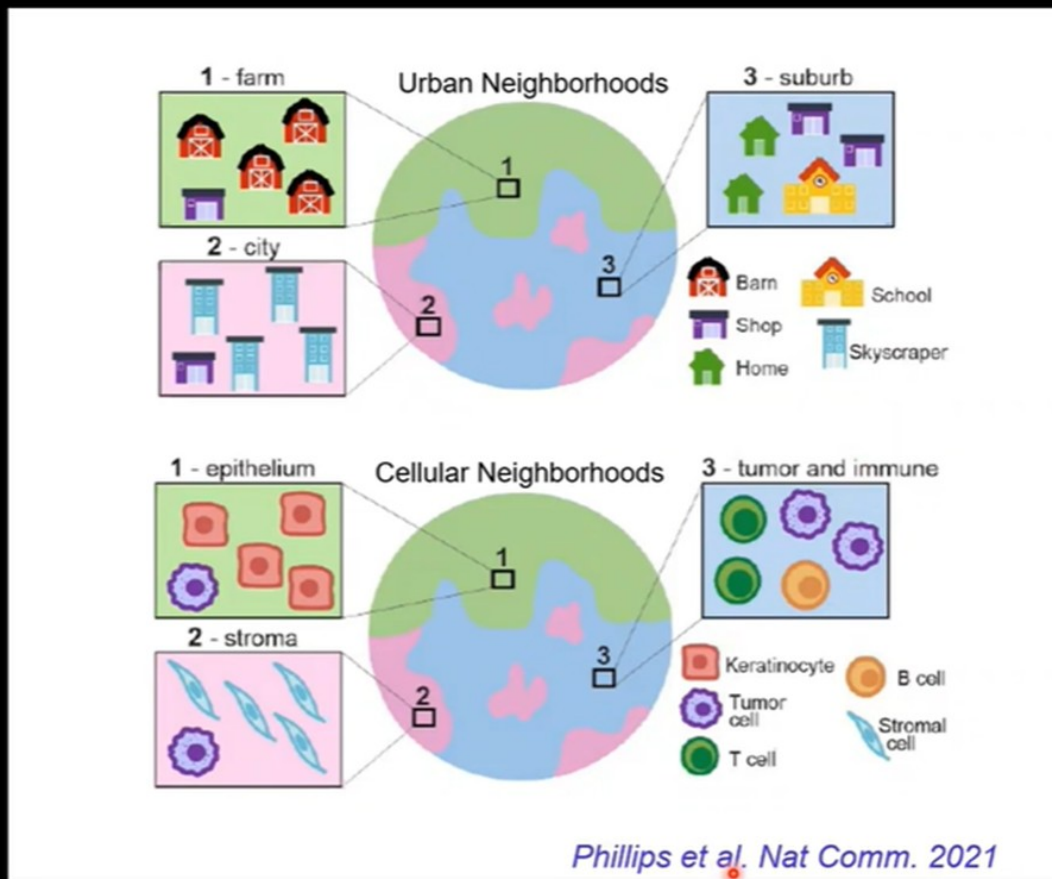
Dr. John Laird, MD 26:14

Presumably, there are programs that indicate what pathways the tumor is using to resist. I just have questions about how clinically useful this is because, as you refer to getting tumor samples, it's challenging depending on where the tumors are. Whether they can be needle biopsied or excised or whatever. I have two questions. One is, how are people at Stanford, clinicians, using your testing technology, and how useful is it clinically? I guess I'd be interested in that. I just have concerns about that on a retail level working with patients.

Wendy Fantl 27:16

The CyTOF technology is really for blood. I'm now moving on to multiplex imaging. Based upon the points you raise about the practicality of getting enough tumor samples for CyTOF, it's very informative, no question, but probably the better way to go for a tumor sample is tissue slices. This is the next part of my talk. I'm going to tell you about CODEX multiplex imaging.

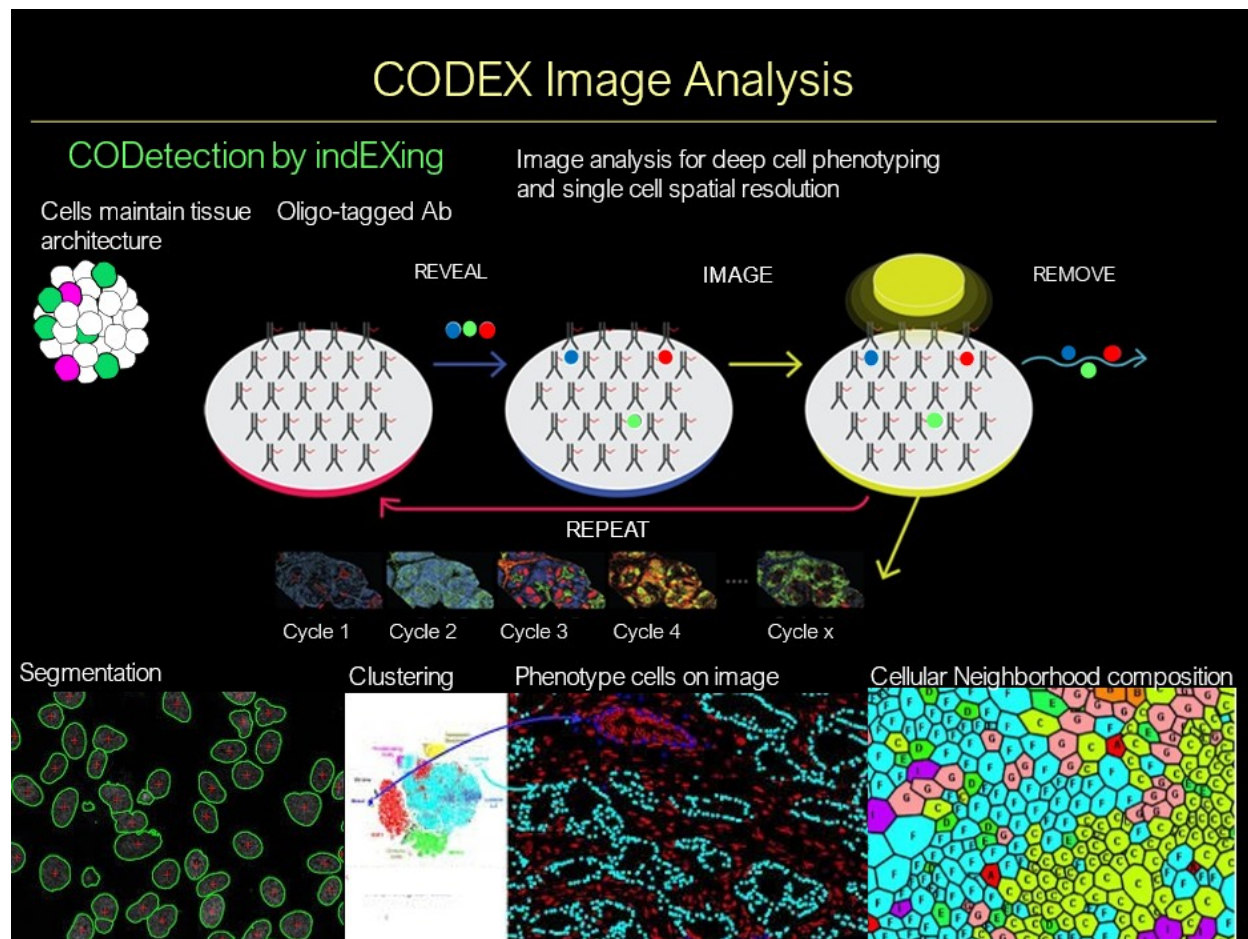
## CODEX Multiplex Imaging: Cellular Neighborhoods



Remember, I told you that CyTOF looks at cells in suspension, so you lose information about which cell is next to which cell. That's important, and that brings up the concept of cellular neighborhoods. I hope this analogy helps you. If we think about urban neighborhoods, you've got a farm, a city, and a suburb, and within each little neighborhood people are going to be talking. The people on the farm who might be growing everything, they're going to have to go to the store and send their kids to a school in the city, or at a school in the suburbs. The neighborhoods interact among themselves, but also with other neighborhoods. This is the analogy that I use to tell people about cells in neighborhoods. Epithelial cells are typically tumor cells. You've got this purple epithelial cell which was skin cancer. This is from Darci Phillip's paper. She's a colleague. You can see there's one tumor cell in there, and then there's a tumor cell in this neighborhood, a stromal neighborhood. I think it's very important for today with the advancement of immunotherapy that you need to know what tumor and immune cells look like. I'm going to stop at this moment to ask if you have questions about that. Within a tumor using these slices, we have areas that are rich in immune cells and tumor cells, or stone cold. They call them hot and cold tumors. A hot tumor has got a lot of immune infiltration and a cold tumor

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does not. But within the same tumor, you get these different neighborhoods. I'm going to just show you how the technology works.



Here's a tumor and it's going to be sliced. We maintain the tissue architecture. Basically, we take antibodies and measure proteins while labeling them with an oligo tag. We can measure 60 proteins in a tissue. Here's the tissue on the slide. We take all the antibodies with different tags on them with different addresses. With an oligonucleotide, you can have a complementary sequence that's the basis of DNA and oligonucleotides. You take three oligonucleotides at a time which have all been labeled with a different fluorochrome. Those oligos will recognize three antibodies based on a complementary sequence of their oligo. Then we image, and we wash off those fluorochromes. We come in with another round, and then you hybridize, reveal, image and remove. We call that a cycle. With more cycles, you get a much more granular picture of the tumor.

Rick Stanton 27:38

Quick question. Could you explain the oligonucleotide aspect? I'm not sure about the binding. Is this an antibody?

Wendy Fantl 32:11

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There's some chemistry that gets the oligo onto the antibody. Think of the oligo as a label. It's a specific sequence. The oligo is being used to identify the antibody and then with DNA.

Rick Stanton 32:36

I'm lost. Is this antibody binding to a protein, is that right?

Wendy Fantl 32:38

The antibody binds to a protein and then it's oligo, which is its address. It's an identity. Oligo is an identifier for the antibody.

Rick Stanton 32:50

The address is not fluorescent?

Wendy Fantl 32:54

It's not fluorescent, until you come in with something that recognizes the address.

Rick Stanton 33:00

Oh, okay. So the address is a nucleotide oligonucleotide sequence, which oligo means "short."

Wendy Fantl 33:11

Yeah, absolutely.

Rick Stanton 33:13

So your antibody is tagged with a short DNA sequence that is later resolved?

Wendy Fantl 33:23

That's correct. Yes.

Rick Stanton 33:26

I did not know that. That's so interesting. Thank you.

Wendy Fantl 33:30

You don't need to go into all the details, but it's computationally then we must know what a cell is, cell segmentation, and clustering. Basically, then we can get to the cellular neighborhoods. I'm not going to go into that, because it's quite complicated. It's even above my pay level because the computation is complicated. But there are now established pipelines. It's getting a lot easier to use.

Anonymous Caregiver 34:13

Wendy, you had me at the very beginning when you started talking about fluorescence in nuclear imaging and the use of radiotracers. Can you talk about how this technology could or could not be used with whole body scans?

Wendy Fantl 34:25

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It could. Absolutely. And people are trying to do that. They're trying to look at body scans and looking at areas that light up on the imaging. An area under research is taking a sample of the tumors that light up on the body scan. That's the idea to integrate the technologies. That's definitely cutting-edge stuff that's underway. I'm not sure I'm not up on it. I know there are a lot of people doing it.

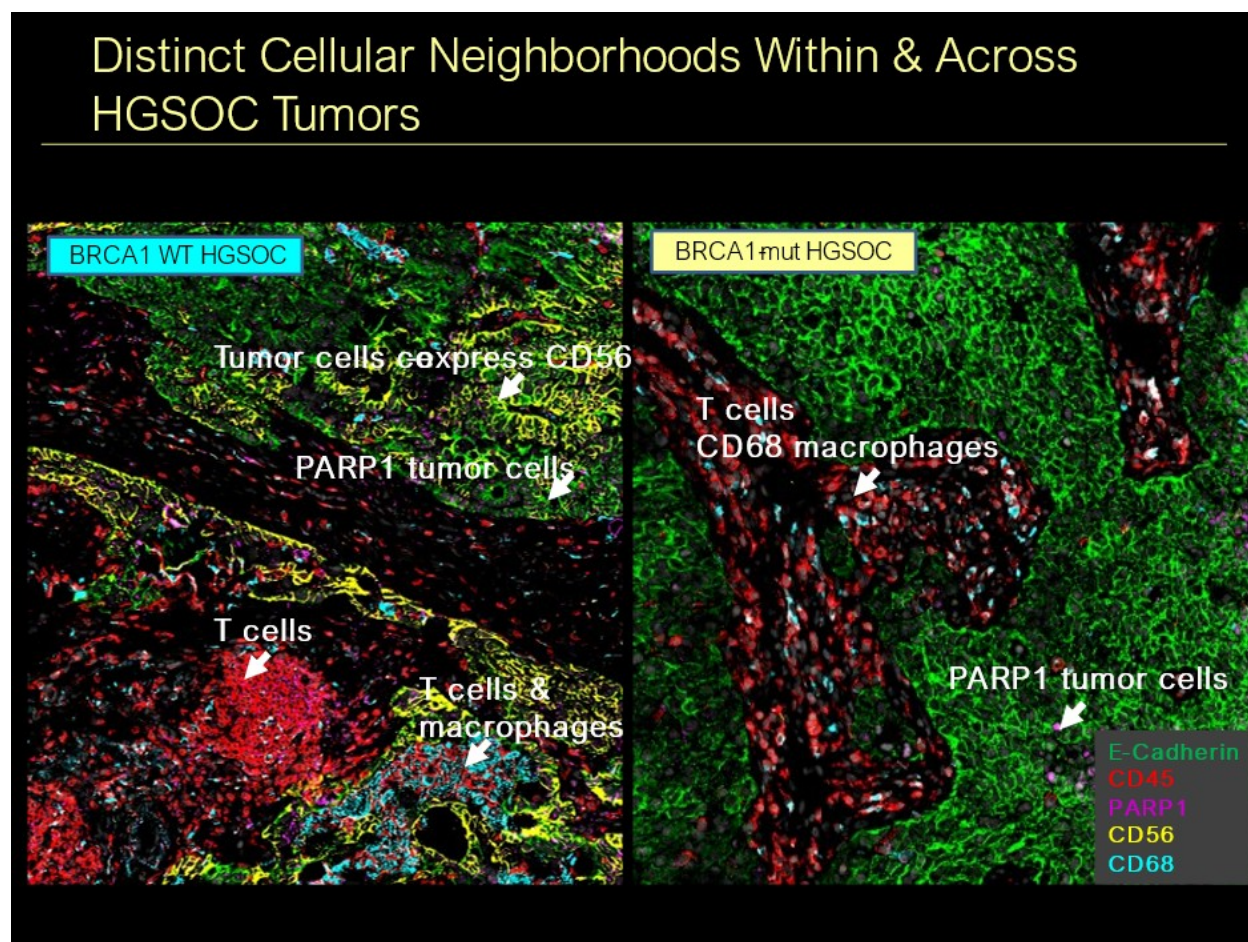
Brian McCloskey 35:00

I'm about ready to go through a battery of imaging. What kind of scans would lend themselves to that type of application? A PET scan?

Wendy Fantl 35:16

I need to check with my clinical colleagues because I don't know. I don't want to say something wrong. I've got a colleague at the University of Cambridge, and I will absolutely ask him. I don't want to say anything wrong as I'm not an MD.

Wendy Fantl 35:38



This is an ovarian tumor cell. You can see neighborhoods, there's no question. There's an immune cell neighborhood. Here's a green neighborhood. It's a tumor cell neighborhood. I really

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just bring that to you to show you that concept. And then yes, and I've labeled them. But you don't have to know the details.

Brad Power 36:10

You should compete with the James Webb photographs. These are beautiful. We should all have those hung on our walls.

Wendy Fantl 36:24

Here's some of the literature that's starting to come out.

### Examples of CNs and their Predictive Value

- Immune cell topography predicts response to PD -1 blockade in cutaneous T cell lymphoma – Phillips et al. Nat Comm. 2021
- Immunogenomic profiling determines responses to combined PARP and PD -1 inhibition in ovarian cancer Färkkilä et al. Nat Comm. 2020
- Analysis of multispectral imaging with the AstroPath platform informs efficacy of PD -1 blockade. Berry S, et al. Science 2021
- Biology confirmed but biomarkers elusive in melanoma immunotherapy. Nat Rev Clin Oncol. 2020. Luke JJ, Ascierto PA.
- Comparison of Biomarker Modalities for Predicting Response to PD -1/PD-L1 Checkpoint Blockade: A Systematic Review and Meta -analysis. JAMA Oncol. 2019. Lu S, Stein JE, Rimm DL, et al.

I want to give another analogy about why I think tissue architecture is so important. If you just took just the healthy tissue, like your liver, your lungs, whatever, and they were just a mass of cells, it's not going to really be doing much. But they are set up to function. Their organization is critical for their function. The same applies for tumors. Their organization is critical for their function. I'm stating that as a hypothesis, but it necessarily has to be so. Here are some papers that show immune cell topography, and predict response to PD1 blockade. Very nice paper.

## Tissue to Use for Ex Vivo Drug Screening

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- Blood from leukemia
- Tissue from solid malignancies
- Blood from patients with solid tumors can be used as a proxy for tumor response

Brad Power 37:24

I have a question about integration. You've got your CyTOF and you've got your CODEX multiplex imaging. CyTOF works on blood. CODEX multiplex imaging works on tissue. They're showing different things. You said one has a unique signature, and the other is giving you some of the neighborhoods, spatial analysis, and the architecture? How do you integrate those together? This could compound on itself. We could also have other tests of various sorts like DNA sequencing, RNA sequencing, etc. If you've got two different tests that are giving you different insights, how do you then integrate them to come up with a more comprehensive view of the patient in the situation?

Wendy Fantl 38:36

Great question. So firstly, with CODEX, you maintain the deep detail that you get about cells from CyTOF. But you add on the tissue architecture, the spatial features, so an additional step. I see the integration if you have tumor tissue and peripheral blood from the same patient. Remember, the immune system is a system. There are interactions between what's going on in the periphery as a proxy for what is going on in the tumor. With CyTOF on the blood, and CODEX on the tumor, you integrate the datasets from the two technologies. The throughput of CyTOF is much greater than CODEX, so you can do millions of cells. Then you can get an idea as a sort of screening, the landscaping, the biology, and so that could help you refine the panel as you're limited. I think you're limited to about 55 antibodies for CODEX and the multiplex

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imaging. It can really help inform the most meaningful panels when this is all proteomic. I've been a protein person since way back when. DNA and RNA are important, but proteins are doing the dirty work. I feel like those are the things you want to really understand and target. RNA expression and DNA can be very, very informative about what transcripts are there that maybe you hadn't noticed proteomically. At the end of the day, it boils down to proteins. People talk about RNA signatures, and they're certainly used in the clinic. For example, RNA signatures can inform a breast cancer patient if she should get chemotherapy. With this deep proteomic phenotyping, we could do even better.

Brian McCloskey 40:59

Let's take that one step forward. I'm being treated at UC San Diego. I had the opportunity to see Akoya as they came down and spoke a couple of months ago. I know that they are setting up their machines at UC San Diego. I want to have more than a philosophical conversation with my doctor about using this technology. I'd like to use it. How do we go about doing that? We've been on this path. We've had DNA and RNA seq. Those are all great proxies for proteins. I don't want to monkey around anymore. I want to get to a good definition of what's happening. What do you recommend is the best way to integrate this capability in my treatment decision making process?

Wendy Fantl 42:09

Does your doctor get what the technologies are doing? Does she understand it? The first place to go would be to tell her about these technologies. This is where things are going. I could even prepare something for you to show if you want that's a little more private. Yes, I will prepare something for you. You've got to use analogies. And I also think with doctors, it's always about time, and how many patients and all the rest of it. Can you imagine them having time to read these papers? This is not bedtime reading. You must immerse yourself. You need somebody that's really, really committed to understanding how new technologies can work. You need really good physician scientists. They need to really get it. Sadly, a lot of physicians don't have time. I am a former patient. I had ovarian cancer. I fired so many doctors you don't even want to know. I told them; “I am the patient from hell. I am going to ask you questions, I am the boss of me. I am a scientist and a cancer biologist, and I am not a dummy.” I did find somebody who was amazing. She's one of my best friends, and we are collaborating. You need somebody who's really going to engage and get what it is, and a lot of them they just don't want to. I'm sorry, this is really bad.

Anonymous Caregiver 44:06

I'll say it for you. They don't want to deal.

Wendy Fantl 44:13

I will tell you one example which I could not believe. I'm a huge advocate for patients. I was at Harvard, and I went to see this very renowned ovarian specialist. I told her about CyTOF and showed her our data, and that you've got this minority cell type that correlates with poor prognosis. It's a metastatic phenotype in the untreated tumor. Could we do a clinical trial? Could we do something? She just sat there and said, "Oh, Wendy, I don't really know what to do

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because ovarian cancer is such a bad disease." I was ready to fling a coffee cup at her. It's about trying to get the doctor to engage and really get it. Stamp your foot on the floor. You are the boss of you. It's your body. It's your life. It's what you want. I know that sounds very philosophical. But there's no harm in it. Number one, you are giving permission for your tissue to be used. That's yours. There's nothing invasive about doing it. I think what they're all terrified of is that you're going to make a decision. They're all terrified that you're going to use the information for informing a clinical decision, and then they'll get sued because it doesn't work. That's very cynical of me, but it's true. I want to say lawsuits don't kill, but cancer does. It starts with getting the physician on board with understanding the power of this. It's not invasive, it's not hurting anybody. It's not just helping you. It's helping everybody - all patients.

Brian McCloskey 46:35

Agree. So given that you're so close to this technology, you have a pretty good idea of what the entire pipeline looks like. What is the path of least resistance? Where do I go to see a clinician who understands this technology and has used it or wants to use it?

Wendy Fantl 47:06

I have a very dear friend at UC San Diego, Dr. Thomas Kipps. I don't know if you've run across him.

Brian McCloskey 47:14

I don't know him.

Wendy Fantl 47:17

We've collaborated. He is a gorgeous person; I just adore him. I could introduce you by email, and you could just go and talk to him. I can write a draft email, and then you can edit it and see how it is. And then let's get him into the picture. He's well known for his work in chronic leukemia, and ovarian cancer. I've known him for years, and he's just a brilliant physician scientist. There could be nobody better, so I will arrange that.

Brad Power 48:05

Fantastic. I'm curious about an argument we hear. Brian's oncologist, and oncologists in general, will say, "Why run a test unless there's a targeted therapy at the end of that rainbow?" But Brian was successful in getting RNA seq data identifying that he had a B7-H3 overexpression, and there was a drug that was targeted for this. It's at the top of his list right now. And his doctor said, "Thank you for doing that RNA seq analysis", which would not have been done without Rick's efforts. It gave insights that have resulted in a treatment option. At the end of the day, the result that they're looking for is how would it guide a treatment decision? Is there an example in your experience in ovarian cancer, where there's a line of sight where we did the CyTOF analysis, and we did the CODEX analysis, and identified something for this patient that then led to a treatment decision we would not have otherwise discovered?

Wendy Fantl 49:17

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Well, I can tell you an example not at the individual patient level. I can tell you that our NK cell work led to cancer research.

Wendy Fantl 50:04

One of my ideas was that in prostate cancer, you could see peripheral decidual-like NK cells in metastatic prostate cancer. Could this be used as a screen for early detection of ovarian cancer? I won't go into all the details, but it's based on that CyTOF finding. Cancer Research UK loved it, and I got funded for a project. So that is one specific example. You can use that. I will write something up for you. But it sounds like Brian, your physician drank the Kool Aid and was very receptive to you doing that?

Brian McCloskey 51:10

Well, it wasn't immediate. I saw her in September of last year, and I shared some of this RNA seq data. She said, "What am I going to do with it?" Three months later, out of the blue, she said, "Hey, I want to thank you for bringing that information to me. It is actionable." I did mention to her in my last meeting just three weeks ago, that I was aware that Akoya had their machines instantiated at UC San Diego. But we didn't get into a very in-depth conversation about testing. Instead, I threw 18 different treatment options at her based upon some great work that this team had done. So we focused more of our attention on treatment options. But I'm going to go back to her on August 1, and I'm going to talk to her about testing. Ideally, before I make a decision on any one of these options, or potentially other treatments, I would really like to have a more precise view of what's happening with my cancer, and not just from two years ago when I had my mets biopsied. I have three tumors right now and caking in the peritoneum, so it's soft tissue, and we can get to it. I want to have a conversation with her about how to use that precious resource. And proteomics to me, is the next stop on this diagnostic journey.

## Suggested Discussion Points With Physician

- New cutting-edge technologies available that are proving to provide an unappreciated level of understanding/insight into cancer.
- The motivation for the development of these technologies was to understand the heterogeneity of tumors
- They look at single intact cells that make up a tumor
- They can measure DNA, RNA or protein.
- Proteins carry out a cell's function and are often targeted by therapeutic agents
- While targeted therapeutics are designed against one protein, it needs to be appreciated that these proteins do not work alone
- They are part of a system, and these new technologies can help understand their participation in the “system”
- These technologies can also identify new protein targets in a particular malignancy and maybe a drug for this protein already exists!!!!
- Some of the new technologies measure the proteins within single cells but with imaging show how they are arranged – perhaps to illustrate this point you can show them slide 15.

Wendy Fantl 52:48

It's about convincing them that it's a good thing to do and there's nothing better than information. I do think some of the papers, for example, show that they are predictive of response to immunotherapy. I think the Journal of Clinical Oncology where they came up with the conclusion that spatial biomarkers were better, or particular cell types were actually better than conventional biomarkers. They need to understand that all these biomarkers aren't working by themselves. It's all about a system, and we need to understand that. Tom Kipps would definitely get that because we publish together, and he gets CyTOF and all that stuff. I think it just boils down to giving a salient, clearly described argument to put in front of their noses that doesn't require them to read tons and tons of papers. Give them a distillation of the key points and why it's important. To me, it's a no brainer but I am very biased.

Brad Power 54:07

We have found that we can talk to you, and if we had your lab's resources to devote to Brian and Rick and Ken and Mike, it would be great. But you're doing your own research, and you've got other masters to serve in a different environment. Someone like Tom Kipps is trying to put it into clinical use, but have you seen any proteomics start-ups that are doing this kind of analysis that's CLIA certified? The kinds that would be pointing to a clinical direction. Coincidentally, this

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past week, we spoke to mProbe. I don't know if you've heard of them. They were willing to run a proteomic analysis for Brian and Rick.

Wendy Fantl 54:55

That's a very hard question. I don't know a lot of that because a lot of it's proprietary information.