Luminex

Whitepaper

Five Ways That Multiplex Technology Accelerates Cancer Research

Introduction

Cancer is a complex group of diseases with many possible causes. The American Cancer Society predicts that half of all men and one-third of all women in the US will develop cancer during their lifetimes, making cancer a high priority for medical research. Due to the nature of the disease, cancer research follows many lines of investigation. A great deal of scientific effort is being focused on research for the identification of biomarkers for cancer and their future use as diagnostics for early detection and for validating clinical trials of new cancer drugs. Much of biomarker research focuses on tyrosine kinases and their phosphorylation states. The correlation between inflammation and cancer, involving a multitude of cytokines, is also an active area of study, as is immune-mediated tumor eradication using genetically engineered receptors to activate T cells. Of course, genetic factors play a significant role in the development of cancer, and there are a multitude of efforts underway to characterize the genetics of cancer.

A Key Consideration

The most striking aspect of cancer may be the extreme complexity of its forms and causes. For example, cancers can develop in a multitude of organs, and a solid tumor can contain numerous cancer cell types. A single cancer type can involve a large number of genes and a correspondingly large number of proteins—many of which may be useful as biomarkers. In fact, cancer research can involve simultaneous analysis of tens and even hundreds of genes and biomarkers. Successful research efforts therefore require the availability of highly multiplexed assays for both protein biomarkers and gene expression. This publication provides several examples of the role that a highly multiplexed bead-based assay technology is playing in the advancement of cutting-edge cancer research.

A Multiplex Assay System that Meets Cancer Researchers' Needs

Traditional ELISA assays cannot provide the required multiplexing to meet cancer researchers' needs. What is needed is a reproducible and sensitive assay system that can simultaneously interrogate large numbers of nucleic acid or protein targets per sample, across a large number of samples, in a short time, and easily adapt to researchers' changing needs.

Rather than rely on single-plex ELISA or low-multiplex real-time PCR assays, a microsphere-based fluorescent system that utilizes a large number of bead types (as many as 500), each carrying a specific antibody or oligonucleotide, can provide the high multiplex capability required for cancer research.

With Luminex® xMAP® Technology, a laser or an LED array is used to interrogate a specific mixture of fluorescent dyes in each bead type that is characteristic for that bead type (Figure 1). Once an analyte is bound to the ligand on each bead type, a reporter dye is used to quantify the amount of analyte bound. Either flow cytometry fluidics or an imaging device is then used to determine which beads are present and the amount of analyte on each bead.

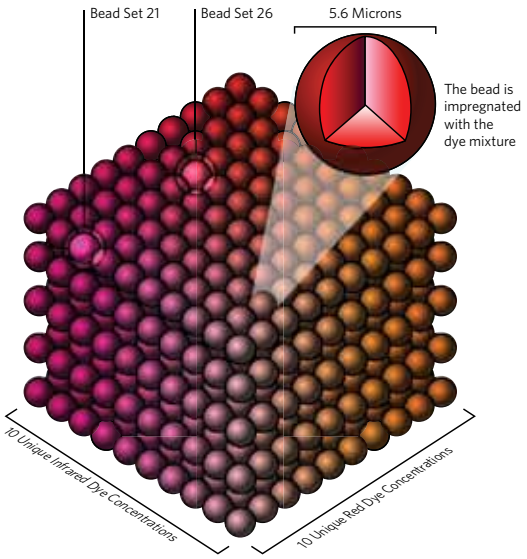

Figure 1. Luminex internally color-codes microspheres with precise concentrations of various florescent dyes—yielding up to 500 distinctly colored bead sets.

Depending upon the instrument used, up to 500 bead types can be used in each well of a 96- or 384-well plate, thus generating a high throughput assessment of a large number of protein or oligonucleotide targets. This microsphere "liquid array" assay system is unique in its ability to provide both high-throughput and high-content data, and the ligands can be readily changed to meet the needs of the researcher. Multiplexing reduces cost and labor, and preserves precious sample. This system is ideally suited to cancer research. It provides the low cost-per-sample, high throughput, high reproducibility and flexible assay design required to help elucidate the many causes of cancer and identify cancer biomarkers.

In this paper, we will discuss how researchers can overcome these limitations by using multiplexing techniques in these five specific areas:

- Biomarker Discovery and Utilization
- Tyrosine Kinases and Cancer
- Inflammation and Cancer
- Combinatorial Antigen Recognition
- Genetic Characterization of Cancer

Biomarker Discovery and Utilization

There is an urgent need for the discovery of biomarkers that can be used in blood-based, non-invasive molecular tests to assist in the detection and diagnosis of cancers, as well as to classify tumors and monitor disease response to treatment and relapse (Hanash et al. 2011). There are currently many efforts underway to identify such biomarkers. For example, a Luminex bead-based multiplexed immunoassay of 40 plasma proteins has been used to determine their potential for distinguishing between early-stage 1 non-small-cell lung cancer (NSCLC) and matched controls without lung cancer. Several of these proteins, including cytokines and chemokines, demonstrated significant differences between stage 1 NSCLC and controls (Hanash et al. 2011). A separate study used a bead-based assay for TGF-ß 1,2,3 and the phosphorylation status of several of the SRC family tyrosine kinases (SFKs) in NSCLC. The results revealed that SRC is activated in Lkb1-deficient primary and metastatic tumors, and that the combined inhibition of SRC, P13K and MEK1/2 resulted in synergistic tumor regression (Carretero et al. 2010).

A similar study linked a decrease in VEGF plasma concentration with disease control in a phase I/II study of a combination of dasatinib and erlonitinib for treatment of NSCLC (Haura et al. 2010). Luminex bead assays were also used as one of the tools to assess 300 serum proteins for predictive value for immune response to a cancer vaccine for renal cell cancer (RCC). Apoliporotein A-1 (APOA1) and chemokine (C-C) motif ligand 17 (CCL17) were both associated with immune response and overall survival (Walter et al. 2012)

Biomarkers that can be associated with positive response to drug therapy are also being identified. A Luminex bead-based multiplexed assay for 97 proteins, for example, was used to identify 5 proteins whose plasma concentrations were associated with the efficacious treatment of colorectal cancer with the drug cetuximab (Tabernero et al. 2010). A decrease in plasma levels of IL-8, macrophage inflammatory protein-1, and the tumor markers carcinoembryonic antigen, CA125, and CA19-9 between baseline and week 4 was significantly associated ($P < .01$) with a positive drug response at week 6.

Biomarkers can also be used for early detection of cancer which can often require multiple biomarkers to assure accuracy of detection. In a study of ovarian cancer, a Luminex bead assay was used to measure the levels of 96 proteins in order to identify and validate a biomarker combination with the highest power to detect early-stage ovarian cancer. The study concluded that a panel of four proteins—CA-125, HE4, CEA, and VCAM-1—provided the highest discriminating power of 86% sensitivity (SN) for early-stage, and 93% SN for late-stage ovarian cancer at 98% specificity (Yurkovetsky et al. 2010).

Circulating angiogenic cytokines (CAC) have been studied in relation to patients' prognoses after resection for pancreatic cancer (Rahbari et al.). CACs are soluble molecules that may reflect the overall angiogenic activity of pancreatic tumors. In patients with primary and metastatic pancreatic cancer, certain CACs were differentially expressed (Figure 2). Circulating levels of P1GF ($p=0.003$) and PDFG-AA ($p=0.02$) were significantly higher in patients with metastatic disease, and these same patients had lower serum concentrations of PDGF-BB ($p=0.0001$). The authors concluded that serum levels of certain CACs correlate with patients' prognosis after resection for pancreatic cancer, and that future research should not be limited to single molecules.

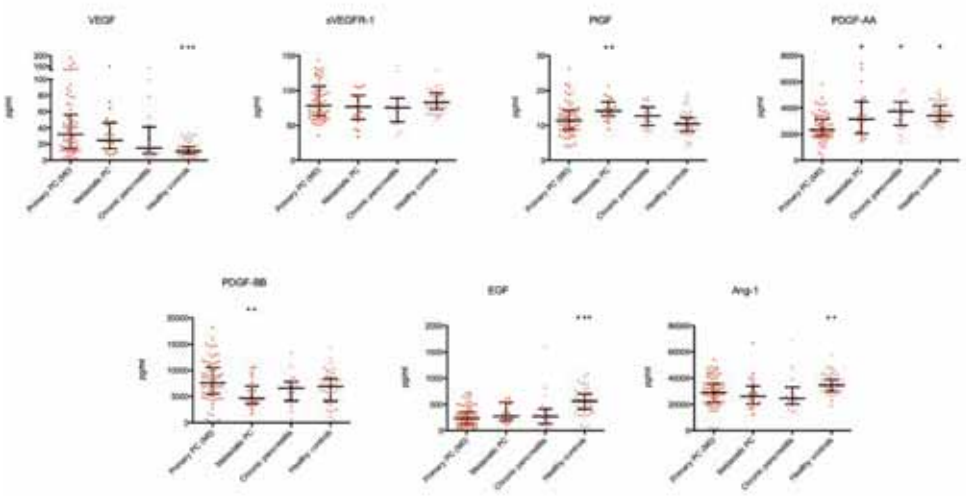

Figure 2. Expression of seven circulating angiogenic cytokines in patients with primary pancreatic cancer (M0), metastatic pancreatic cancer, chronic pancreatitis and healthy control subjects. Data are presented as median and interquartile range (black bars). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (Mann-Whitney U test). Comparisons refer to the group of patients with primary pancreatic cancer.

Figure reproduced from Rahbari et al. 2011 under the Creative Commons Licence.

Tyrosine Kinases and Cancer

As previously noted, tyrosine kinases and their respective phosphorylation states can be used as biomarkers for cancer detection and monitoring. One study used a Luminex bead-based assay to detect phosphorylation of 70 wild-type and mutant tyrosine kinases across 130 human cancer cell lines (Du et al. 2009). In addition to confirming many of the known kinase activation events associated with cancer, the study identified SRC kinase phosphorylation in both glioblastoma cell lines and primary patient samples, making it a potential therapeutic target. Notably, the role of SRC in glioblastoma was not suggested by recent DNA sequence or copy-number analysis (Cancer Genome Atlas Research Network 2008), but instead required protein phosphorylation data for its discovery.

A separate study using the same bead-based assay demonstrated increased SRC activity (phosphorylation) in invasive thyroid tumors, relative to non-invasive tumors (Figure 3). In addition, SRC inhibition in cell lines decreased cell invasion and proliferation (Cho et al. 2012).

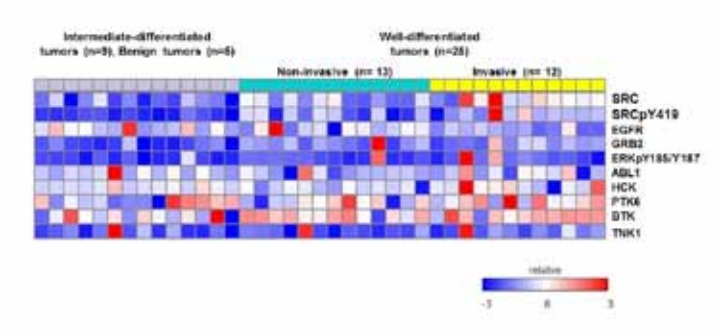

Figure 3. Src is activated in invasive thyroid cancer specimens. Paired normal and thyroid tumor protein lysates were collected and subjected to kinome array analysis. Tyrosine kinase phosphorylation levels were tested by single or multiple antibodies. The raw data was processed and positive signals were normalized to fold-over-backgrounds. The kinase phosphorylation value of each tumor specimen was divided by the matched normal specimen. The normalized readings were log-transformed and converted into a .gct file, and the HeatMap diagram was generated using the GenePattern 3.0 software. Figure courtesy of Dr. Nancy Cho.

Inflammation and Cancer

The human immune system can both promote and suppress cancer. Chronic inflammation and proinflammatory cytokines such as interleukin (IL-1) and IL-6 are considered to be tumor promoting, while the exact nature of protective anti-tumor immunity remains obscure (Haabeth et.al). Several multiplex cytokine assays using Luminex bead technology have been used to elucidate the relationship between inflammation and cancer.

The tumor microenvironment is characterized by chronic inflammation represented by infiltrating leukocytes and soluble mediators, which leads to a local and systemic immunosuppression associated with cancer progression. One study of a rat melanoma model showed increased levels of several cytokines correlated with tumor progression (Meyer et al. 2011). It also linked a decrease in numerous inflammatory mediators with a concomitant decrease in the level of myeloid-derived suppressor cells (MDSC) known to inhibit tumor-reactive T cells.

Another study used the mulitplex cytokine assay to determine the ability of several phenylglycolipids to stimulate murine and human iNKT cells to secrete T helper 1 (Th-1) skewed cytokines and exhibit anticancer efficacy (Wu et al. 2011). In addition, the study found that Cd1d-phenyl glycolipid complexes interact with iNKT cells with higher avidity and stability to drive Th-1 polarization.

The bead-based multiplex cytokine assay platform was also used to measure the levels of 21 cytokines (Rakhra et al.). The results demonstrated that an intact immune system—specifically CD4+ T cells—is required for the induction of cellular senescence, shutdown of angiogenesis, and chemokine expression resulting in sustained tumor regression upon oncogene inactivation

A Luminex bead-based multiplexed assay for 33 cytokines was used to quantify locally secreted cytokines during primary immune responses against myeloma and B-cell lymphoma in mice (Haabeth et al.). Successful cancer immunosurveillance mediated by tumor-specific CD4 + T cells was consistently associated with elevated local levels of both proinflammatory (IL-1α, IL-1β and IL-6) and Th1-associated cytokines (interferon-γ (IFN-γ), IL-2 and IL-12). Thus inflammation, when driven by tumor-specific Th1 cells, may prevent rather than promote cancer.

Combinatorial Antigen Recognition

Current T-cell engineering approaches redirect patient T cells to tumors by transducing them with antigen-specific T-cell receptors (TCRs) or chimeric antigen receptors (CARs) that target a single antigen. However, few truly tumor-specific antigens have been identified. For this reason, a strategy to render T cells specific for a tumor in the absence of a truly tumor-restricted antigen has been developed (Kloss et al.2013). T cells are transduced with both a CAR that provides suboptimal activation upon binding of one antigen, and a chimeric costimulatory receptor (CCR) that recognizes a second antigen. A multiplexed bead-based cytokine assay was used in this study to demonstrate that T cells transduced with both a CAR and a CCR produced relatively high amounts of Th1 and Th2 cytokines responsible for tumor eradication, whereas T cells expressing only CARs produced lower amounts of cytokines. The T cells transduced with both a CAR and a CCR destroyed tumors that expressed both transduced antigens, but did not affect tumors that expressed either antigen alone.

A second study investigated the effect of adding combined CD28 and 4-1BB costimulatory signaling domains to a CAR specific for prostate-specific membrane antigen (PSMA) (Zhong et al. 2010). A CAR including all three signaling domains was superior to receptors that only include one or two of these domains in promoting cytokine release—as measured using a bead-based cytokine assay as well as ELISAs. This CAR also provided superior *in vivo* T-cell survival and tumor elimination following intravenous T-cell administration to tumor-bearing severe combined immunodeficient (SCID)/beige mice.

Genetic Characterization of Cancer

Linking the molecular aberrations of cancer to drug responses could guide treatment choice and identify new therapeutic applications, but

until recently there has been no systematic approach for analyzing gene-drug interactions in human cells. Luminex bead-based technology has been used to build a multiplexed assay to study the cellular fitness of a panel of engineered isogenic cancer cells in response to a collection of drugs, enabling the systematic analysis of thousands of gene-drug interactions (Muellner et al. 2011).

The method uses molecular barcodes, short, non-transcribed stretches of DNA introduced into the genomic DNA of cell lines that can be selectively quantified by PCR. In a mixed population of cells that each contain a unique barcode, the relative number of cells containing a particular vector can therefore be determined by quantification of the barcodes. Antisense DNA barcodes attached to Luminex beads were used to quantify 70 barcodes simultaneously. This approach has been used to query a 70 × 87 drug-gene interaction matrix in breast cancer cells, which allowed the interrogation of over 6,000 drug-gene pairs. Applying this approach to breast cancer revealed various synthetic-lethal interactions and drug-resistance mechanisms, some of which were known, thereby validating the method (Figure 4). The data revealed a new mechanism of resistance to PI3K inhibitors with direct clinical implications.

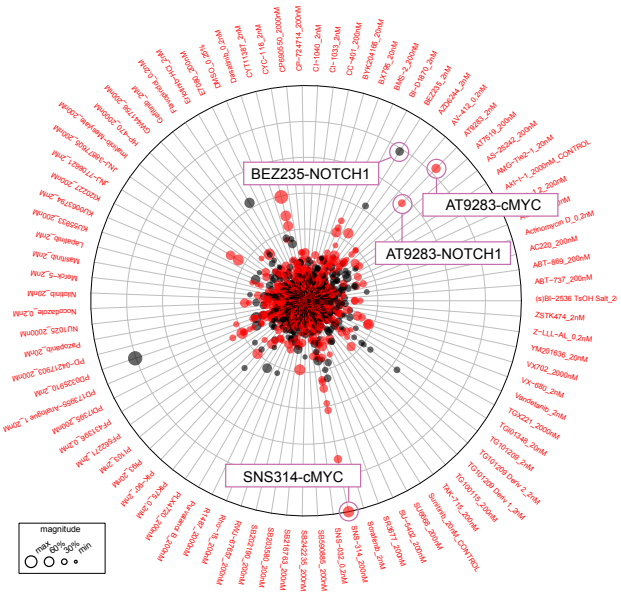

Figure 4. Combinatorial breast cancer gene small compound screen. Radial gene-drug interaction plot displaying pairwise drug-gene measurements. Distance from the center indicates significance, and dot size is proportional to the magnitude of the drug versus control effect. Figure provided courtesy of Dr. Sebastian Nijman.

Luminex bead-based multiplex technology has also been used systematically to assess the expression of 217 mammalian miRNAs across a large panel of samples representing diverse human tissues and tumor types (Lu et al. 2005). The miRNA profiles reflected the developmental lineage and differentiation state of the tumors, with a general down-regulation of miRNAs in tumors compared with normal tissues. The method enabled the classification of poorly differentiated tumors, whereas messenger RNA profiles were highly inaccurate when applied to the same samples.

Luminex xMAP Technology

Luminex provides the only flexible and open multiplexing technology used by several market leaders to provide assays for both gene and protein expression. Unlike conventional technologies that can only measure one or a few biomarkers, researchers have the capability to easily scale up or down the number of biomarkers measured and to customize assays. xMAP Technology combines advanced fluidics, optics, and digital signal processing with proprietary microsphere technology. Featuring a flexible, open-architecture design, xMAP Technology can be configured to perform a wide variety of bioassays quickly, cost-effectively and accurately. Focused, flexible multiplexing of 1 to 500 analytes meets the needs of a wide variety of applications, including genotyping, protein expression profiling, and gene expression profiling.

All of the microsphere bead assays described in this white paper were developed using xMAP Technology to provide highly-multiplexed genotyping and protein biomarker capabilities. The open architecture of the system made it feasible for the researchers to create their own protein assessment (e.g., tyrosine kinases) and genotyping assays, while commercially available kits were used to monitor cytokine levels. An ever-expanding menu of assays for other applications is also available from Luminex and its commercial partners. See www.luminexcorp.com for the latest information.

Partial List of Institutions using Luminex xMAP Technology for Cancer Research

Broad Institute
Dana-Farber Cancer Institute
Vall d'Hebron University Hospital
H. Lee Moffitt Cancer Center and Research Institute
Fred Hutchinson Cancer Research Center
City of Hope Comprehensive Medical Center
German Cancer Research Center and University Hospital Mannheim
Division of Oncology, Stanford University School of Medicine
Centre for Immune Regulation, University of Oslo
Research Center for Molecular Medicine of the Austrian Academy of Sciences
Memorial Sloan-Kettering Cancer Center
University of Heidelberg

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