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At the Single Cell Level Cellular Noise Changes Normalization

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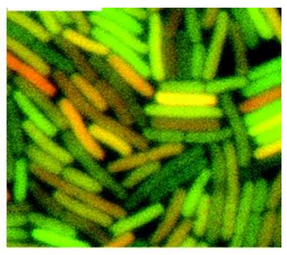
ABSTRACT -

To look at the effect of noise on gene expression in a large number of genes we used the Cell Sensor AP-1-*bla* ME-180 cell line as a model system. We treated cells with epidermal growth factor to stimulate the RAS and MAPK pathways and chose target genes predicted to be up- and down-regulated. In order to analyze gene expression profiles from a large number of individual cells we used the Single Cell-to-CT kit. This kit includes optimized reagents for sample preparation, reverse transcription, preamplification and qPCR. We compared normalized expression levels of a number of target genes (normalized to a variety of control genes), with non-normalized expression levels. We then compared this data to samples of cells prepared *en masse*. We found that for samples prepared *en masse*, gene expression levels varied from sample to sample. However, expression levels of all genes changed equally between samples. This allowed sample to sample differences to be eliminated when target genes were normalized to control genes. When analyzing single cells we also found that expression levels of genes varied from cell to cell, however, in contrast to cells analyzed *en masse*, expression of all genes did not change equally from cell to cell. This caused the normalized expression profiles to vary widely from cell to cell, and change depending on which genes were used to normalize.

INTRODUCTION -

As expected, biological systems such as tissues exhibit considerable phenotypic variation from cell to cell. Surprisingly, cellular variation even occurs in “clonal” populations. Until now this heterogeneity has been attributed to differences in cell cycle, microenvironments, or cell type. These differences would be expected to affect genes within a cell in a similar manner, i.e. cells at the same point in the cell cycle would have the same gene profile. However, it has now been shown that there are also differences within identical biological processes and this has been referred to as “noise” (1).



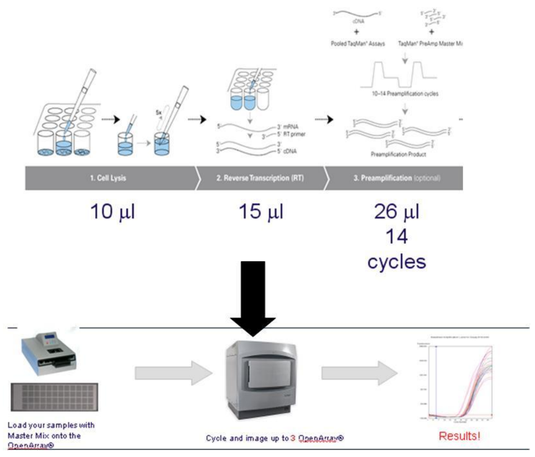
These differences likely result from stochastic events within a cell due to the limited numbers of components such as RNA polymerases. For example, cells with CFP and YFP under identical promoters give different levels of total fluorescence from cell to cell, however, vastly different levels of each protein within each cell are also seen.

MATERIALS AND METHODS -

Me-180-AP1-bla cells were cultured in assay media overnight at 37C. Cells were kept unstained or stained with CentriRed® stain (live cell permeable stain) alone or in combination with Ethidium Homodimer-A (a viability stain). Cell sorting was done using a FACSAria I Flow Cytometer, gated by side scatter vs forward scatter (P1), then side scatter-height vs side scatter-weight (P2) and finally forward scatter-height vs forward scatter-weight (P3) to eliminate possible cell doublets. Cells were then sorted on the basis of CentriRed alone or in combination with EthHD-A fluorescence. Cells were lysed using a TaqMan® Gene Expression Single Cell-to-CT, a kit to perform lysis in 10 µl, Reverse Transcription in 15 µl and PreAmp in 26 µl reaction volumes. Preamplification was done for 14 cylces. Samples with 56 Assays on Demand were then run on an Open Array using GeneFast Taqman PCR mixture.

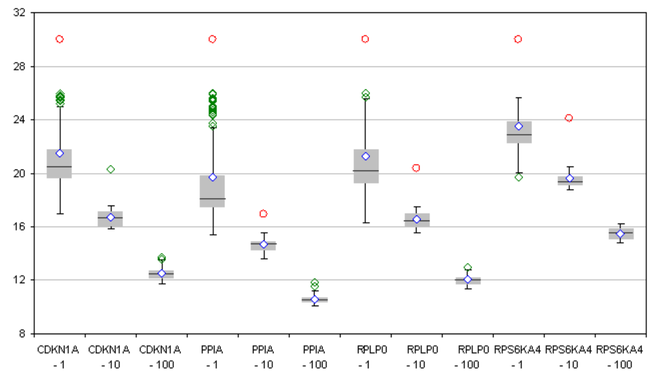
RESULTS -

Figure 1. Single Cell analysis and Open Array workflow



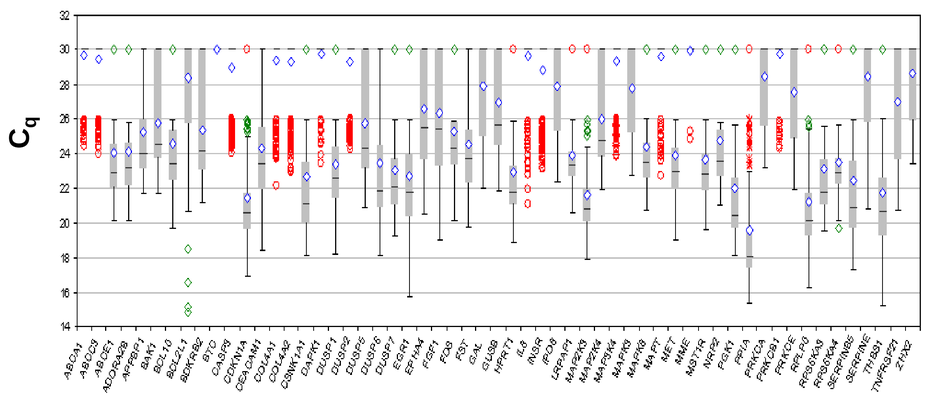
After selecting cells by FACS, cells were lysed, reverse transcribed and preamplified. 960 samples with 56 genes were run on the Open Array.

Figure 2. qPCR from single, 10 and 100 cell samples



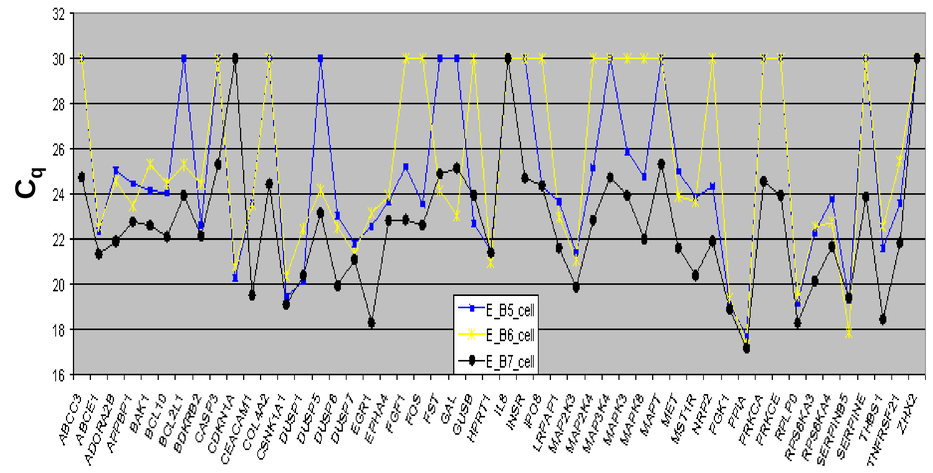
Single cell samples show a large range in expression levels. 360 single cell samples, 20 ten cell samples and 20 one hundred cell samples are shown for 4 control genes. Differences for each 10 fold change in cell number range from 3.9 to 5.0 Cts. This is higher than the expected 3.6 Cts, however, is likely due to the bias of non-expressers (set at 30).

Figure 3. Large variation in expression level in single cells



A large range in expression level is seen in each gene from 360 single cells, and the spread is not equal for each gene. For some genes, such as IL8, cells exist which express at a high level even though the majority of cells do not express at all. Samples which did not express any of the genes were removed (60 out of 420). Blue diamond represents the mean, grey box is the center quartile (50%), whiskers represent 1.5 quartiles outside of the center quartile, and green diamonds and red circles represent outliers outside of 1.5 and 3.0 quartiles respectively.

Figure 4. Three cells shows different expression patterns

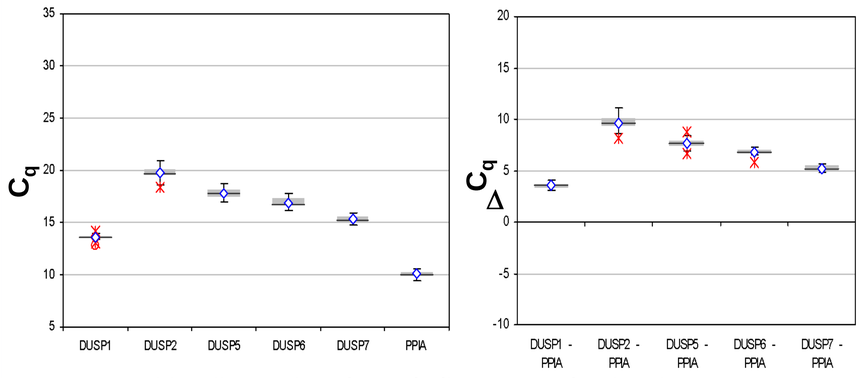


Three cells were randomly selected to demonstrate different expression patterns between cells. Expression levels for some genes such as DUSP7, PGK1 and PPIA are similar in all three cells, however other genes such as BCL2L1 vary widely in each cell. Also notable is that even though cell B7 appears to have a higher expression level for most genes, this is not the case for genes like CDKN1A. Inversely, cell B6 appears to express most gene at a lower level, however, this cell expresses some genes, such as SerpinB5, at a very high level.

REFERENCES -

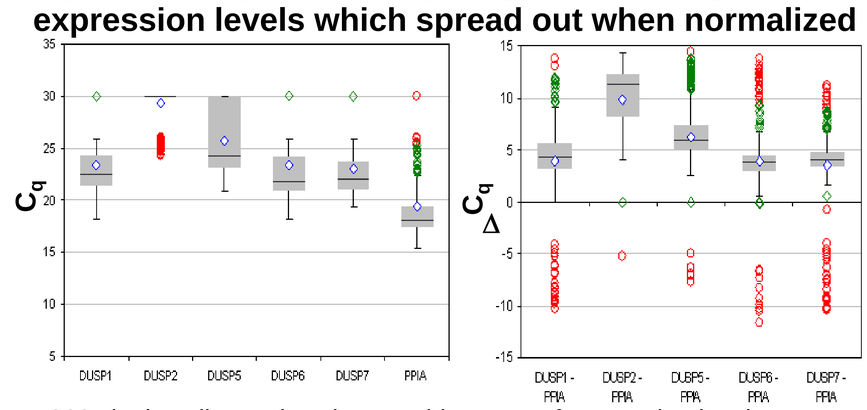
1. Elowitz, et al., Stochastic Gene Expression in a Single Cell. 2002. Science, **297**:5584,1183-6.

Figure 5. 100 cell samples have similar expression levels which tighten when normalized



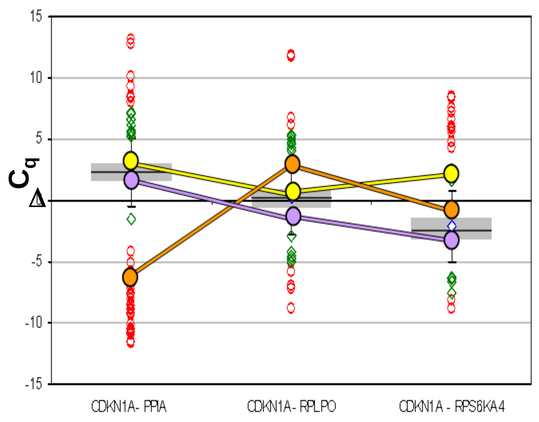
Twenty 100 cell samples show similar expression levels as demonstrated by small center quartiles (left). Normalized expression levels of the DUSP genes to PPIA remove some of the sample to sample variability as shown by smaller box and whiskers (right) and show that the gene “profiles” of each sample are very similar.

Figure 6. Single cell samples give wide range of expression levels which spread out when normalized



360 single cell samples show a wide range of expression levels shown by the large box and whiskers (left). After normalization (right), box and whisker sizes do not significantly decrease in size, and the number of outliers (green and red) dramatically increase.

Figure 7. Normalizing skews gene relationships and profiles



For 360 single cells, CDKN1A was normalized two endogenous control genes (PPIA and RPLP0) and a randomly chosen gene (RPS6KA4). The ΔCts were then plotted in order to see the large variation in relative expression levels (as in Figure 6). 3 cells were then selected (yellow orange and purple circles) and their plotted values show that they do not have similar ΔCt patterns.

CONCLUSIONS -

- This simplified workflow allows a large number of single cells and genes to be analyzed simultaneously (Figure 1)
- Average expression patterns of single cell populations are similar to 10 and 100 cells demonstrating the sensitivity and accuracy of this protocol (Figure 2)
- Single cells have a large range of gene expression levels (Figure 3)
- Expression levels of each gene vary independently within a single cell (Figure 4) and may be a result of microenvironments and noise
- When analyzed *en masse*, expression levels vary from sample to sample, however, these differences are reduced when results are normalized to control genes (Figure 5).
- In single cells normalization actually increases the spread of calculated expression levels due to differences in the amount of each gene within the same cell (Figure 6)
- Similarly, these normalized values are not the same within each cell and vary depending on the genes compared. This gives different “profiles” for every pair of genes in each cell (Figure 7)
- Together, these results suggest that normalizing actually complicates analysis and gives biased relationships between genes. This suggests that for single cells normalizing is not an accurate method of analysis.