Reproducible, quantitative and flexible molecular sub-typing of clinical DLBCL samples using the NanoString nCounter system

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Abbreviations
ABC; Activated B-cell
CC; Consensus Clustering
COO; Cell of Origin
DLBCL; Diffuse Large B-cell Lymphoma
FFPE; Formalin Fixed Paraffin Embedded
GCB; Germinal Center B-cell
GEP; Gene Expression Profiling
ICC; intraclass correlation coefficient
IHC; immunohistochemistry
qNPA; quantitative Nuclease Protection Assay

Word Count: 5000
Translational Relevance

Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous disease that can be classified into 2 major molecular subtypes: activated B-cell-like (ABC) and germinal center B-cell-like (GCB) DLBCL. Clinical trials of targeted therapies are now underway using ABC/GCB segmentation as a patient stratification approach. Both gene expression profiling (GEP) and immunohistochemistry (IHC) have historically been used to segment ABC from GCB-type DLBCL. While the accurate classification of patients is essential for data interpretation, commonly used GEP and IHC methodologies suffer from several limitations. We developed a DLBCL assay on the NanoString gene expression system that accurately and reproducibly categorized multiple DLBCL sample types including both fresh and formalin-fixed patient tumor tissue. After comparing against IHC and several other standard gene expression methodologies, the NanoString offered several key advantages including sensitivity, flexibility, and clinical applicability. The NanoString platform should be strongly considered for both DLBCL research and patient management activities.
Abstract

Purpose:
Diffuse large B-cell lymphoma (DLBCL) is a heterogeneous disease with distinct molecular subtypes. The most-established subtyping approach, the ‘Cell of Origin’ (COO) algorithm, categorizes DLBCL into activated B-cell (ABC) and germinal center B-cell (GCB)-like subgroups through gene expression profiling. Recently developed immunohistochemical (IHC) techniques and other established methodologies can deliver discordant results and have various technical limitations. We evaluated the NanoString nCounter™ gene expression system to address issues with current platforms.

Experimental Design:
We devised a scoring system using 145 genes from published datasets to categorize DLBCL samples. After cell line validation, clinical tissue segmentation was tested using commercially-available diagnostic DLBCL samples. Finally, we profiled biopsies from relapsed/refractory DLBCL patients enrolled in the fostamatinib phase IIb clinical trial using three independent RNA expression platforms: NanoString, Affymetrix, and qNPA.

Results:
Diagnostic samples showed a typical spread of subtypes with consistent gene expression profiles across matched fresh, frozen and formalin-fixed paraffin-embedded tissues. Results from biopsy samples across platforms were remarkably consistent, in contrast to published IHC data. Interestingly, COO segmentation of longitudinal fostamatinib biopsies taken at initial diagnosis and then again at primary relapse showed 88% concordance (15/17), suggesting that COO designation remains stable over the course of disease progression.

Conclusion:
DLBCL segmentation of patient tumor samples is possible using a number of expression platforms. However, we found that NanoString offers the most flexibility and fewest limitations in regards to robust clinical tissue subtype characterization. These subtype distinctions should help guide disease prognosis and treatment options within DLBCL clinical practice.
Introduction

Diffuse large B-cell lymphoma (DLBCL) is the most common type of lymphoma, accounting for nearly 40% of newly diagnosed cases (1). While approximately 50% of DLBCL is curable through standard treatment, there is an urgent need for new therapies as most relapsed patients will eventually die from their disease. DLBCL has been long recognized as a heterogeneous disease with diverse genetic features and variable clinical outcomes. Gene expression profiling (GEP) techniques have been used for over a decade to classify DLBCL into distinct molecular subtypes, some of which carry significant prognostic value (2-5). The most well-established expression signature is the ‘Cell of Origin’ (COO) algorithm, which divides DLBCL into activated B-cell (ABC) and germinal center B-cell (GCB)-like subgroups. The COO signature can be refined to 12 distinct genes capable of accurately subtyping DLBCL with little loss of specificity or sensitivity (4). The continued refinement and potential prognostic value of COO profiling has led to its incorporation into patient selection strategies for emerging targeted therapeutics. Both ibrutinib (Pharmacyclics, Sunnyvale, CA) and bortezomib (Millennium, Cambridge, MA) have applied retrospective ABC/GCB patient profiling to phase I and II clinical trials (6, 7).

COO (and other) expression profiling approaches will undoubtedly lead to continued investment in personalized medicine opportunities in DLBCL (8). An obstacle to more widespread clinical profiling has been the limited availability of snap frozen DLBCL biopsies that yield high quality tumor RNA. Standard clinical practice usually results in collection of formalin-fixed, paraffin-embedded (FFPE) diagnostic tissue that yields fragmented RNA. Such RNA is not the preferred input type for microarray-based approaches (i.e. Affymetrix) historically used for COO profiling. Because of this limitation, alternatives to classic microarrays have emerged to enable COO profiling of diagnostic FFPE specimens. Numerous immunohistochemical (IHC) classifiers have been developed over the last decade (9). The original IHC algorithm is the ‘Hans’ approach based on expression of three protein markers that have been widely adopted within segments of the clinical DLBCL community (10). Being an IHC-based assay, the Hans methodology is subject to antibody limitations, pathologist subjectivity, and is relatively non-quantitative in its scoring. Not surprisingly, Hans IHC and Affymetrix GEP have been shown to lack full concordance when tested concurrently on a blinded set of biopsies (11). More recently, the quantitative nucleic acid protection assay (qNPA) (HTG Molecular Diagnostics, Tucson, AZ) has emerged as another technology for DLBCL segmentation as it can measure gene expression from FFPE material in a fully quantitative manner (12, 13). While qNPA has some distinct advantages, the current platform is limited in its ability for multiplex analysis of a large number of genes. While Affymetrix can monitor thousands of genes, qNPA monitors less than 50 in a single run.

The desire to develop an approach that provides a flexible, robust, and fully quantitative method for DLBCL segmentation led us to evaluate the recently described NanoString system (Seattle, WA) for gene expression (14-16). NanoString technology uses digital, color-coded barcodes (codesets) that are attached to sequence specific probes, allowing for fully quantitative and direct measurement of mRNA without amplification. The non-enzymatic nature of NanoString allows for accurate and reproducible quantification of as little as 100 ng of input mRNA from FFPE clinical samples. A key differentiating feature of the NanoString system from IHC and qNPA-based
approaches is its ability for multiplex analyses of up to 800 distinct targets. To evaluate the NanoString system for DLBCL subtyping, we designed a custom codeset of over 300 genes, consisting of 145 COO signature genes and additional genes from alternative DLBCL segmentation signatures. We tested these codesets on the NanoString against DLBCL cell lines with known COO designation and then against a set of commercially procured de novo DLBCL patient samples. Finally, we applied the NanoString codeset to biopsies from an ongoing 60 patient clinical trial evaluating the effectiveness of fostamatinib (Syk inhibitor) in relapsed-refractory DLBCL (17). Through the course of the evaluation, we directly tested many samples against other relevant platforms, including Affymetrix microarray and qNPA, to allow for both relative expression data and COO designation comparisons.

Materials and Methods

Cell line and tissue samples

DLBCL cell lines were purchased from Cambridge Enterprise (DSMZ) in 2010 unless otherwise noted. HBL-1 (2010; Professor Masafumi Abe, under license from Tokyo Medical and Dental University), SU-DHL-10, Karpas 422, RC-K8 and SU-DHL-4 were cultured in RPMI with 10–15% fetal bovine serum and 1% L-Glutamine. TMD8 (2010; Daniel Krappmann, German Research Center for Environmental Health) and OCI-Ly19 were cultured in alpha MEM with 10% fetal bovine serum and 1% L-Glutamine. OCI-Ly4 (2009; Mark Minden) and OCI-Ly10 (2010; Louis Staudt, Center for Cancer Research, NCI) were cultured in IMDM with 20% fetal bovine serum, 1% L-Glutamine and 50 μM β-mercaptoethanol. Each vial of cells was used for no more than six passages. Cells were maintained in 5% CO₂ at 37°C. All cell lines were tested for authenticity by genotyping in 2010 prior to use; only those cell lines confirmed to carry the genetic profiles as described previously were used in data presentation.

Commercially available DLBCL tissue samples (RNA; designated as fresh, frozen and FFPE) were purchased from OriGene (Rockville, MD). All required consents for these exploratory analyses were acquired. Prior to processing, each sample was reviewed by an internal certified pathologist to confirm disease diagnosis and verify tumor content.

Fostamatinib phase IIb clinical trial (NCT01499303) pre-treatment frozen core needle biopsies and archival FFPE tissue sections were shipped from recruitment centers with required consent and handled according to AstraZeneca Human Biological Samples policies and procedures. Prior to analysis, an H&E stained section from each sample was assessed by a certified pathologist to confirm disease diagnoses and verify tumor content. A titration study between two frozen DLBCL biopsies (one of each subtype) and benign lymphoid tissue showed a linear relationship between reduction in tumor content and the effect on the gene signature score (data not shown). These results support an arbitrary minimum cut-off of 70% tumor, with samples deemed unevaluable if containing less than
70% tumor tissue. All clinical samples used throughout this study were determined to contain at least 70% DLBCL.

Sample preparation and RNA extraction

Under TMA agreement, total RNA from all nine DLBCL cell lines was isolated using standard procedures and provided by colleagues at AstraZeneca Gatehouse Park, USA. Replicate batches of total RNA were isolated from six DLBCL cell lines (HBL-1, TMD8, SU-DHL-4, SU-DHL-10, OCI-Ly19 and Karpas 422) using the RNeasy mini kit (QIAGEN, Valencia, CA). Frozen tissues obtained from OriGene were processed as 2x8 µm curls. Fostamatinib phase IIb trial frozen core needle biopsies were sectioned to at least 5 mm in length and, if size allowed, processed and analysed in biological duplicates. Processed frozen tissue sections were homogenized on the TissueLyser II system (QIAGEN). Total RNA was isolated using the RNeasy mini kit. FFPE tissues were processed as 2–5 10 µm sections and total RNA isolated using the RecoverAll FFPE RNA isolation kit (Ambion, Austin, TX). Quality of tissue RNA was assessed using the RNA 6000 Nano Kit (Agilent, Santa Clara, CA) and the quantity assessed on the NanoDrop 2000.

NanoString codeset design and expression quantification

COO and consensus clustering (CC) signature and housekeeping genes were included in the codeset based on previously described publications (Supplemental Table 1). Input total RNA amount was determined by titration studies to show comparable and reliable gene expression data at 100 ng for high quality (cell lines, fresh and frozen tissue) and at 400 ng for low quality (FFPE tissue) samples based on detection levels, linearity of genes, and binding density (data not shown). Data was normalized through an internally developed Pipeline Pilot Tool (publicly available for use on the Comprehensive R Archive Network, CRAN). In brief, data were log2 transformed after being normalized in two steps: raw NanoString counts were first background adjusted with a Truncated Poisson correction using negative control spikes followed by a technical normalization using positive control spikes. Data was then corrected for input amount variation through a Sigmoid shrunken slope normalization step using the GEO mean expression of housekeeping genes. A transcript was designated as not expressed if the raw count was below the average of the internal negative control raw counts plus 2 standard deviations.

Microarray (Affymetrix) quantification

Total RNA was extracted from fresh and frozen samples using a standard protocol. At least 250 ng of total RNA from each sample with OD 260/280 ratio between 1.68 and 2.08 and concentration >50 ng/µl was submitted to Almac Diagnostics (Craigavon, UK). Gene expression profiling was conducted using Affymetrix U133 plus 2.0 chip according to manufacturer recommendations. The CEL files were analyzed using Bioconductor’s Affy package in R. Expression was normalized using MAS5.0 method with scaling factor set to 100. The signals were then log2 transformed before downstream analysis. When a gene had more than one probeset, the probeset with the highest mean signal was selected to represent the gene.
qNPA

Two 5 µM FFPE sections per patient were delivered to HTG Molecular Diagnostics (Tucson, AZ) for processing and qNPA analysis. All samples were run against HTG’s 12 gene COO signature array consisting of the following genes: CD10, LRMP, CCND2, ITPKB, PIM1, IL16, IRF4, FUT8, BCL6, LMO2, CD39, and MYBL1. All processing and data analyses were conducted as described (13).

Quantitative real-time RT-PCR

Gene expression assays for 12 genes [FUT8, IL16, IRF4, CCND2, PIM1, CD39 (ENTPD1), ITPKB, LMO2, LRMP, CD10 (MME), MYBL1, and BCL6] were ordered from Applied Biosystems (Carlsbad, CA) (Supplemental Figure 1A). For normalization purposes, IPO8 was selected from a screen of 16 housekeeping genes based on its robust stability and low standard deviation across a panel of six DLBCL cell lines (Supplemental Figures 1B, 1C). Reverse transcription of 100 ng RNA was performed using the Superscript Vilo kit (Invitrogen, Carlsbad, CA) and quantitative real-time PCR amplification of cDNA was performed on the 7900HT Taqman (Applied Biosystems) in 10 µl reactions containing Taqman Gene Expression master mix and assays (Applied Biosystems). Samples were amplified with three experimental replicates. No template controls were reliably negative.

DLBCL COO subtype classification using signature scores

We calculated a composite score of our COO signature, using a method previously described (18). The unweighted average score was calculated from gene expressions within a cohort after housekeeping gene normalization and log2 transformation. Signature scores were then calculated as the mean expression of genes associated with ABC subtype minus the mean expression of genes associated with GCB subtype; a higher score thus indicating a more ABC-like sample.

We then assessed the ability of our COO signature scores to predict the COO subtypes in 32 DLBCL cell lines and publicly available datasets of DLBCL patient expression profiles. High signature score cell lines were almost exclusively ABC, with TOLEDO the only exception (Supplemental Figure 2A). We further applied the method to two datasets (GSE10846 and GSE4732, available in GEO), and found them to be correlated well with ABC and GCB subtypes assigned by study authors (Supplemental Figures 2B, 2C). We also found unclassified DLBCL to be mainly concentrated in the middle. We thus decided to assign an empirical cut-off for samples to ABC if the signature scores were above 0.7, to GCB if below 0, and unclassified if between 0 and 0.7.

Results

Definition of NanoString and RT-PCR gene lists for DLBCL segmentation

A comprehensive list of 307 genes was compiled from two literature-established methodologies: COO and CC (Figure 1). For COO segmentation, multiple predictors have been
described in the literature (4, 13, 19). By combining unique predictors from these studies, a list of 51 distinct genes was obtained (Figure 1A). From four publicly available gene expression profiles of DLBCL patients with ABC and GCB annotations [Lymphoma (3); Lymphoma 2-GSE4732 (20); Lymphoma-GSE4475 (21); Lymphoma-GSE10874 (5)] in Oncomine (22), we selected 126 genes that were differentially expressed in at least two of the four studies (Figure 1B). Combined with the 51 predictor genes, we derived 145 unique genes that were associated with either ABC or GCB subtypes. For CC classification, we relied on the original publication (23), which used 150 probes, representing 133 unique genes (Figure 1C). In order to correct for batch effects and normalize samples analyzed over time in several different test sets, we included in the codeset 33 housekeeping genes whose expressions were moderate to high and showed little variance across datasets (Figure 1D). The final NanoString gene list (codeset) contained 307 unique genes (Supplemental Table 1). To compare NanoString with an established quantitative technique, real-time PCR (RT-PCR) primer-probe sets were created for a 12 gene subset of the most refined version of the COO signature, as previously described (4, 13) (Supplemental Table 1, Supplemental Figure 1B).

Validation of NanoString Codeset for disease segmentation in DLBCL cell lines and clinical samples

We examined nine DLBCL cell lines with literature-established COO designations (24, 25). To confirm that in-house cell lines were representative of those in the literature, we used a previously described (18) RT-PCR method and applied a COO signature score (detailed in Methods); a high scoring sample (>0.7) signified an ABC subtype, low scoring samples (<0) a GCB-subtype, and those in between (>0 to <0.7) as unclassified. Reverse transcription and RT-PCR was performed using the refined 12 gene COO subset on eight DLBCL cell lines and dCt values (mean of triplicate) were scored and ranked. The results confirmed the expected COO designation (Supplemental Figure 2D) and correlated well to NanoString data (Supplemental Figures 2E and 2F). We then assessed the reproducibility of the NanoString platform. Replicates of all nine DLBCL cell lines were used to investigate technical reproducibility (intra- and inter-assay) and correlations were found to be extremely tight, with biological reproducibility only slightly inferior (Figures 2A and B). In addition, agreement between replicates was confirmed using an intraclass correlation coefficient (ICC) pooled over genes from a mixed effects model with gene as a fixed effect and cell line within gene and residual variation as random effects (ICC technical replicates 0.965, ICC biological replicates 0.873). From this data, we concluded that biological replicates should be used in favor of technical replicates whenever possible.

After establishing the reproducibility of the platform and verifying the DLBCL cell line COO designations, cellular RNA was profiled using the complete DLBCL codeset. By examining relative expression levels of 145 COO genes as measured by NanoString, two distinct subgroups of cell lines (ABC and GCB) were clearly identifiable (Figure 2C). The COO signature scores of the nine DLBCL cell lines show near-perfect correlation with each cell line’s designation in the literature and by RT-PCR (Figure 2D). One potential outlier, OCI-Ly19, appeared to have elements of both sub-types according to the 145 gene COO signature score, but has been called GCB by various methodologies in the literature. Culturing our OCI-Ly19 cell line in the presence of FBS rather than human plasma as...
previously described (26) could have resulted in slightly altered gene expression and an affected COO profile. However, publicly available RNA-seq data also clustered OCI-Ly19 between ABC and GCB subtypes, corroborating our findings (27). The DLBCL cell lines were also simultaneously profiled against the 133 genes reported to define the CC algorithm (23). Despite repeated attempts, we were unable to segregate the cell lines into their reported CC bins (BCR, HR and OxPhos) using the 133-gene NanoString expression data. Because early attempts with clinical samples also failed, we decided to abandon additional efforts related to the CC algorithm.

To evaluate NanoString and the DLBCL codeset, we examined a set of commercially-available diagnostic clinical DLBCL samples, which included 14 FFPE tissue blocks (FFPE), 36 RNA samples prepared from fresh DLCBL biopsies (fresh), and 24 flash-frozen excisional DLBCL biopsies (frozen) (Figure 3A). Importantly, these three sample types were almost entirely paired and patient-matched, enabling cross-matrix comparisons. First we profiled the 36 fresh RNA samples and generated COO signature scores on the NanoString platform. These scores showed the expected subtype distribution of a pre-therapy (diagnostic) DLBCL population with 47% GCB, 25% ABC, and 28% unclassified (Figure 3B). Together with the cell line dataset, this gave confidence in the ability of the NanoString platform to identify and classify DLBCL subgroups using fresh RNA. Patient biopsy material is most often collected as FFPE tissue at clinical sites so we proceeded to profile RNA extracted from FFPE tissue of 13 patients alongside matched fresh RNA from the same patients (Figure 3C) and five matched frozen biopsies (Supplemental Figure 3A). The quantitative correlation was shown to be very robust, establishing that NanoString could generate high quality data on FFPE material to allow for clinically relevant DLBCL segmentation. The same sample set was also evaluated using RT-PCR (12 COO signature genes) with strong correlations observed between the two platforms further validating the NanoString results (data not shown). These data demonstrate that tissue preparation and processing does not compromise the gene expression signatures generated from NanoString.

**Correlation between NanoString quantification and alternative platforms using OriGene samples**

There are several existing and emerging methodologies for COO classification of clinical DLBCL tissue (Figure 4A). After validating the NanoString platform as another viable approach, we compared NanoString outputs to other established technologies using the OriGene clinical DLBCL matched sample set. The Hans IHC algorithm, the most widely-used clinical COO segmentation tool, is based on three antibodies (CD10, Bcl-6, Mum1) and uses FFPE diagnostic tissue (10). We profiled the FFPE samples from ten different patients through both the Hans IHC (Phenopath Inc., Seattle, WA) and NanoString platforms and observed a 90% concordance rate of finalized COO signature calls (Supplemental Figure 3B).

Although not often employed for clinical segmentation, Affymetrix GEP is the most well-established methodology for COO subtyping and is an important comparator for NanoString evaluation (28). Thirty-four fresh RNA samples were profiled concurrently across NanoString and
Affymetrix using the full NanoString DLBCL codeset and the U133 plus 2.0 array (Affymetrix, Santa Clara, CA). In contrast to Hans IHC, both Affymetrix and NanoString provide fully quantitative outputs, thus allowing for more robust analyses. The COO score correlations derived from the same 145 COO genes were impressive across the two platforms (Figure 4B), as were the gene expression correlations and COO designations (Supplemental Figures 3A, 3C).

A quantitative nuclease protection assay (qNPA) from HTG Molecular Diagnostics is another COO segmentation methodology that has been described in the literature (12). Because qNPA has already been used to segment DLBCL patients in a clinical trial, we wished to directly compare the two methodologies (13, 29-31). Thirty-one samples (18 fresh, 13 FFPE), representing 26 distinct patients, were profiled through HTG’s COO array consisting of the same 12 COO genes used for the RT-PCR profiling described previously. The quantitative correlations between NanoString and qNPA datasets were very robust and, importantly, tight correlations were observed for both FFPE and fresh RNA samples (Figure 4C, Supplemental Figure 3D).

Any misclassifications between platforms can be explained by the use of different classifiers to create COO scores (12 vs 145 genes). As expected, the correlation is better between matching and larger classifiers (qNPA vs RT-PCR, NanoString vs Affymetrix). In addition, the target RNA sequences used were not identical between platforms, which can lead to different expression patterns that affect the COO designation. Of note, many discordant cases were due to those classifications bordering the cut-off criteria.

**Application of NanoString-based DLBCL segmentation to fostamatinib relapsed-refractory DLBCL phase IIb trial samples**

The NanoString platform and DLBCL codeset were applied to biopsy samples from an ongoing clinical trial of the Syk inhibitor, fostamatinib. The randomized, double-blind phase IIb study, is a 60 patient trial designed to evaluate the efficacy of fostamatinib in patients with relapsed or refractory DLBCL (31). Based on emerging preclinical data, an objective of the study was to explore if DLBCL subtype might predict response to fostamatinib. Flash frozen core needle tumor biopsies were collected from all relapsed/refractory participants prior to fostamatinib dosing, as well as the original diagnostic (FFPE) biopsy. Because pre-dose biopsies would best represent the patient’s tumor biology at the start of fostamatinib therapy (following R-CHOP relapse), RNA was first extracted from the evaluable core needle biopsies (n=59) and subsequently profiled on NanoString to enable COO segmentation. The COO signature scores of these relapsed/refractory DLBCL patients (Figure 5A) displayed a distribution similar to that seen from diagnostic (pre-therapy) biopsies described in the literature (32). The same RNA from 48 patients that passed QC criteria and profiled across the Affymetrix U133 2.0 gene chip displayed strong quantitative and COO score concordance with the NanoString output (Figure 5B). Diagnostic tumor material (FFPE slide or block) was available for a subset of the patients on the fostamatinib study providing the opportunity to explore whether COO designation might evolve during the course of R-CHOP therapy in patient-matched tissue. Eighteen patients provided evaluable diagnostic (FFPE) and fresh pre-fostamatinib biopsy material that were run on the qNPA and NanoString platforms, respectively. An 88% concordance (15/17) was observed.
in the COO calls between the two sample types (Figure 5C), suggesting that COO designation remains stable from initial diagnosis through primary relapse. In addition to COO profiling, specific 5’ and 3’ NanoString probes were designed to detect the presence of BCL2-IGH t(14:18) gene fusions known to occur in DLBCL (33). Using the OCI-Ly8 cell line with known BCL2-IGH fusion as a positive control, we detected putative BCL2 gene fusion events in 25% of fostamatinib DLBCL samples (Figure 6, Supplemental Table 2).

Discussion

The heterogeneous nature of DLBCL has prompted many efforts at disease segmentation to inform prognosis or predict efficacy of specific therapies. The original description of the COO algorithm, indicating that GCB patients showed significantly better overall survival than ABC patients, led to numerous reports aimed at confirming and extending the original findings (3). In that time, several COO profiling methodologies have emerged that have influenced both research and clinical practice. Use of microarrays is well-established at the research level but high quality microarray data requires RNA isolated from frozen tissue. Because the majority of clinical DLBCL diagnostic tissues are FFPE samples, microarray-based COO profiling can be challenging. As a way to enable widespread clinical utility, many IHC-based approaches for COO segmentation have been developed and adopted by clinical practitioners. IHC is attractive as a rapid, cost effective and accessible platform, commonly available in most clinical centers. While IHC algorithms for COO segmentation have shown reasonable concordance with microarray-based approaches (10, 39, 40), the subjective nature of IHC scoring allows for inherent variability. Indeed, recent data have shown that various iterations of nine related IHC algorithms do not correlate well with one another (9). A robust and reliable methodology for COO profiling, applicable to both research and clinical samples, is required to enable the successful discovery and development of targeted therapies for DLBCL.

In this study, we evaluated the NanoString gene expression platform for the molecular classification of DLBCL specimens. The NanoString system generated high quality, reproducible, and fully quantitative results on a range of samples, including cell lines and clinical specimens. Importantly, inclusion of paired sample sets allowed us to demonstrate a strong concordance between patient-matched frozen and FFPE material, showing the applicability of the NanoString platform to the most commonly available type of DLBCL patient tissue. Sample processing and turnaround time were user-friendly; RNA input requirements were minimal and achievable from a single 10 micron clinical section.

Affymetrix microarray profiling is the most well-established COO sub-classification methodology and considered the gold standard. To compare NanoString to Affymetrix, we profiled a set of 34 well-annotated DLBCL clinical samples, with resulting COO scores showing remarkable concordance. We extended this comparative analysis by profiling 48 additional patient samples from the fostamatinib clinical trial across both platforms with similarly concordant COO scores. Any discordant COO designations were due to small changes in the COO score around the cut-offs and
highlights the importance of considering the score in conjunction with the designation to distinguish between clear and marginal GCB/ABC classifications. These data are in close agreement with a recent report using a small 20 gene NanoString codeset (34).

The frequent clinical use of the Hans IHC algorithm prompted us to compare a subset of 10 FFPE samples across both NanoString and IHC. The Hans COO designations showed a 90% concordance with designations from NanoString profiling of 145 genes. While concordance in this small sample set is high, it is important to recognize differences in the data output between these two platforms despite the identical sample input demand (one FFPE section). The Hans approach delivered qualitative expression on just three proteins while NanoString returned fully quantitative data on more than 300 genes. Our cross-platform results comparing NanoString with IHC and Affymetrix are consistent with a recently published report using a smaller DLBCL sample set (35).

NanoString is not the only quantitative, FFPE-applicable technology that has been used for COO segmentation of DLBCL. The qNPA platform uses a 12 gene COO panel and requires similar sample inputs as NanoString without needing RNA extraction (13). To compare qNPA and NanoString directly, 31 clinical samples (frozen and FFPE) were evaluated. COO scores were tightly correlated in both FFPE and frozen tissue and unlike the Affymetrix platform, relative gene expression data also showed strong correlation, even for genes at lower expression level (data not shown). While qNPA data compared favorably to NanoString, the setup of the current qNPA array is limited in its ability to multiplex large numbers of genes. At present, single qNPA arrays are restricted to a maximum of 48 genes while NanoString’s upper limit is 800 and Affymetrix arrays can multiplex thousands. Depending on the application, the ability to profile large numbers of genes may be an advantage of NanoString, especially for exploratory approaches. As COO signatures have already been refined multiple times over the past years, this particular feature may be less important for DLBCL segmentation. To ensure increased coverage of biology, we have chosen to be inclusive with our 145 gene COO NanoString codeset. Scott et al have recently published a 20 gene version of a NanoString codeset for COO segmentation (34) and our analyses of 14 overlapping genes common to both codesets showed highly correlated COO scores (Supplemental Figure 4), suggesting our 145 gene codeset could be further refined.

The robust pilot data and promising comparative findings of NanoString versus other established methodologies gave us the confidence to profile patient samples from an active DLBCL clinical trial. The clinical activity of fostamatinib is being assessed in DLBCL patients who have progressed on R-CHOP therapy in a 60 patient phase IIb study (17). As preclinical data suggested that ABC-type cell lines may be preferentially sensitive to fostamatinib, COO segmentation of patient DLBCL tissue was considered key to a possible patient selection strategy [(25), AstraZeneca unpublished data]. Fresh, pre-dose DLBCL tumor biopsies (core needle) were successfully collected from nearly all patients during pre-screening. These flash-frozen tissues were processed for NanoString and Affymetrix analyses. Fifty-nine samples were successfully profiled on NanoString using the complete 307 gene codeset. The NanoString-derived COO designations of these 59
relapsed/refractory samples showed a 31% ABC and 56% GCB split, numbers comparable to those previously reported for diagnostic (pre-R-CHOP) DLBCL tissue. To our knowledge, these represent the largest COO dataset reported in tumor tissue from second line R-CHOP relapsed DLBCL. With the caveats associated with a sample set of only 59 patients, these data could suggest that R-CHOP treatment may not selectively enrich for ABC-type disease. Additional profiling of relapsed biopsies will be needed to corroborate this hypothesis. Furthermore, by comparing diagnostic tissue (pre-R-CHOP) from 18 patients to patient-matched re-biopsies (post-R-CHOP), we were able to show that nearly 90% maintained a stable COO designation during the course of extended R-CHOP therapy. This observation could have implications for future targeted agents that may intend to selectively treat ABC or GCB-type patients upon relapse to R-CHOP (i.e. ibrutinib). In this setting, patient re-biopsies may be requested. Our data suggest that diagnostic tissue may represent a suitable alternative to an invasive re-biopsy, at least in regards to COO classification.

In addition to expression profiling, the flexibility of the NanoString system allows for detection of disease-relevant gene fusions/translocations. Several groups have reported successful fusion detection using NanoString with 5’ and 3’ probes that span the specific breakpoint of the gene under investigation (36, 37). Using a similar strategy, we designed NanoString probes aimed at detecting BCL2 translocation events within the fostamatinib clinical trial sample set. The BCL2-IGH t (14;18) fusion is known to occur in DLBCL with an incidence of up to 30%; the majority are associated with the GCB subtype (33, 38). We detected putative BCL2 gene fusion events in 25% of fostamatinib DLBCL samples, prevalence similar to that previously reported for BCL2-IGH (Figure 6). All but two of these occurred in a sample with a GCB subtype, again consistent with the literature. The specific BCL2 NanoString assay used to detect BCL2 in the fostamatinib samples was designed to detect only major break points within the 3’UTR that result in truncated BCL2 transcripts. Because the assay was not designed to detect intermediate and minor break points, we cannot rule out the possibility that other BCL2 translocations may have gone undetected. While additional profiling is underway to confirm the specific BCL2 fusion partner and expand the analyses, these observations demonstrate the flexibility of the NanoString platform compared to other methodologies.

Additional NanoString analyses from fostamatinib biopsies, including COO subtype, BCL2 fusion incidence and/or baseline gene expression correlation to clinical response are ongoing and will be reported separately.

The findings from this study indicate that the NanoString system is a robust platform for molecular classification of DLBCL. NanoString offers several advantages to established techniques for COO segmentation, including multiplexing a large number of genes, low RNA input requirements, good reproducibility, complex genomic analysis (fusions) and sample type flexibility (FFPE and frozen) giving access to greater numbers of clinical samples. We have successfully applied NanoString to a large set of relapsed/refractory DLBCL biopsies obtained from an ongoing clinical trial. The resulting data confirmed the robust COO classification outputs from NanoString and demonstrated the promise of the system to generate data not otherwise achievable using other
techniques. The NanoString system should be strongly considered alongside other established approaches for the molecular characterization of DLBCL.

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References

31. Eastern Cooperative Oncology Group ; National Cancer Institute. Biomarkers in Patients With Diffuse Large B-Cell Lymphoma Treated With Combination Chemotherapy With or Without Rituximab. In: ClinicalTrials.gov [Internet]. Bethesda (MD): National
Figure Legends

Figure 1. The process of deriving the 307 gene list for NanoString profiling. Numbers in brackets indicate the number of genes identified from each search; numbers in boxes show the number of unique genes from pooled datasets with overlapping genes omitted. Abbreviations: ABC = Activated B-cell; COO = Cell of Origin; CC = Consensus Clustering; BCR = B-Cell Receptor; HR = Host Response; OxPhos = Oxidative Phosphorylation

Figure 2A. NanoString correlation plot showing technical replicates of six cell line samples. Normalized log2 values of 287 genes are shown. Level of Detection (LOD) was set at mean + 2 standard deviations of the 8 negative spiked-in controls. Pearson correlation is 0.981.

Figure 2B. NanoString correlation plot showing biological replicates of six cell line samples. Normalized log2 values of 287 genes are shown. Level of detection (LOD) was set at mean + 2 standard deviations of the 8 negative spiked-in controls. Pearson correlation is 0.933.

Figure 2C. Heatmap of NanoString relative expression data illustrating unsupervised hierarchical clustering of nine DLBCL cell lines (four ABC and five GCB subtypes): horizontal columns represent individual genes (log2 count of 145 gene codeset), vertical columns individual cell lines. ABC and GCB DLBCL cell lines with known COO designations are clustered together as expected except for OCI-Ly19, which is classified as a GCB subtype but is clustered together with the ABC cell lines.

Figure 2D. Waterfall plot of NanoString data illustrating 145 gene COO scores of nine DLBCL cell lines with literature-associated designations (red = ABC, blue = GCB). Shaded area shows arbitrary scoring cut-off (> 0.7 = ABC, < 0.0 = GCB, 0.0 – 0.7 = unclassified).

Figure 3A. Overview of OriGene clinical DLBCL samples: 14 FFPE blocks (14/14 have matched fresh RNA, 13/14 have matched frozen RNA), 24 frozen tissues (24/24 have matched RNA, 13/24 have matched FFPE blocks) and 36 fresh RNA samples (13/36 have both matched FFPE and frozen).

Figure 3B. Pie chart showing expected spread of DLBCL classifications of 36 fresh RNA samples based on 145 gene COO scores.

Figure 3C. Scatter plot showing NanoString data correlation between freshly prepared RNA and FFPE-derived RNA. Normalized log2 values of 287 genes from 13 OriGene sample pairs are shown in different shades of grey. Pearson correlation is 0.954.

Figure 4A. Comparison of platforms used for COO profiling. FF = Fresh Frozen, FFPE = Formalin-Fixed, Paraffin-Embedded, GEP = Gene Expression Profiling.

Figure 4B. Correlation plot between NanoString and Affymetrix platforms based on 145 gene COO scores calculated from 34 fresh RNA samples. Pearson correlation is 0.993.
Figure 4C. Correlation plot between NanoString and qNPA platforms based on 12 gene COO scores calculated from 31 OriGene samples. Connected lines indicate samples were derived from the same patient. Pearson correlation is 0.956.

Figure 5A. Waterfall plot of NanoString data illustrating 145 gene COO scores and subtype designations of 59 patients. Arbitrary scoring cut-off was set at > 0.7 = ABC, < 0.0 = GCB and 0.0 – 0.7 = unclassified.

Figure 5B. Correlation between NanoString and Affymetrix platforms based on 145 gene COO scores calculated from 48 patients. Pearson correlation is 0.995.

Figure 5C. COO designation is maintained over the course of standard DLBCL therapy. Patients marked in bold show discordant COO designation and patients marked in italics could not be correlated due to sample QC issues. ND = Not Determined.

Figure 6. Identification of potential BCL2 translocation events from 59 patients in fostamatinib phase IIb trial using NanoString technology. Two probes spanning common BCL2 fusion breakpoints were designed to identify patients with potential BCL2 translocation, which typically results in the loss of the BCL2 3' UTR. The scatter plot shows the expression of these two probes, where x-axis is the probe upstream of the breakpoints, and the y-axis is the probe at the 3' UTR downstream of the breakpoints. The solid line is the correlation between these two probes when there is no translocation. The dashed lines indicate 4-fold deviation from the regression line. Each point represents a patient. Patients at the lower right outside the dashed line are potential BCL2 translocation candidates. Cell line OCI-Ly8, which is known to carry the BCL2-IGH translocation, is highlighted. Thirteen out of the 15 BCL2 translocations are classified as GCB subtype, which is consistent with the literature.
A) COO: ABC/GCB Predictors
- Rimsza 2011 (14)
- Blenk 2007 (24)
- Wright 2003 LC (26)
- Wright 2003 Affy (30)

B) COO: ABC/GCB Differentiators From Microarray Profiling
- Alizadeh 2000 (51)
- Dave 2006 GSE4732 (200)
- Hummel 2007 GSE4475 (188)
- Lenz 2008 GSE10874 (200)

C) CC: BCR Consensus Clustering
- Monti 2005 (133; 37 BCR, 50 HR, 46 OxPhos)

D) Housekeeping Genes
- Various (33; selected on expression and low variance in DLBCL)

Figure 1

Total Unique genes: 307
NanoString normalized expression level for Technical replicate 2 (log2 scale)

Color Key
- Green: Detected in both
- Pink: Detected in one or the other
- Yellow: Not detected in both

Figure 2A
Figure 2B
Figure 2D

145 Gene COO score

 OCI-Ly10
 HBL-1
 TMD8
 RCK8
 OCI-Ly19
 OCI-Ly4
 SU-DHL-10
 SU-DHL-4
 Karpas 422

 Subtypes
 ABC / GCB
ABC (9/36 = 25.0%)
GCB (17/36 = 47.2%)
Unclassified (10/36 = 27.8%)

Frozen (24)
Fresh (36)
FFPE (13)

Figure 3A

Figure 3B
<table>
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<th>Vendor</th>
<th>Input</th>
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<th>Preferred sample type</th>
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<td>GEP</td>
<td>FF</td>
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Figure 4B

Color Key:
- ABC
- GCB
- Unclassified
Figure 5A

NanoString 145 gene COO scores

Color Key
- ABC
- GCB
- Unclassified

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Figure 5B

NanoString 145 gene COO scores

Affymetrix 145 gene COO scores

Color Key
- ABC
- Discordant
- GCB
- Unclassified

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Figure 6
Reproducible, quantitative and flexible molecular sub-typing of clinical DLBCL samples using the NanoString nCounter system

Margaret Veldman-Jones, Zhongwu Lai, Mark Wappett, et al.

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