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Direct multiplexed measurement of gene expression with color-coded probe pairs

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We describe a technology, the NanoString nCounter gene expression system, which captures and counts individual mRNA transcripts. Advantages over existing platforms include direct measurement of mRNA expression levels without enzymatic reactions or bias, sensitivity coupled with high multiplex capability, and digital readout. Experiments performed on 509 human genes yielded a replicate correlation coefficient of 0.999, a detection limit between 0.1 fM and 0.5 fM, and a linear dynamic range of over 500-fold. Comparison of the NanoString nCounter gene expression system with microarrays and TaqMan PCR demonstrated that the nCounter system is more sensitive than microarrays and similar in sensitivity to real-time PCR. Finally, a comparison of transcript levels for 21 genes across seven samples measured by the nCounter system and SYBR Green real-time PCR demonstrated similar patterns of gene expression at all transcript levels.

We have developed a technology to capture and count specific nucleic acid molecules in a complex mixture. In principle, the NanoString nCounter system can be used to detect any type of nucleic acid in solution and could be modified with appropriate recognition probes to detect other biological molecules as well. We focus here on mRNA expression profiling. In brief, a multiplexed probe library is made with two sequence-specific probes for each gene of interest. The first probe, a capture probe (**Fig. 1a**), contains a 35- to 50-base sequence complementary to a particular target mRNA plus a short common sequence coupled to an affinity tag such as biotin. The second probe, the reporter probe, contains a second 35- to 50-base sequence complementary to the target mRNA, which is coupled to a color-coded tag that provides the detection signal. The tag consists of a single-stranded DNA molecule, the backbone, annealed to a series of complementary *in vitro* transcribed RNA segments each labeled with a specific fluorophore (**Fig. 1a**). The linear order of these differently colored RNA segments creates a unique code for each gene of interest.

Unique pairs of capture and reporter probes are constructed to detect transcripts for each gene of interest. All probes are mixed together with total RNA in a single hybridization reaction that proceeds in solution. Hybridization results in the formation of tripartite structures, each comprised of a target mRNA bound to its specific reporter and capture probes (**Fig. 1a**). Unhybridized reporter and capture probes are removed by affinity purification, and the remaining complexes are washed across a surface that is coated with the appropriate capture reagent (e.g., streptavidin). After capture on the surface, an applied electric field extends and orients each complex in the solution in the same direction. The complexes are then immobilized in an elongated state (**Fig. 1b**) and imaged (**Fig. 1c**). Each target molecule of interest is identified by the color code generated by the ordered fluorescent segments present on the reporter probe. The level of expression is measured by counting the number of codes for each mRNA.

We show here the linearity, reproducibility and sensitivity of the nCounter system and demonstrate that fold-change measurements of statistically regulated genes ($P \leq 0.05$) correlated closely with microarrays. In addition, we show that the nCounter system can detect low abundance mRNAs that are declared "absent" by DNA microarrays. The validity of this detection was confirmed for a subset of genes using real-time PCR. We suggest that this technology can fill an immediate niche in the expression analysis of hundreds of genes across many samples. Applications include translational medical studies, research involving gene regulatory systems, diagnostic fingerprinting and validation of high-throughput gene expression experiments.

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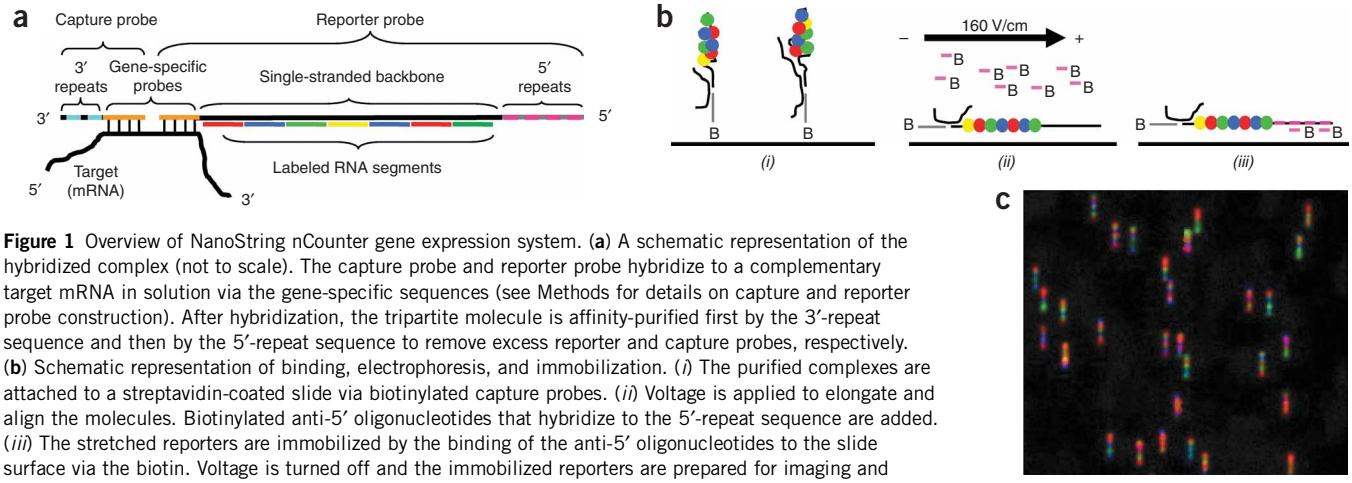

Figure 1 Overview of NanoString nCounter gene expression system. (**a**) A schematic representation of the hybridized complex (not to scale). The capture probe and reporter probe hybridize to a complementary target mRNA in solution via the gene-specific sequences (see Methods for details on capture and reporter probe construction). After hybridization, the tripartite molecule is affinity-purified first by the 3′-repeat sequence and then by the 5′-repeat sequence to remove excess reporter and capture probes, respectively. (**b**) Schematic representation of binding, electrophoresis, and immobilization. (*i*) The purified complexes are attached to a streptavidin-coated slide via biotinylated capture probes. (*ii*) Voltage is applied to elongate and align the molecules. Biotinylated anti-5′ oligonucleotides that hybridize to the 5′-repeat sequence are added. (*iii*) The stretched reporters are immobilized by the binding of the anti-5′ oligonucleotides to the slide surface via the biotin. Voltage is turned off and the immobilized reporters are prepared for imaging and counting. (**c**) False-color image of immobilized reporter probes.

RESULTS

nCounter gene expression system overview

The basis of the nCounter system is the unique code assigned to each gene to be assayed. We used seven positions (visualized as spots) and four colors. The four colors were chosen to minimize spectral overlap during imaging. The number of positions was based on a combination of factors that include the length of the DNA backbone, the minimum spot size that can be resolved under current imaging conditions, flexibility in code selection for modest-sized gene sets (that is, $<1{,}000$ genes) and the number of potential codes for future versions of the system ($4^7 = 16{,}384$ if all possible combinations of codes are used). The total number of codes required was 524 (15 controls and 509 genes) or roughly 3% of the available codes in a seven-spot system.

Specific reporter and capture probes were synthesized in 96-well plates using a semi-automated process (see Methods). Briefly, gene-specific probes were ligated to reporter backbones, and each ligated backbone was annealed to a unique pool of seven dye-coupled RNA segments corresponding to a single code. The reporter probes were then pooled and purified using a common sequence at the end of each backbone (the 5′-repeat sequence; **Fig. 1a**) to remove excess probe oligonucleotides and dye-coupled RNA segments. Capture probes were made by ligating a second sequence-specific oligonucleotide for each gene to a universal sequence containing biotin (**Fig. 1a**). After ligation, the capture probes were also pooled and affinity purified using the universal sequence to remove the excess unligated gene-specific oligonucleotides. Reporter and capture probes were combined into a single 'library' and used as a single reagent in subsequent hybridizations. All reagents will be commercially available in a ready-to-use format.

The expression levels of all selected mRNAs were measured in a single multiplexed hybridization reaction. The sample was combined with the probe library, and hybridization occurred in solution. The tripartite hybridized complexes (**Fig. 1a**) were purified in a two-step procedure using magnetic beads linked to oligonucleotides complementary to universal sequences present on the capture and reporter probes. This dual purification process allowed the hybridization reaction to be driven to completion with a large excess of gene-specific probes, as they were ultimately removed and thus did not interfere with binding and imaging of the sample. All post-hybridization steps were handled on Prep Station, a custom liquid-handling robot.

Purified reactions were deposited by the Prep Station into individual flow cells of a sample cartridge, bound to a streptavidin-coated surface by the capture probe, electrophoresed to elongate the reporter probes and immobilized (**Fig. 1**). After processing, the sample cartridge was transferred to the Digital Analyzer, a fully automated imaging and data collection device. The expression level of a gene was measured by imaging each sample in four colors and counting the number of times the code for that gene was detected. For each sample, 600 fields-of-view (FOV) were imaged ($1{,}376 \times 1{,}024$ pixels), representing $\sim$10 mm^2 of the binding surface.

Image processing and code counting were performed with custom software. A reporter was required to meet stringent criteria concerning the number, size, brightness and spacing of the spots to ensure that the code was interpreted correctly and to minimize false positives. Reporters that did not meet all of these criteria were discarded. Using these criteria, $\sim$20% of the detected molecules were counted. No parity schemes or error correction were used in the current system. Data were output in simple spreadsheet format listing the number of counts per gene per sample.

Experimental design

We performed a series of experiments in which the expression levels of 509 genes were assayed with the nCounter system. We selected 347 of these genes from previous microarray studies of poliovirus-infected A549 cells and the remaining 162 genes were selected from previously designed probes and added to bring the multiplex total to over 500. Additional experiments with other probe libraries were performed with commercially available RNAs and total RNA isolated from developing sea urchin embryos. We compared the nCounter results to those obtained with the Affymetrix GeneChip system and with real-time PCR measuring the same total RNA samples.

NanoString nCounter gene expression system performance

Hybridization reactions were performed in triplicate with total RNA samples isolated from mock- and poliovirus-infected A549 cells. Each reaction contained 100 ng of total RNA plus reporter and capture probes for 509 human mRNAs contained in the RefSeq database[1]. In addition, six pairs of positive-control and two pairs of negative-control reporter and capture probes were included in every reaction. The spike-in controls produce a standard concentration curve for every

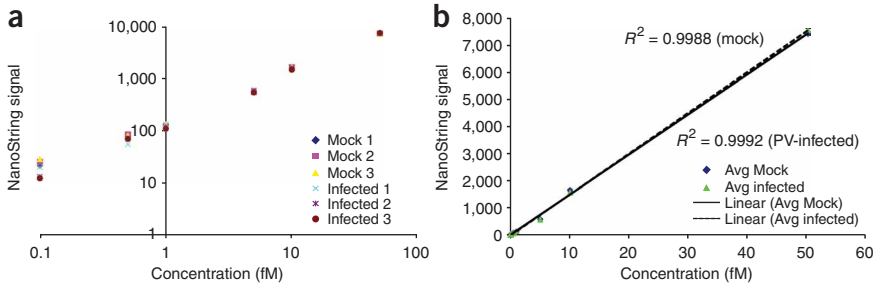

Figure 2 The linearity and reproducibility of the NanoString spike-in controls. Non-human DNA oligonucleotide targets were spiked into each sample at concentrations of 0.1, 0.5, 1, 5, 10 and 50 fM. No target was added for the two negative control probe pairs. (**a**) Signal (counts) on a log scale versus concentration of the spike on a log scale. Each of three replicate measurements for each spike in Mock- and poliovirus-infected RNA is shown. At this scale, the replicate measurements lie essentially on top of each other except at the lowest spike-in concentration. (**b**) Average signal *vs.* concentration on a linear scale for spikes in both mock- and poliovirus-infected samples. The correlation coefficients (R^2 values) of a linear fit to the average signal are 0.9988 and 0.9992 for mock and poliovirus-infected samples respectively. The normalized counts used to construct both graphs are available in **Supplementary Table 2**.

hybridization reaction and were used to normalize the data for slight differences in hybridization, purification and capture efficiencies.

We first examined the linearity, dynamic range and reproducibility of the six positive controls. **Figure 2a** shows the results of the control measurements from each hybridization reaction with RNA from mock- and poliovirus-infected cells ($n = 6$). The control signal values (counts) for each replicate were very reproducible between 0.5 fM and 50 fM as indicated by overlapping points on the log-log plot. The assay was also highly linear over 2.5 logs of concentration with linear regression correlation coefficients of counts versus concentration at $\geq$0.998 (**Fig. 2b**).

We then examined the sampling efficiency and the lower limit of detection. The sampling efficiency of the system can be estimated by dividing the number of counts for a spike-in target by the theoretical number of molecules of that target in the reaction. For example, there were a total of ~1,800 molecules of the 0.1 fM spike-in target in each reaction. The average measurement for this target in the mock sample was 25 counts, resulting in a sampling efficiency of ~1%. The limit of detection of the assay was determined by comparing the counts for the positive control at the lowest concentration to the counts of the negative controls using a Student's *t*-test. The lowest concentration of controls detected in the context of the 500-plex hybridization reaction was between 0.1 fM and 0.5 fM in a total volume of 30 µl containing 100 ng of total RNA. Background signal for the two negative controls averaged 14.4 ± 6.5 and 10.2 ± 3.5 for the mock and poliovirus-infected cells, respectively. Assuming 10 pg of total RNA per cell (that is, 10,000 cells in 100 ng), the limit of detection corresponds to between 0.2 to 1 molecule of control target per cell.

The reproducibility of the nCounter system in measuring the 509 mRNAs was also examined. The normalized counts for all 509 genes from two independent hybridizations of RNA from poliovirus-infected cells (technical replicates) are shown on a log-log scale (**Fig. 3a**). The data demonstrate that the nCounter system is reproducible: a linear fit to the data results in a correlation coefficient of 0.9999. The average correlation coefficient of each pair-wise combination of replicate assays is 0.9995 ± 0.0004 (**Supplementary Table 1** online). This is slightly higher than that obtained from the same analysis of genes on the DNA microarray (average correlation coefficient = 0.9934 ± 0.0059). In addition, endogenous genes were detected with signals ranging from ~25 counts to >50,000 counts, which suggests that the dynamic range of the system is larger than the 2.5 logs tested with the positive spike-in controls (**Fig. 3a**).

An important feature of any gene expression technology is determining the relative difference in gene expression between two or more samples. We measured change in expression levels for the 509 genes in the reporter library between mock- and poliovirus-infected cells (**Fig. 3b**). Using cutoff criteria of a twofold change in expression with $P \leq 0.05$, there were 28 genes that were induced and 115 genes that were repressed by poliovirus infection as indicated by the red and green lines in **Figure 3b**. These results demonstrate the nCounter system can be used to measure gene expression of >500 genes in a single assay and identify those genes that change significantly between samples.

Comparisons between nCounter and microarrays

We compared the ability of the nCounter system to detect and measure the level of endogenous transcripts against microarrays; we chose the widely used Affymetrix GeneChip system as a representative microarray platform. As described above, nCounter assays were performed directly on 100 ng of total RNA without amplification. The same samples and amount of RNA were also analyzed with Affymetrix U133Plus2 arrays, using the two-cycle amplification and labeling protocol recommended by the manufacturer.

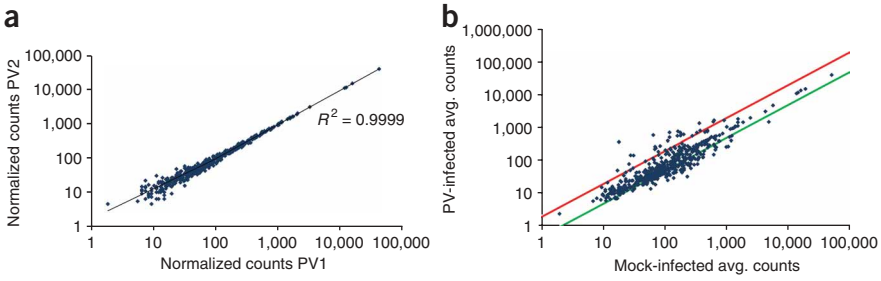

Figure 3 Reproducibility and differential gene expression plots for 509 genes on the NanoString nCounter platform. (**a**) Scatter plot of normalized signal for all 509 genes assayed is shown in log scale for technical replicates. Genes were not filtered based on detection. The R^2 value of a linear fit to this data are 0.9999 +/− 0.0002. The R^2 value for all pairwise comparisons of technical replicates for both NanoString and Affymetrix systems are shown in **Supplementary Table 1**. (**b**) Scatter plot of mock-infected versus poliovirus-infected counts for 509 genes. The normalized average counts for the triplicate assays are shown. The red and green lines represent twofold increase and decrease in expression levels, respectively. All 509 data points are shown without filtering.

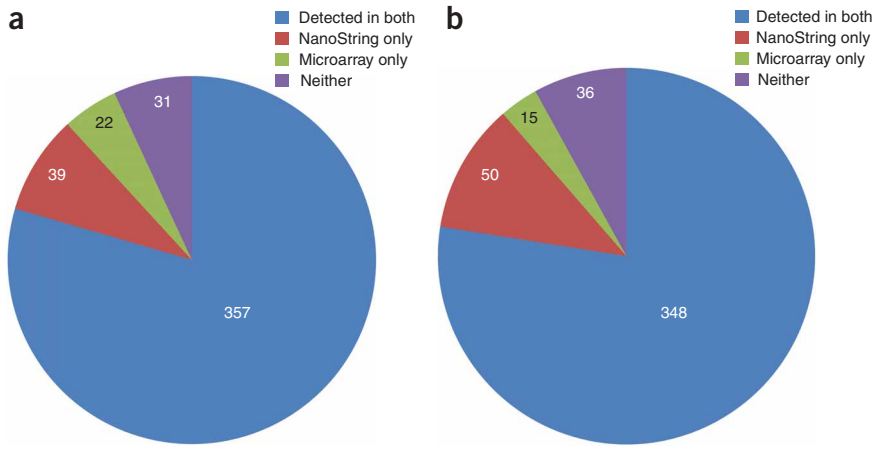

Figure 4 Comparison of detected/undetected calls for the NanoString and Affymetrix assays. A set of 449 RefSeq mRNAs that had corresponding Affymetrix probe sets was used in this analysis. (**a**,**b**) Mock-infected (**a**) and poliovirus-infected samples (**b**). For the NanoString assay, a gene was considered detected if the average normalized signal for the three replicates was significantly above that of the negative controls ($P \leq 0.05$). For Affymetrix assay, a gene was considered detected if any one of the three replicates was called "Present" or "Marginal" based on MAS 5.0 analysis.

To determine how the nCounter system compares in sensitivity to microarrays, we examined the number of genes detected in each platform. Of the 509 genes assayed, there were 60 for which there was no acceptable corresponding Affymetrix Probe ID (based on ref. 2, Supplementary Table 2). For the remaining 449 genes, we examined how many were called detected by each platform. The nCounter system uses a Student's *t*-test of the replicate values for each gene compared to two negative controls ($n = 6$) to determine the presence or absence of each gene, whereas the Affymetrix MAS 5.0 algorithm is based on the relationship between the Perfect Match and Mismatch probe sets. The average percentage of detected transcripts in both samples was higher in the NanoString assay than in the DNA microarray assay (88.4% versus 82.6%; **Fig. 4a**,**b**, respectively), and the boundary between detected and undetected calls was more distinct (**Supplementary Table 2** online). The accuracy of the NanoString detection calls for several genes was further validated in TaqMan assays (**Table 1**).

The correlation of fold-change measurements for genes that change significantly in both the NanoString and Affymetrix platforms was assessed. After normalization and preprocessing of data (see Methods), the mean $\log_2$ fold-change between poliovirus-infected and mock-infected samples was calculated for both platforms. A Student's *t*-test for differential expression was performed between the samples. A threshold *P*-value of 0.05 without multiple testing correction was used to identify significantly regulated genes. This analysis resulted in four classes of genes: those that are determined to be regulated by both platforms (202 genes), by NanoString only (55 genes) or by microarray only (78 genes), and those that are not found to be regulated by either platform (114 genes). A plot of $\log_2$ ratios for all 449 genes with their significance in each platform is given in **Supplementary Figure 1** online. **Figure 5a** shows a comparison of $\log_2$ ratios for the 202 genes that were found to be significantly regulated in both the NanoString and microarray assays. The two platforms agree well for these 202 genes; only four were found to be regulated in opposite directions (blue diamonds in the upper left and lower right quadrants of **Fig. 5a**). The correlation coefficient of a linear fit to $\log_2$ ratios between the assays was 0.788. This correlation coefficient is similar to previous results comparing different array

Table 1 Comparison of signal levels and detected/undetected calls for 14 genes on the nCounter, GeneChip and TaqMan platforms

				NanoString			Affymetrix			TaqMan		
				Mock	PV	Mock/PV	Mock	PV	Mock/PV	Mock	PV	Mock/PV
Accession#	Gene Name	Affymetrix Probeset ID	TaqMan ID	signal		Detection	signal		Detection	Ct		Detection
NM_005570	*LMAN1*	203293_s_at	Hs00194366_m1	669 (63)	224 (10)	D/D	61 (11)	45 (9)	D/U	25.5 (0.17)	27.2 (0.11)	D/D
NM_020726	*NLN*	225943_at	Hs00252959_m1	428 (78)	164 (10)	D/D	545 (54)	443 (47)	D/D	25.7 (0.03)	27.4 (0.09)	D/D
NM_015884	*MBTPS2*	206473_at	Hs00210639_m1	347 (37)	111 (9)	D/D	48 (3)	48 (8)	U/U	26.7 (0.04)	28.5 (0.09)	D/D
NM_002895	*RBL1*	1555004_a_at	Hs00161234_m1	270 (39)	108 (7)	D/D	62 (8)	51 (3)	D/D	27.4 (0.05)	28.8 (0.10)	D/D
NM_006219	*PIK3CB*	217620_s_at	Hs00178872_m1	204 (31)	73 (9)	D/D	23 (0)	24 (3)	U/U	28.0 (0.07)	29.5 (0.15)	D/D
NM_016436	*PHF20*	209423_s_at	Hs00363134_m1	195 (19)	70 (8)	D/D	47 (4)	54 (9)	U/U	27.9 (0.02)	28.8 (0.05)	D/D
NM_014484	*MOCS3*	206141_at	Hs00819330_s1	183 (6)	83 (9)	D/D	42 (2)	40 (2)	D/U	28.5 (0.15)	29.1 (0.30)	D/D
NM_025209	*EPC1*	223875_s_at	Hs00228677_m1	111 (22)	57 (6)	D/D	30 (1)	31 (2)	U/U	27.2 (0.07)	28.9 (0.07)	D/D
NM_018094	*GSPT2*	205541_s_at	Hs00250696_s1	100 (43)	76 (3)	D/D	214 (12)	102 (18)	D/D	30.3 (0.22)	30.7 (0.04)	D/D
NM_006420	*ARFGEF2*	215931_s_at	Hs00197455_m1	77 (2)	29 (1)	D/D	42 (5)	47 (6)	U/U	26.6 (0.12)	28.3 (0.23)	D/D
NM_007211	*RASSF8*	207754_at	Hs00200537_m1	62 (13)	31 (7)	D/D	37 (2)	37 (2)	D/U	27.3 (0.05)	28.5 (0.09)	D/D
NM_020800	*IFT80*	226098_at	Hs00398803_m1	41 (6)	29 (5)	D/D	321 (15)	123 (22)	D/D	29.0 (0.06)	29.8 (0.41)	D/D
NM_015139	*SLC35D1*	209713_s_at	Hs00209446_m1	38 (1)	20 (3)	D/D	42 (7)	43 (0)	U/U	27.8 (0.03)	29.1 (0.16)	D/D
NM_153034	*ZNF488*	229901_at	Hs00399237_m1	31 (8)	13 (4)	D/U	114 (7)	92 (15)	D/D	29.1 (0.09)	30.0 (0.80)	D/D

A set of 14 genes were tested on all three platforms. They are listed by RefSeq Accession numbers, Probeset ID and TaqMan ID. Signal levels for both samples in all three platforms are shown with s.d. in parentheses. Values shown correspond to normalized counts for the nCounter system, RMA normalized intensity for Affymetrix's GeneChip, and cycle threshold (Ct) for ABI TaqMan assay. Detected (D) and Undetected (U) calls are based on platform-specific criteria. For the Affymetrix platform a gene was only considered undetected if all three replicates for each sample were called "Absent" by the MAS 5 algorithm. All genes were detected by the TaqMan assay based on a cutoff of less than 35 cycles. Fold-change comparisons are shown in **Figure 5b**. PV, poliovirus.

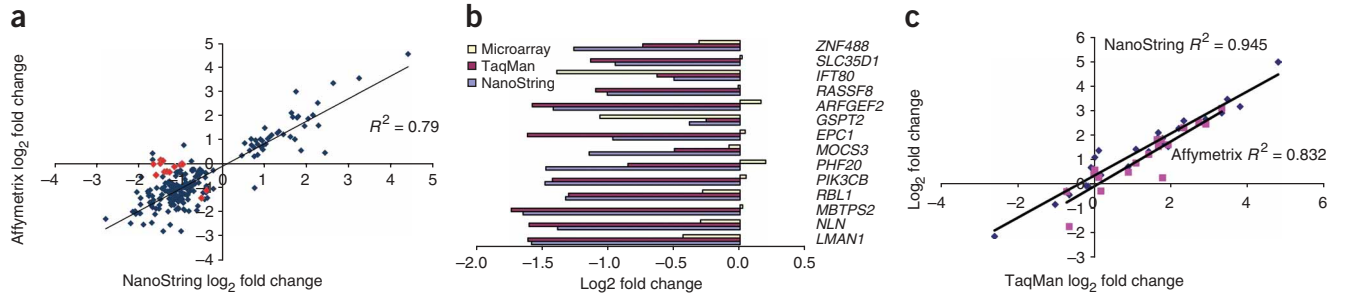

Figure 5 Comparison plots of NanoString nCounter to Affymetrix GeneChip and Applied BioSystems TaqMan platforms. (**a**) Log_2 (poliovirus-infected/mock-infected) ratios as measured by NanoString assay (*x*-axis) and Affymetrix arrays (*y*-axis). Genes were considered differentially regulated for $P \leq 0.05$ in a Student's *t*-test performed on replicate data ($n = 3$). Affymetrix ratios were based on RMA normalized data. A linear fit to the ratios that are deemed statistically significant in both assays (blue ♦) yields a correlation coefficient of 0.79. Genes were not filtered based on the magnitude of fold-change or the detected/undetected calls for this analysis. A set of 14 genes whose expression levels were discordant between the two platforms and were selected for real-time PCR analysis are also shown (red ♦). Genes were selected based on criteria outlined in Methods. (**b**) The discordant 14 genes shown in **a** were analyzed by TaqMan real-time PCR performed in triplicate on 100ng of the same mock and poliovirus-infected samples. The bar graph shows log_2 ratios (poliovirus-infected/mock-infected) for the NanoString (■), TaqMan (■) and Affymetrix (■) platforms in triplicate. The root mean square deviation of log_2 ratios between NanoString to TaqMan was 0.34, DNA microarray to TaqMan was 1.20. (**c**) A library of probe pairs to 35 RefSeq mRNAs that overlapped with the published MAQC consortium study was hybridized to commercially available reference RNAs. Data were filtered to remove genes that were not detected in all samples (see Methods). The Affymetrix data shown here were downloaded from the MAQC study and represents data from a single site (site 1, Affymetrix). TaqMan real-time PCR data were obtained at Applied Biosystems Inc. The R^2 values for 27 NanoString genes (♦) and 18 Affymetrix genes (■) that met the selection criteria were 0.95 and 0.83, respectively. The overall correlation of Affymetrix data for 469 genes (site 1) in the original study was 0.92.

platforms, as well as comparisons with other quantitative measurement technologies such as real-time PCR[2–4], suggesting the results can be extrapolated to other microarray platforms.

TaqMan analysis of selected genes

As mentioned above, there were a number a genes in which the measured log_2 fold-change was significant in one platform but not in the other. We selected a subset of 14 of these genes (see Methods) for further analysis by TaqMan real-time PCR. Selection criteria are described in Methods and the genes are indicated in **Figure 5a**. Twelve genes were determined to be differentially expressed by the NanoString assay and two by the microarray assay. TaqMan real-time PCR was performed using RNA from the same master stock of mock- and poliovirus-infected samples, and log_2 fold-changes were calculated. Overall, the NanoString assay shows much higher concordance with the TaqMan assay than does the DNA microarray assay (**Fig. 5b**). Nine of the 12 genes met the same fold-change criteria by real-time PCR and the other three showed similar trends but had slightly higher *P*-value (*ZNF488*) or missed the twofold cutoff criteria (*MOCS3* and *PHF20*). In contrast, neither of the two genes determined to be regulated by the Affymetrix GeneChip system alone (*GSPT2* and *IFT80*) were validated by the TaqMan assay.

Using the same set of 14 genes, we also compared the sensitivity of each platform by its ability to detect each gene in the two samples (**Table 1**). All 14 genes were detected in both samples by real-time PCR in less than 35 cycles. The results were similar for the nCounter system, with 13 of the 14 samples being detected in both samples and one gene (*ZNF488*) detected in mock-infected but not the poliovirus-infected sample. In contrast, six genes were declared absent in both samples by microarrays and another three genes were declared absent in poliovirus-infected cells. Hence, in these experiments the sensitivity of the nCounter system is superior to that of microarrays and similar to that of real-time PCR.

Comparison of nCounter system with MAQC data set

Recently a series of studies performed by members of the microarray quality control (MAQC) consortium used commercially available reference RNA samples to compare the performance of different microarray platforms[2,4], as well as several quantitative gene expression technologies[5], using TaqMan real-time PCR as the benchmark technology.

An nCounter probe library was constructed that was specific for 35 RefSeq mRNAs that overlapped with the MAQC gene set. The library was hybridized to human reference RNA and human brain reference RNA samples used by the MAQC consortium to determine log_2 fold-change values. After eliminating genes declared absent in either sample by either the nCounter or the TaqMan data[2], we compared the log_2 fold-change values for the remaining 27 genes. There was excellent correlation between the NanoString and TaqMan platforms ($R^2 = 0.945$; **Fig. 5c**). A similar analysis of Affymetrix microarray data (site 1, Affymetrix[2]) from the same study revealed a significantly lower correlation of $R^2 = 0.832$ for the 18 genes that met the same criteria (**Fig. 5c**).

Comparison of nCounter system and SYBR Green real-time PCR

To further demonstrate the sensitivity, accuracy and dynamic range of the nCounter system, we compared it to real-time PCR in a different biological system. Total RNA was isolated from sea urchin embryos at seven time points of development (from egg to 70 h) and either analyzed directly with the nCounter system or converted into cDNA and analyzed by real-time PCR. The transcript levels of 21 genes were examined at each time point. For the nCounter assay, all genes were combined in one library and analyzed in a multiplexed reaction. Each hybridization was performed in triplicate on 100 ng of total RNA (21 assays). For real-time PCR each gene was assayed individually in quadruplicate for each time point from 2.8 ng of starting material (588 assays). For both assays the data were normalized to ubiquitin[6].

A correlation in the relative expression patterns was observed between nCounter and real-time PCR data across the time course for all 21 genes (**Supplementary Fig. 2** online). The correlation was consistent for genes that are expressed at both low (e.g., *Snail*, *Pmar 1*) and high (e.g., *Est*, *Dri*) transcript levels per embryo as well as those whose expression levels changed over 3 logs during the time course

(e.g., *Tgif*, *Msp130*). These results confirm that the nCoutner system produced real-time PCR quality data without enzymatic or signal amplification.

DISCUSSION

We have developed a gene expression analysis system that is sensitive (0.1–0.5 fM detection limit), reproducible (replicates averaging R^2 of 0.999 over a 3-log dynamic range) and simple to use. We have demonstrated that the nCounter system is capable of a high degree of multiplexing, measuring over 500 genes in a single reaction starting with just 100 ng of total RNA sample. The overall performance of the nCounter gene expression system correlated well with both microarrays ($R^2 = 0.79$ over 202 genes) and real-time PCR ($R^2 = 0.95$ in MAQC gene set) in head-to-head comparisons with the same total RNA samples. In addition, our data indicate that the nCounter gene expression system is more sensitive than microarrays and similar in sensitivity and accuracy to real-time PCR (**Table 1**).

The nCounter system has advantages over the major existing gene expression technologies. First, the sample RNA is measured directly without amplification or cloning. Thus, no gene-specific or 3′ biases are introduced, and the levels of each transcript within a sample can be established by counting the number of molecules of each sequence type and calculating concentration with reference to internal standards. In contrast, in real-time PCR transcript concentration is calculated from the number of enzymatic steps required to attain a threshold level of product. Second, both the probe and target are in solution rather than bound to a surface. The reaction is driven to completion (data not shown), allowing for a higher level of sensitivity than in microarrays across many target genes with lower amounts of starting material. Third, nCounter provides a digital readout of the amount of transcript in a sample. A pure digital readout of transcript counts is linear across a large dynamic range, exhibits less background noise and is less ambiguous for downstream analysis than technologies that use analog signals. Finally, the time, effort and sample requirements of the nCounter system are more scalable than real-time PCR or microarrays. For example, to measure 500 genes using 2 ng of RNA per real-time PCR reaction in triplicate, one would need 3 μg of total RNA and 1,500 reactions whereas the same experiment could be performed using the nCounter system with 300 ng of total RNA in three reactions.

There are many applications for this technology. For example, estimates of mRNA expression levels in both mouse and human cells suggest that the vast majority of the genes in the transcriptome are expressed at or below 20 transcripts per cell[7,8]. Currently, real-time PCR is the most widely accepted platform for measuring low-abundance messages. We have shown the nCounter system yields remarkable similar results. Another potential application of the technology is to measure expression profiles in clinical settings. Several studies have used expression arrays to identify a set of genes whose expression pattern or 'signature' can serve as a clinical diagnostic or prognostic indicator. Classic examples of such studies include the AML/ALL work[9] and breast cancer classification studies[10,11]. After identifying a set of predictive genes by full genome arrays, one would like to validate their expression profile on a large number of patients and ultimately develop a diagnostic assay (see ref. 12 for a recent review). Typically these clinical signatures involve >30, but <500, genes. The nCounter system is ideally suited for profiling such clinically relevant signatures, particularly from small samples with limited amounts of RNA such as tissue biopsies, micro-dissected or laser-captured samples, and cells sorted by flow cytometry.

METHODS

Cell culture, infection and RNA isolation. A549 cells, a human lung epithelial cell line, were purchased from ATCC. Poliovirus (PV) stocks were the kind gift of Kurt Gustin's laboratory. Sub-confluent A549 cells were either mock-infected or infected with poliovirus at a multiplicity of infection of 50. Virus was adsorbed for 30 min at 32 °C in PBS supplemented with 10 mM $MgCl_2$ and 10 mM $CaCl_2$. After adsorption, residual virus was removed and DMEM with 10% FBS, 2mM L-glutamine and penicillin-streptomycin was added. After 5 h of infection, the total RNA was extracted using Qiagen RNeasy mini-spin columns, according to the manufacturer's protocols. Two independent mock- and poliovirus-infections were performed. After RNA isolation, the RNA from the replicates was pooled to create one sample of RNA from poliovirus-infected cells and another from mock-infected cells. Aliquots of these two RNAs were used in all subsequent microarray, real-time PCR and nCounter analyses.

Control target preparation. Targets for spike-in controls consist of 100-base high-performance liquid chromatography (HPLC)-purified oligonucleotides that are complementary to the spike-in reporter and capture probes. These and all other oligonucleotides were purchased from Integrated DNA Technologies. They were generated to specific 100-base regions of the following non-human sequences and arbitrarily named A-H (spikes A, E and F, (accession number AY058658.1), spikes B-D (accession number AY058560.1), and spikes G and H (accession number DQ412624)).

Generation of fluorescent RNA segments. To prepare the RNA segments for reporter probe synthesis, PCR fragments for each segment were generated using primers specific to M13 and containing either T7, T3 or SP6 RNA polymerase promoters. RNA transcripts were *in vitro* transcribed from these templates using the Megascript kit (Ambion) in the presence of 50% amino-allyl UTP (Sigma). Each of the seven resulting amino-allyl labeled RNA transcripts was coupled to one of 4 NHS-ester fluorophores (Alexa 488, Alexa 594, Alexa 647 (Invitrogen) or Cy3 (GE Healthcare)).

NanoString reporter preparation. NanoString reporters consist of linearized single-stranded M13 DNA, referred to as backbone, annealed to fluorescently labeled, *in vitro* transcribed RNA segments. Using standard molecular biology protocols, circular single-stranded M13 (United States Biological) was linearized, and an oligonucleotide containing four 15-base repeats, referred to as the 5′-repeat, was ligated on to the 5′-end of the backbone. Using a Hamilton STAR liquid-handling robot, a master mix containing a universal oligonucleotide that serves as a ligation 'bridge' plus ligase buffer was added to individual wells of 96-well plates containing normalized (10 μM) gene-specific oligonucleotide probes (35–50 bases). After a short incubation at 37 °C to anneal the probe oligonucleotide to the complementary portion of the bridge oligonucleotide, ligation was initialized by addition of another master mix containing the equivalent of 1.2 pmoles of M13 backbone per well, additional ligation buffer and T4 ligase. Plates were incubated at 37 °C in a 96-well thermocycler for 2 h. The efficiency of the ligation reactions was assessed by cutting the backbone ~600 bases away from the ligation site using short oligonucleotides to generate double-stranded restriction sites and analyzing the size of the resulting fragments by PAGE. Ligation reactions were desalted by centrifugation through G-50 Sephadex columns in a 96-well format.

Each gene-specific backbone was assigned a unique code consisting of an ordered series of differently colored RNA segments annealed to the backbone. Sets of seven ~900-base fluorescently labeled RNA transcripts complementary to distinct sequences on the backbone were created in 96-well plates using a Hamilton STAR robot. Each well received a unique combination of RNA segments that, when annealed to the M13 backbone and visualized in linear sequence, resulted in a unique code. Plates containing RNA segment pools were mixed with probe-ligated M13 backbones in a 2:1 molar ratio. Annealing of segments to the backbone was performed in individual wells of a 96-well PCR plate. At the same time, one unlabeled RNA segment was also annealed to each reporter to cover the remaining single-stranded region of the backbone, leaving only the probe at one end and the 5′-repeat at the other as single-stranded DNA. The rest of the reporter is a double-stranded DNA/RNA hybrid. To remove excess RNA transcripts and unligated probes, the reporters were then pooled and affinity-purified over magnetic beads (Dynal, Invitrogen) coupled

to oligonucleotides complementary to the 5′-repeat sequence on the 5′ end of each backbone. The final reporter molecules had seven labeled regions in a linear sequence each of which resulted in a ~300 nm spot when imaged by an epi-fluorescent microscope under the conditions described below.

NanoString capture probe preparation. The capture probe consisted of a 35- to 50-base gene-specific sequence attached to a capture-oligonucleotide comprised of two 15-base repeats, referred to as 3′-repeats, linked to a biotin molecule. In a process similar to reporter probe synthesis, normalized gene-specific oliogonucleotides were annealed to a short universal bridge oligonucleotide in ligation buffer. A master mix containing the 3′-repeat oligonucleotide, additional ligation buffer and T4 ligase was added. The 3′-repeat oligonucleotide was present in fourfold excess. Ligation reactions were performed in 96-well plates in a thermocycler for 2 h at 37 °C. The efficiency of each ligation was assessed by PAGE. After ligation there are three potential species of molecules in the reaction: the 3′-repeat ligated to the gene-specific probe (the "capture probe" in **Fig. 1**), the excess unligated 3′-repeat, and any residual unligated probe oligonucleotide if the reaction did not go to completion. Excess free probe is the only species that negatively affects the hybridization results as it competes for target with the fully ligated capture probe. Therefore, after ligation the capture probes were pooled and purified over magnetic beads coupled to an oligonucleotide complementary to the 3′-repeat to remove free probe oligonucleotide. A later post-hybridization purification step removed excess unligated 3′-repeat oligonucleotide (see the anti-5′-repeat post-hybridization purification, below).

Probe design and selection. Potential pairs of 50-base probes were chosen by first screening 100-base target regions of the mRNA to eliminate long direct and inverted repeats, high GC content and long poly-C stretches (due to the difficulty in synthesizing poly-G sequences in probe oligonucleotides). The refined list of target regions was then screened for cross-hybridization using NCBI BLAST[13] (version 2.2.14) to align them against the Human RefSeq mRNA database[1] (Hs: release 17). These 100-base target BLAST alignments were used to filter out targets that resulted in either 50-base probe having >85% identity or stretches >15 contiguous bases complementary to any non-target mRNA. The cross-hybridization cutoffs were chosen based on prior 50-base hybridization and probe design studies[14,15]. Probes were then screened for inter- and intra-reporter and capture probe interactions and selected for probe pairs with calculated melting temperatures (T_m) between 78–83 °C, with an ideal target of 80.5 °C. In the last stage of selection, probes that met all requirements but had a calculated T_m > 83 °C were dynamically trimmed until the T_m was calculated to be ≤83 °C with a minimum-length cutoff of 35 bases. Final probe-pair selection was based on a score calculated from cross-hybridization and T_m screens, with preference given to probes which did not need to be trimmed to meet T_m requirements.

NanoString reporter gene libraries. The reporter library for the A549 cell study contained probes to 509 human genes. The majority of these genes (347) were selected based on previous microarray studies on poliovirus-infected A549 cells (R.E.B., unpublished data) using the Limma package in Bioconductor[16] to identify genes with a false detection rate of less than 0.05. The remaining 162 genes were collected from a variety of other studies; they have no particular biological relevance to the poliovirus study, but were added to evaluate the ability of the nCounter assay to multiplex more than 500 genes. The list of 509 RefSeq mRNAs was based on the current human genome organization (HUGO) gene name associated with the list of Affymetrix probe set IDs. Note that not all of the target regions for the Affymetrix probe sets overlap completely with the RefSeq mRNAs. The reporter library for the MAQC-consortium study contained probes to 35 human genes that were selected based on the RefSeq gene list published in the MAQC consortium study[2]. The list of RefSeq mRNAs and summary data for both libraries can be found in **Supplementary Tables 2** (509 gene library) and **3** online (MAQC library). The probe library for the *Strongylocentrotus purpuratus* study contained probes to 55 *S. purpuratus* genes including polyubiquitin, which was used for normalization purposes, and seven probes to *Homo sapiens* genes, which were used as the negative controls. The analysis described in this paper only includes the 21 *S. purpuratus* genes for which there were comparable real-time PCR data available. The list of *S. purpuratus* genes and summary data used in the analysis are available in **Supplementary Table 4** online. All libraries described also contained eight non-human control probe pairs (spike-ins) and multiple control reporters that did not contain gene-specific probes, but were used to assess purification and binding efficiencies.

Hybridization reactions. Detection of cellular transcripts was carried out in multiplexed hybridization reactions. Each sample was hybridized in triplicate with final concentrations of the hybridization reagents as follows: 200 pM each capture probe, 40 pM each reporter probe, 5× SSPE (pH 7.5), 5× Denhardt's reagent (Sigma), 100 ng/μl sheared salmon sperm DNA (Sigma) and 0.1% Tween-20. Each 30 μl hybridization reaction also contained 100 ng total RNA at a final concentration of 3.3 ng/μl. In addition, six positive and two negative-control probe-pairs to non-human sequences were added to each reaction. Final concentrations of the 100-base control targets were 50 fM spike A target, 10 fM spike B target, 5 fM spike C target, 1 fM spike D target, 0.5 fM spike E target and 0.1 fM spike F target. No target was added for spikes G and H (negative controls). Reagents were mixed and incubated at 65 °C in a thermocycler block with a heated lid for 20 h.

Post-hybridization purification. All post-hybridization steps are handled robotically on a custom liquid-handling robot (Prep Station, NanoString Technologies). The Prep Station can process 12 samples in 2.5 h for a total of 48 assays per instrument in 10 h. To remove unhybridized reporters, reactions were purified over magnetic beads (Invitrogen) coupled to oligonucleotides complementary to the 3′-repeat sequence contained on every capture probe. Reactions were first diluted to 1× SSPE in 0.1% Tween-20/TE and allowed to bind to beads at 22.5 °C for 30 min with continuous rotation. The beads were washed three times in 150 μl of 0.1× SSPE/0.1% Tween-20 and the hybridized complexes eluted in 100 μl of 0.1× SSPE/0.1% Tween-20 for 15 min at 45 °C. After elution, samples were purified a second time to remove excess capture probes by binding to magnetic beads coupled to oligonucleotides complementary to the 5′-repeat sequence contained on every reporter probe. The elutions from the anti-3′-repeat beads were brought to a final concentration of 1× SSPE by addition of 50 μl of 3× SSPE/0.1% Tween-20 and bound for 15 min at 22.5 °C with rotation. Beads were washed as above and eluted in 30 μl of 0.1× SSPE/0.1% Tween-20 at 45 °C. The doubly purified samples were then prepared for capture as described below.

NanoString reporter capture, stretching and imaging. One microliter of 1:5,000 dilution of a 0.1% solids solution of a custom-formulation of Tetraspeck fluorescent microspheres (Invitrogen) was added to each sample. Samples were loaded into a NanoString fluidic device made by lamination of laser-machined cast acrylic with a coverslip coated with streptavidin (Optichem, Accelr8 Technology Corporation) using a laser-cut double-sided adhesive layer (Fralock) to generate 30-μm deep microfluidic channels. The samples were driven through the channel by hydrostatic pressure and bound specifically by the biotinylated 3′-end of the capture probe. After capture, the surface was washed once with 90 μl of 1× TAE and prepared for stretching by the addition of 40 μl of TAE to each well. Reporter probes were stretched and aligned by applying 160 V/cm for 1 min along the fluidic channel. Stretched reporters were then immobilized to the surface by addition of 60 μl of a 500 nM solution of a biotinylated oligonucleotide complementary to the 5′-repeats present on the 5′-end of all reporter probes. The current remained on for 5 min, throughout the immobilization process. After immobilization, the TAE solution was removed and replaced with a custom formulation of the anti-photobleaching reagent SlowFade (Invitrogen) for imaging.

Slides were imaged on a Nikon Eclipse TE2000E equipped with Perfect Focus, a 1.4 NA Plan Apo VC 60X oil-immersion lens (Nikon), anX-cite 120 metal halide light source (Exfo Corporation), an automated H117 stage (Prior Scientific) and aSmartShutter (Sutter Instrument). Typical imaging density is 100-200 counted reporters per field of view depending on the degree of multiplexing, the amount of RNA and overall gene expression levels. However, the system is capable of operating at densities five- to tenfold higher. The Digital Analyzer (NanoString Technologies) can accommodate up to six cartridges at once and current scan times for 600 FOV are 4 h per sample cartridge. Unattended, it can process 72 samples in 24-h per instrument. For

each field of view, four images at different excitation wavelengths (480, 545, 580 and 622) were acquired with an Orca Ag CCD camera (Hamamatsu) under control of either Metamorph (Universal Imaging Corporation) or custom software.

Image processing. Image processing is performed on four images (one for each wavelength) on a FOV-by-FOV basis. The custom algorithm treats each FOV as a fundamental block in which the following basic steps are performed: (i) spot identification, (ii) image registration, (iii) spatial clustering to produce strings and (iv) string classification.

In the first step of the algorithm, spots are identified. The background intensity level of each channel is computed and used to threshold the image into signal and background, where signal regions are the result of a specific wavelength of light observed as a point spread function (PSF). The signal mask is segmented using a custom Watershed algorithm. The segmented regions are then labeled, parameterized and filtered to remove non-PSF spots. The remaining spots are centrally archived for use in registration and reporter calling.

Image registration is performed on each FOV based on archived spots that correspond to fluorescent beads (fiducials) that are bound to the imaging surface (see NanoString reporter capture, stretching and imaging). The archived spots are crossreferenced to identify interchannel clusters of spots that meet fiducial requirements (interchannel intensity thresholds and ratios). Clusters that meet requirements are archived as fiducials. The final list of fiducials represents the spatial transforms that occurred between channels during image acquisition. Spatial offsets can be as large as 5–6 pixels. The spatial transform is solved for using the observed fiducial centroids and their pretransform (assumed) coincident centroids ($X_2 = X_1 * T$). The inverse transform is then applied to all identified spots to restore their original centroids.

After spot identification and image registration spots are assembled into "strings" via clustering. At this point, each string is filtered to remove any spots attributed to bleed-though signal. The filtered strings are then classified as reporters or non-reporters. To be classified as a reporter, the string must contain the correct number of spots, meet specific spot-to-spot spacing thresholds (1.2–2.9 pixels) and meet acceptable linearity and orientation requirements. Clusters that are classified as reporters are then counted and summed for each gene over all FOVs.

NanoString data normalization and analysis. To account for slight differences in hybridization and purification efficiency, data were normalized to the average counts for all control spikes in each sample. To determine if a gene was "detected" by the nCounter system, the triplicate measurements obtained for each experimental gene were compared to triplicate measurements for the two negative controls. For a gene to be categorized as detected, the average counts for the experimental gene had to be greater than the average counts for the two negative controls, and the Student's *t*-test P-value had to be <0.05. For the *S. purpuratus* study, the data were normalized to the polyubiquitin gene and detected genes were determined by a Student's *t*-test against the seven human negatives.

Production of Affymetrix array data. Aliquots of the same RNA samples analyzed by the NanoString nCounter system were also analyzed by microarray. In brief, triplicate samples of 100 ng of total RNA were provided to the University of Washington's Center for Array Technologies for analysis on Human U133 Plus 2 arrays. Since 1–2 μg of total RNA is typically required for the standard Affymetrix single amplification protocol, the RNA expression data were produced following the manufacturer's standard protocol using the GeneChip Two-Cycle Target Labeling kit (Affymetrix). Hybridization, washing and staining were carried out using the manufacturer's standard protocols. Data were normalized using RMA. Affymetrix "presence/absence" calls were obtained by independently processing the data with MAS 5.0 algorithm. The array and NanoString data have been made public via the Array Express database (E-MEXP-1072)[17]. For data in **Figure 4**, an Affymetrix probe set was declared detected if any one of the three replicates was called "present" or "marginal."

TaqMan real-time PCR data. Genes which showed discordant levels of expression between the NanoString and microarray systems were selected based on the following criteria: (i) genes had to be significantly differentially expressed in one platform (greater than twofold, $P < 0.05$) and not in the other platform (less than 1.5-fold, $P > 0.05$); (ii) both the Affymetrix and NanoString probe sets had to map to the same RefSeq mRNA; and (iii) an inventoried ABI TaqMan probe set had to be available. The product numbers for the TaqMan assays are listed in **Table 1**. All TaqMan assays were performed by University of Washington Gene Sequencing and Analysis Center. For each sample, 4 μg of total RNA was reverse-transcribed using random hexamers in a final volume of 40 μl. The reactions were diluted to 200 μl in TE and then 5 μl, equivalent to 100 ng of total RNA, was used in each real-time PCR reaction. All assays were performed in triplicate. The data were normalized to Beta-glucuronidase (*GUS*).

MAQC comparisons. A library of 35 RefSeq mRNAs that were also listed in the MAQC TaqMan real-time PCR data set[2] was used to analyze differential gene expression between the two commercially available reference RNAs, Human Reference total RNA (Stratagene) and Human Brain Reference total RNA (Ambion). As described in the original study[2], genes that were not detected in all samples for both the NanoString and TaqMan platforms were removed from further analysis. *STAT5A* was removed from the NanoString data due to a known cross-hybridization issue with *STAT5B*. Fold-change correlation of NanoString results with MAQC Taqman real-time PCR data for the remaining 27 genes was determined by plotting the $\log_2$ ratio of normalized signal values (Human Reference RNA versus Human Brain Reference RNA) and calculating the linear correlation coefficient for that plot.

SYBR Green real-time PCR methods. *S. purpuratus* total RNA isolation, cDNA synthesis and real-time PCR was carried out as described[6,18]. Twenty-one *S. purpuratus* genes were assayed by quantitative real-time PCR. The primers used for PCR are available at http://sugp.caltech.edu/resources/methods/q-pcr.php. All genes were assayed in quadruplicate.

Note: Supplementary information is available on the Nature Biotechnology website.

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AUTHOR CONTRIBUTIONS

G.K.G. designed the experiments, analyzed data and prepared the manuscript. D.L.D., S.F., P.J.W. and G.K.G. designed and developed the NanoString system described. T.D. and J.D.M. developed probe selection and image analysis software, respectively. N.D. performed all nCounter assays described. B.B., R.D.G., T.G., J.J.J., M.M., J.L.O. and A.L.R. provided ideas and technical support. T.P. and P.O. provided microarray and real-time PCR data and samples. R.E.B. contributed to experimental design, scientific direction and manuscript preparation. H.P.F., E.H.D. and L.H. provided scientific direction and experimental concepts. K.D. provided the ideas and design of this technology and participated in the initial research.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturebiotechnology/.

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1. Pruitt, K.D., Tatusova, T. & Maglott, D.R. NCBI Reference Sequence (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. *Nucleic Acids Res.* **33**, D501–D504 (2005).
2. Shi, L. *et al.* The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. *Nat. Biotechnol.* **24**, 1151–1161 (2006).
3. Kuo, W.P. *et al.* A sequence-oriented comparison of gene expression measurements across different hybridization-based technologies. *Nat. Biotechnol.* **24**, 832–840 (2006).
4. Patterson, T.A. *et al.* Performance comparison of one-color and two-color platforms within the MicroArray Quality Control (MAQC) project. *Nat. Biotechnol.* **24**, 1140–1150 (2006).

5. Canales, R.D. *et al.* Evaluation of DNA microarray results with quantitative gene expression platforms. *Nat. Biotechnol.* **24**, 1115–1122 (2006).
6. Oliveri, P., Carrick, D.M. & Davidson, E.H. A regulatory gene network that directs micromere specification in the sea urchin embryo. *Dev. Biol.* **246**, 209–228 (2002).
7. Hastie, N.D. & Bishop, J.O. The expression of three abundance classes of messenger RNA in mouse tissues. *Cell* **9**, 761–774 (1976).
8. Velculescu, V.E. *et al.* Analysis of human transcriptomes. *Nat. Genet.* **23**, 387–388 (1999).
9. Golub, T.R. *et al.* Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science* **286**, 531–537 (1999).
10. van't Veer, L.J. *et al.* Gene expression profiling predicts clinical outcome of breast cancer. *Nature* **415**, 530–536 (2002).
11. van de Vijver, M.J. *et al.* A gene-expression signature as a predictor of survival in breast cancer. *N. Engl. J. Med.* **347**, 1999–2009 (2002).
12. Simon, R. Roadmap for developing and validating therapeutically relevant genomic classifiers. *J. Clin. Oncol.* **23**, 7332–7341 (2005).
13. Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410 (1990).
14. Kane, M.D. *et al.* Assessment of the sensitivity and specificity of oligonucleotide (50mer) microarrays. *Nucleic Acids Res.* **28**, 4552–4557 (2000).
15. Li, X., He, Z. & Zhou, J. Selection of optimal oligonucleotide probes for microarrays using multiple criteria, global alignment and parameter estimation. *Nucleic Acids Res.* **33**, 6114–6123 (2005).
16. Gentleman, R.C. *et al.* Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol.* **5**, R80 (2004).
17. Brazma, A. *et al.* ArrayExpress–a public repository for microarray gene expression data at the EBI. *Nucleic Acids Res.* **31**, 68–71 (2003).
18. Rast, J.P. *et al.* Recovery of developmentally defined gene sets from high-density cDNA macroarrays. *Dev. Biol.* **228**, 270–286 (2000).

Corrigendum: Direct multiplexed measurement of gene expression with color-coded probe pairs

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