Community Perspective

Integrated Biospecimens and Multi-omic Technologies:

A Process to Accelerate Biomarker Discovery and Companion Diagnostic Approval

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Introduction

The realization of personalized medicine relies on the ability to select the patients most likely to respond to treatment. Selecting patients can be efficiently accomplished using biomarkers, typically discovered and developed as the drugs go through clinical development. Clinical trials in which biomarker targeted therapies were utilized were found to have the highest rate of success.[1] When an estimated 90% of clinical trials fail,[2] it's crucial to understand how to optimize drug programs including target validation and identifying the patient population most likely to respond.

Some research facilities that carry out biomarker discovery programs lack the ability to validate findings and achieve the statistical power needed to demonstrate the association between biomarker expression and treatment response.[3,4] This lack of a coordinated systems-based approach has been identified as one of the major pitfalls preventing the approval of many companion diagnostics.[4] Furthermore, biospecimen bias also contributes to the difficulty faced in translating biomarkers from preclinical research to the clinical setting. This bias can be especially prevalent when biospecimens from multiple sources are used to increase the sample size of a study; the bias may be even more severe if these samples are processed

and stored according to different methods. Such disparities in multiple sample sources, processing, and storage can dramatically affect the levels of biomarkers detected.[5]

A standardized, multi-systems approach is needed to enhance drug and biomarker discovery and increase the efficacy of moving companion biomarkers from the bench into the clinic. About twenty-five years ago, this approach would have been carried out starting with an extensive literature search followed by massive cell lines screenings to identify potential drug target or biomarker candidates. These approaches were arduous, ineffective, and prone to bias. With the advent of high-throughput, cutting-edge screening technology and informatics-supported searches, we can tackle the process in a more comprehensive way that will increase the pool of targets to be investigated downstream.[6]

Current approaches to simultaneous drug and biomarker discovery programs

Three processes are necessary to identify and establish a biomarker as a potential companion diagnostic: first, streamlining biospecimen procurement; second, utilizing a multiomic approach that facilitates target recognition and profiling (e.g., next-generation sequencing); and, third, confirming the marker using traditional methods such immunohistochemistry (IHC) and flow cytometry.

Applying Multi-omics to biomarker discovery

Combining our expertise in biospecimen procurement and a variety of technologies, we have developed a process for the discovery of targets expressed on the surface of tumor cells (**Figure 1**). We evaluate those targets as potential leads to new drug targets or to new biomarkers for already existing drugs. We begin the discovery process with human tumors collected in our procurement network. RNA extracted from the tumor samples are then subjected to bulk sequencing to profile the expression of all genes using an unbiased method. This sample is further characterized by dissociation of single cells from the tumors into single cells for evaluation via Citeseq* analysis, allowing immunophenotyping of the cells and unbiased transcriptome analysis.[7]

In theory, the target of interest could be any type of receptor that is expressed in the cell membrane of tumor cells. Of special interest are the ones that regulate the interactions of immune cells and tumors. Then, a more in-depth evaluation is carried out in each of the tumors to home in on interesting receptors and ligands. Single cell sequencing is performed in those tissues, and the transcripts used to distinguish the different cell types. We have concentrated on B cells, NK cells, CD4+ or CD8+ T cells, fibroblasts, normal epithelial and malignant epithelial cells. Once targets of interest are identified, we then further characterize and analyze those markers via flow cytometry. A 14-color flow cytometry assay has been developed to highlight a targeted receptor ligand population to understand cell surface expression on specific cell populations. The flow cytometry data can then be correlated with IHC data to allow for visual and spatial representation of protein expression.

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Moving from receptor-based cell selection to RNA spatial profiling

At this point in the process, the expression of specific receptors and their ligands is re-evaluated. From here, we then look at the spatial distribution of the RNA expression in the tumors of interest. This spatial profiling can be done with a couple of technologies, like Visium from 10X Genomics, or Digital Spatial Profiling from Nanostring. It is important to be able to come back and place the specific cells in their tumor microenvironment, to make sure the signals we are seeing correspond to intrinsic parts of the tissue. For example, the signals could be coming from the immune cells outside the tumor, or fibroblasts outside the tumor, or immune cells in contact with tumor cells. This last category is of more interest when one is looking for targets or predictive biomarkers. This visualization and selection process can then help facilitate choosing specific targets or biomarkers that are present in tumors of patients for drug targeting or for biomarker clinical trial assay development.

Correlating targets, tumors, and patients

If this approach is done with multiple patients with different tumor types, one can find the tumors that express the targets of interest and thus concentrate on those tumor types for the future clinical trials that target that specific biological axis. Furthermore, tumors arising from the same tissue can have different genetic profiles. In breast cancer, for instance, tumors may be HR+ or ER+, have Her2Neu positivity, be triple negative, and so forth. Each of these genetic variants may be addressed separately as targets in our drug development.

If one needed to develop a predictive biomarker, a clinical trial-robust assay must then also be developed. Due to the length of time for the described process, one cannot develop the biomarker and its assay coincident with an ongoing clinical drug trial. This type of approach must be carried out during the research phase using technology that may not be optimal for a clinical trial deployment. Therefore, an orthogonal

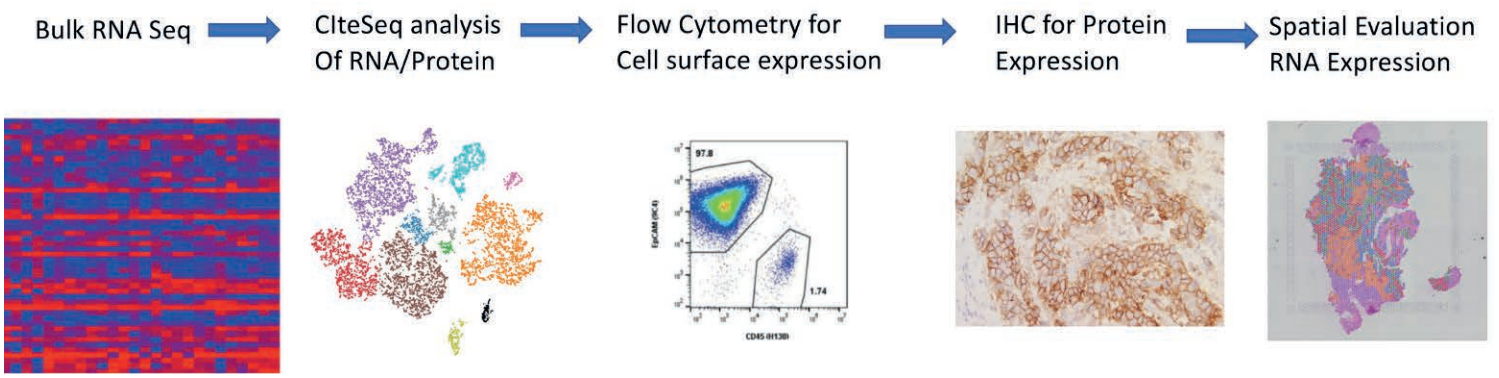

Figure 1: Flow through of the biomarker discovery process.

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About DLS Capabilities

Discovery Life Sciences (DLS) combines one of the world's largest commercial biospecimen inventory and procurement network with multi-omic service laboratories to accelerate precision medicine programs for cancer, infectious diseases, and other complex conditions. In addition to housing over 10 million research quality samples (one of the world's largest repositories of biosamples), DLS also has a large toolbox of technologies, including toxicology services, genomics, proteomics, and pathology, in addition to its extensive tissue and blood/bone marrow inventory that makes it a value-added partner in drug discovery and biomarker development. This unique combination of capabilities allows us to begin with the most appropriate samples (be that blood or tissues,) and then identify new biomarkers or new targets for drug development through cutting-edge, multi-omics, and high-throughput techniques.

DLS Capabilities

Diseased Biospecimens	Cell & Gene Therapy	ADME-Tox	Genomics	Proteomics	Pathology
Blood/Bone Marrow • PBMCs • Plasma/Sera • Buffy Coat Tissue • FFPE • Flash Frozen • DTCs **Cell Biology Services:** • Flow Cytometry • Single Cell Genomics • Spatial Gene Expression	Leukopaks • Mobilized Leukopaks • Clinical-Grade Leukopaks • Mononuclear Cells **CGT Services:** • Cell Isolations • Donor Screening	• Hepatocytes • Enterocytes • Microsomes • Supersomes • TransportoCells **ADME-Tox Services:** • Enzyme Induction and Inhibition • Transporter Interaction Studies • Reaction Phenotyping • Metabolic Stability Permeability • Plasma Protein Binding • MetMax Metabolism-dependent Cytotoxicity Assay	• RNA Sequencing • Whole Exome Sequencing • Whole Genome Sequencing • Single Cell Sequencing • Targeted Sequencing • Pan-cancer Sequencing • Long Read Sequencing • Epigenomic Sequencing • NGS Bioinformatics	• Proteogenomics • Mass Spectrometry • Targeted Multiplexed Immunoassays	• Immunohistochemistry • Multiplex Immunofluorescence • In Situ Hybridization • Histology and Digital Pathology • Molecular Pathology • Quantitative Gene Expression Analysis

method needs to be developed that can easily identify the biomarker of interest. Something like IHC, a method that is already in place in many hospitals in the world, shows where the marker is present, or a qPCR assay that can easily detect gene mutations or translocations, are two of the most robust ways to test in clinical trials.

If during this process, a biomarker is found and confirmed in the phase I clinical trial, a companion diagnostic prototype could then be considered a candidate for further development. This preliminary diagnostic test can be later confirmed during development and later validated as a companion diagnostic when it is run simultaneously with the Phase II/III or registrational trial, according to FDA guidance.[8] One advantage of this approach is the ability to find the most informative biomarkers in human tissues, develop an accurate, robust assay and then have it ready to be a CDx, while keeping pace with the clinical trial. Another advantage is that one can accelerate clinical drug development by concentrating on patients that will respond and avoiding unnecessary adverse events in those unlikely to respond. A specific case is highlighted below describing the strategy that was employed to accomplish this in our company.

PDL1 IHC, a case study in CDx development

Pembrolizumab, also known by a Merck brand name as Keytruda,** is a member of the class of drugs that revolutionized the immune-oncology field; of note, Keytruda is the first FDA approved immunotherapy with a biomarker. Tumor cells express PD-L1 to mask themselves from the immune system, evading detection and thus preventing tumor cell killing by immune cells. Pembrolizumab works by binding to PD-1, an inhibitory signaling receptor expressed on the surface of activated T cells, thereby blocking the T cell interaction with PD-L1 on tumor cells. This blockade helps to restore the anti-tumor immune response and T cell-mediated cell death. Since this therapy targets a specific molecule, a companion diagnostic (CDx) was developed to ensure detection of PD-L1 expressed on tumor cells. The presence of this biomarker indicates that these tumors would likely benefit from a drug promoting T cell activation, and absence of the biomarker would indicate another treatment option may be more efficacious.

Developing this type of CDx requires a multi-systems approach, including biospecimen

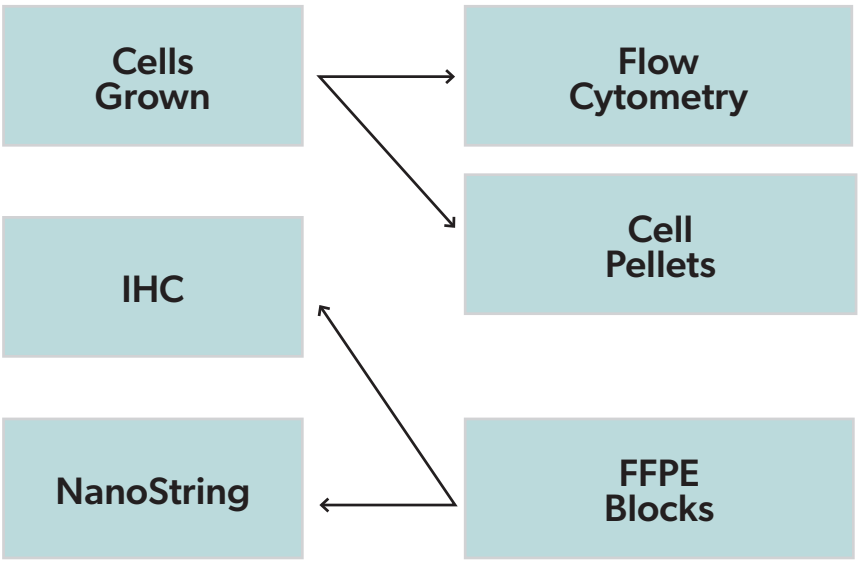

Figure 2: Confirmatory methods for controls for antibodies.

procurement and an accurate, reproducible, and simple method to measure protein levels. Immunohistochemistry (IHC) based assays are well-established and widely accepted as a method to detect protein levels in clinical trials as it enables evaluation of cells in the tumor microenvironment and is commonly available in clinical settings.

Determining the specificity and sensitivity of the antibody is of initial importance when developing an IHC assay to detect biomarkers. In fact, the use of a less sensitive assay is a potential cause of the ill-fated CheckMate-026 clinical trial which evaluated the efficacy of another PD-1 inhibitor, nivolumab, in NSCLC.[9] This underscores the importance of testing multiple antibody clones against the target and evaluating the performance in proper controls so that the optimal assay is selected, and the potential of non-specificity and cross-reactivity may be reduced.[10] Our on-site team of board-certified pathologists further assisted in confirming diagnoses of patient samples stored in our biorepository, developing the optimal assay to be used in biomarker detection, as well as provide expert insight into devising the scoring method and cut-off values that are then used in patient screening.

Here, the proprietary PD-L1 clone 22C3 was investigated for additional metrics of specificity to correlate expression levels of the marker and subsequently confirmed in a control material (**Figure 2**). These confirmatory methods may include flow cytometry, RNA sequencing, and in situ hybridization, techniques available at DLS, that can increase confidence that the antibody is accurately detecting the antigen of interest. Furthermore, the suitability of the ideal antibody is largely predicated on its ability to detect a dynamic range of expression. The Keynote-010 study, evaluating the efficacy of Pembrolizumab in NSCLC, demonstrated that the benefit of PD-1 inhibition was correlated with PD-L1 expression.[9]

In our development of the prototype CDx, we established that the assay could detect negative, low, moderate, and high PD-L1 expression (**Figure 3**) allowing for the clinical trial to select and stratify patients based on a range of expression, not just the presence or absence of the marker.

Once the assay has been established, the next crucial stage is to draw samples from DLS's repository to determine the prevalence of the marker in the patient population, which allow for the strategic design of clinical trials. These research samples have detailed patient history, including treatment and lifestyle history, as well as diagnosis, stage, and genetic mutations. The

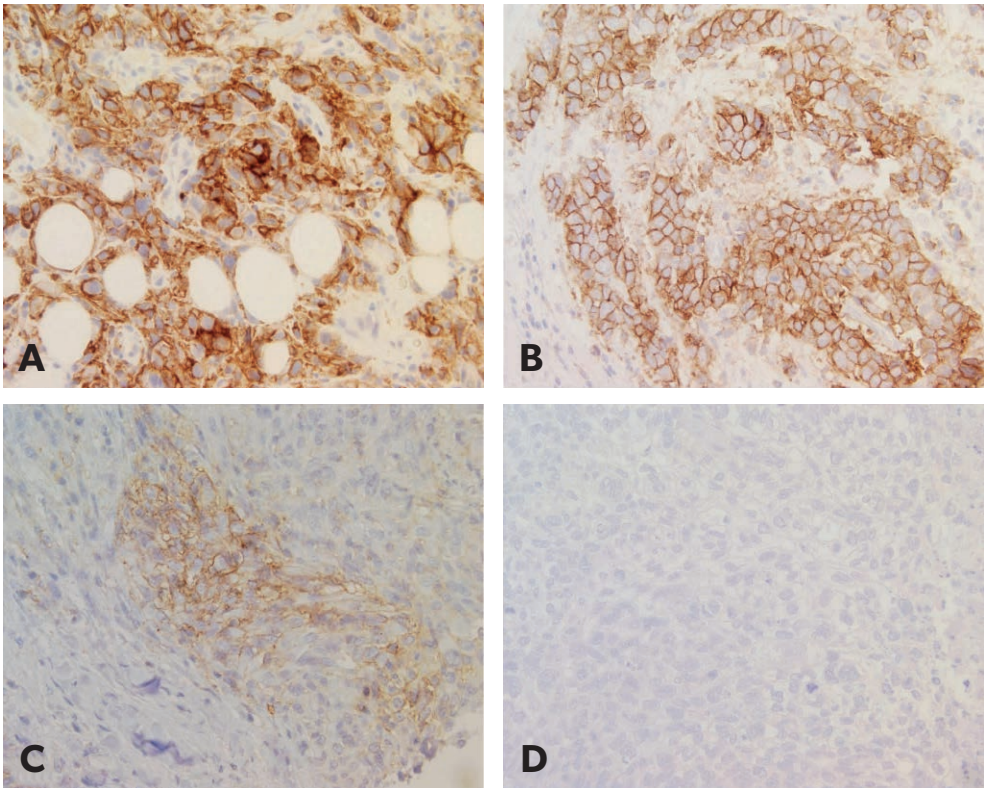

Figure 3: Lung cancer tissues showing **(A)** high, **(B)** moderate, **(C)** low, and **(D)** negative PD-L1 membrane expression on tumor cells. Images were taken at 40x magnification.

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DLS repository typically has an adequate supply of suitable tissues to reach the statistical power necessary to understand expression patterns.

One approach utilizes a sensitivity screen performed in multiple tumor types to understand the expression of the marker within and among tumor indications. For example, in one study performed, we tested up to 20 different tumor types and found the gonadotropin receptor (GR) was highly expressed in melanoma, renal cell, sarcoma, and head and neck tumors, but expressed at very low levels in colon, gastric, and endometrial cancer.[10] During development of the pembrolizumab prototype CDx, archived tissue from NSCLC and melanoma was examined to understand the prevalence of PD-L1 in these indications, as well as begin developing the scoring scheme to be used in clinical trials. Researchers examined 142 NSCLC and 79 melanoma archived samples and found that 56% of NSCLC samples and 53% of melanoma samples were classified as PD-L1+ using a preliminary scoring method,[11] indicating what subtype of patients express the targeted biomarker and may respond to PD-1 inhibitor therapy.

A prototype CDx serves multiple functions, including establishing the scoring scheme, validating the assay in the intended to treat (ITT) populations, determining a specific cut-off for the biomarker expression level that denotes positivity, understanding if the biomarker is predicting treatment response, and screening patients for prospective clinical trial enrollment. The scoring method used for the prototype 22C3 assay laid much of the groundwork for the scoring method employed with the 22C3 CDx in use today. Furthermore, the prototype 22C3 assay was used to establish the cut-off limit to help understand what population of potential patients would be considered eligible for enrollment, was subsequently used to enroll patients in prospective clinical trials and is still used to determine eligibility in select clinical studies. This foundation led to the PD-L1 22C3 assay becoming the first companion Dx approved in lung cancer for patients whose tumors express PD-L1, followed by its approval as first-line treatment in metastatic NSCLC, metastatic melanoma, and metastatic HNSCC, in addition to several other indications.[12]

Lastly, an often-overlooked aspect of preclinical and biomarker research is the lab accreditation performing the experiments. CAP (College of

American Pathologists)/CLIA (Clinical Laboratory Improvement Amendments of 1988) accredited laboratories ensure that results are in line with industry standards and regulatory guidelines for clinical diagnostic testing and may potentially aid in the FDA approval process. Without this foresight, approval delays and/or reproducibility issues may occur that undermine the confidence of the research and prevent timely and cost-effective progression into the clinic.

Summary

Discovering novel biomarkers for companion diagnostics program starts with identifying a biological target with a critical role in disease, and then identifying a candidate molecule that highlights the target. Investing resources in such programs faces the risk of the low success rate of drug discovery programs that underscores the complexity of the task – approximately 0.04% of preclinical drug development programs yield licensed drugs.[13] The primary method of finding potential drug targets has historically been combing through the literature and large cell line screens, but the advent of omics technology has introduced a more powerful and comprehensive system. Utilizing a high-throughput multi-omics approach has allowed us to streamline the process of target identification, with the recent success of identifying immunomodulatory receptors. Spatial technology can then be used to understand the expression of the marker of interest within the context of cell-type localization. Once this preclinical research has been carried out and a target identified, one is able to move that biomarker into the clinic in the form of a robust clinical assay.

Should the assay be an indication for the response of the associated clinical trial drug, a prototype companion diagnostic can then be developed. At this stage, it is critical to confirm the specificity and sensitivity of the assay, which DLS accomplishes by screening a large number of patient samples to understand prevalence of the marker within, and among tumor types. This information is key to inform clinical trial design. Throughout the clinical trial process, the assay may be used to evaluate its predictive potential and potentially correlate expression with treatment response. This approach was successfully employed in our development of the pembrolizumab and trastuzumab prototype CDx,[14] arguably among the most influential oncology drugs. By combining the technologies and the resources described in this article, we posit that one can find biomarkers of interest and rapidly incorporate them in the clinical trial with a high likelihood that a positive signal in the assay with the drug could lead to a companion diagnostic assay. PMQ

Frank Lynch, PhD

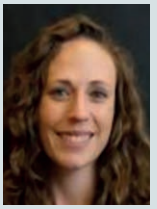
Executive Vice President Business Development, Tissue Biomarkers Discovery Life Sciences

Frank co-founded QualTek Molecular Laboratories in 1997 and developed an IHC services platform. He has overseen over 1,000 studies and hundreds of oncology clinical studies using IHC biomarkers, including the development of a crucial, high profile, PD-L1 prototype companion diagnostic test. QualTek merged with Discovery Life Sciences in 2019. Prior to QualTek, Frank helped grow start-up company BioTek Solutions (later merging with Ventana) where he assisted in automating clinical IHC assays in hundreds of hospitals and anatomic pathology labs around the world. At BioTek, he developed the first widely accepted automated IHC test for estrogen receptor (H222 clone) in FFPE breast cancer using the TechMate immunostainer. Frank received his PhD from Rutgers University.

Shawn Fahl, PhD

VP Lab Operations, Cell Services & R&D, Biospecimens

Shawn Fahl received his PhD in Microbiology from the University of Virginia and completed his postdoctoral fellowship at Fox Chase Cancer Center. During his graduate and postgraduate career, Shawn focused on the transcriptional regulation of conventional and non-conventional lymphocyte development and function. Shawn joined Discovery Life Sciences in 2017 to establish the analytical pipelines required for single cell evaluation of complex human biospecimens.



Noreen McBrearty, PhD

Dr McBrearty is an Associate Director at Discovery Life Sciences, specializing in biomarker assay development. She earned a PhD in Biology at Temple University where she studied early and novel treatment strategies in the context of Hepatitis B Virus-related Hepatocellular Carcinoma. She then moved to the University of Pennsylvania for her postdoctoral training in immuno-oncology. There, she studied mechanisms of immune evasion in pancreatic tumors and worked on improving current CAR T-cell therapies targeting solid tumors. Moving more towards the clinical side of translational medicine, she has spent the last several years working on developing companion diagnostics to be used in clinical trials.

Suso Platero, PhD

Dr Platero is Chief Scientific Officer at Discovery Life Sciences. Prior to this role, he worked in the pharmaceutical industry and in the diagnostic industry. He has developed multiple biomarkers for clinical trials in the areas of Oncology, Immunology, Immuno-Oncology, Cell and Gene Therapy and Rare Diseases. His experience in drug development includes small molecules, antibodies, gene therapy and vaccines. Among the drugs he worked with include Erbitux, Ixabepilone, Sprycel, Yervoy and Erdafitinib. Work in the Erdafitinib clinical trial led to the approval of a companion diagnostic for the drug. While in the diagnostic industry, he developed diagnostic tests and companion diagnostics for several indications, including Cell and Gene Therapies. He obtained his PhD from St Louis University Medical School and he postdoc at the Fred Hutchinson Cancer Research Center.

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* cellular indexing of transcriptomes and epitopes by sequencing, see also https://cite-seq.com/.

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