Is Z' factor the best assessment for the quality of cellular assays delivering higher content?

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Abstract
As high throughput imaging systems, analysis algorithms and associated cellular assays have begun to be used in profiling and secondary analysis, scientists have used tools derived from primary screening such as Z' factor to assess assay quality. Image-based cellular assays may be better suited to lead profiling, where the needs are different and modified criteria should be applied in assessing assay quality. We ask the question what statistical metrics most accurately reflect the nature and use of cell-based assays in lead profiling? We present data from a variety of such assays we have developed, such as GFP translocation assays, including simple Z' factor analysis and propose multiple statistical approaches to assess assay quality that may be better suited to high information content assays.

Introduction
We have developed a number of live-cell translocation assays that are compatible with high-throughput micro-imaging platforms such as the IN Cell Analyzer 3000 and the IN Cell Analyzer 1000. To assess assay quality during assay development and for subsequent QA, we initially used the well known measure of assay performance, Z' factor. While Z' factor is of value in assessing the size of the reading window for a screening assay, we observed that in some cases Z' factor analysis obscured meaningful differences in assay performance. Consequently, we have explored the use of signal-to-noise as a statistical metric more appropriate to the needs of image-based cellular assays.

Results
A common characteristic of cell-based assays (in contrast to many *in vitro* assays) is that the statistical variation associated with control and treated sample populations is often significantly different (Figure 1). This phenomenon may be both cell type- and target-dependent. The causes may be manifold, but inherent cell population heterogeneity, particularly with respect to cell cycle postion, and methods of image analysis have been noted as potential contributory factors.

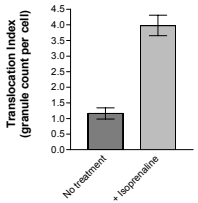


Figure 1: Magnitude of standard deviation (SD) associated with negative and positive controls is not always equal. *Data are from a CypHer™ assay for agonist-induced activation and internalization of the β2-Adrenergic Receptor.*

Assay performance assessments based on a signal-to-noise (S:N) method that does not take into account the standard deviation of both control and treated samples will be prone to error. For example, a signal-to-noise metric still cited in screening literature for assessment of *in vitro* assays involves dividing the magnitude of assay response by the standard deviation of the control (untreated) sample:

Method A

$$S:N = \frac{|\mu_p - \mu_n|}{\sigma_n}$$

where
μ = mean signal
σ = standard deviation
p = positive control
n = negative control

We used this S:N calculation to assess assay performance of eight replicate plates imaged and analyzed using the IN Cell Analyzer 1000. The S:N values obtained using this method varied greatly (Figure 2, S:N, method A).

By contrast, S:N values were much more consistent between replicate plates (Figure 2, method B) when we used an alternative method for S:N calculation that takes into account variation of both the control and responding sample populations:

Method B

$$S:N = \frac{|\mu_p - \mu_n|}{\sqrt{\sigma_p^{\,2} + \sigma_n^{\,2}}}$$

where
μ = mean signal
σ = standard deviation
p = positive control
n = negative control

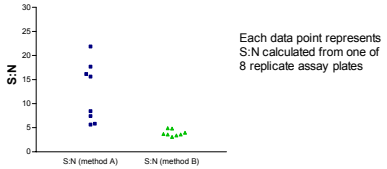
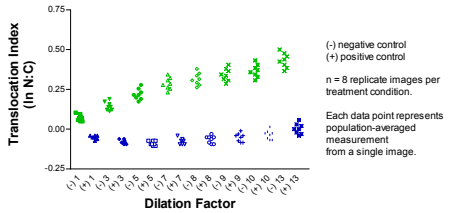


Figure 2: *Variation of assay performance as assessed by different methods of S:N calculation. Data are from a live-cell GFP-MAPKAP-k2 translocation assay (code 25-8008-82) performed on the IN Cell Analyzer 1000.*

During assay optimization, S:N (method B) can be a more sensitive indicator of assay performance than the Z' factor. This is demonstrated by the data shown in Figure 3, where Z' factor and S:N values of the same assay performance data are compared.

A



B

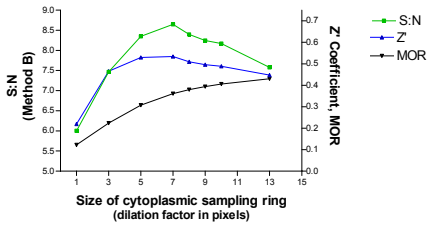


Figure 3: Affect of varying a primary analysis parameter (cytoplasmic sampling ring dilation factor) on the assay window of an antagonist-format GFP-MAPKAP-k2 assay. ***A.*** *Increasing the dilation factor increases the assay magnitude of response (MOR) but also leads to an increase in noise associated with both control and treated samples.* ***B.*** *Comparison of S:N and Z' factor values from the same data set as in A. S:N analysis indicates that a dilation factor of 7 provides the largest assay window, while Z' factor analysis of the same dataset fails to distinguish adequately between dilation factors of 5 and 7.*

Since both S:N (method B) and Z' factor are based on the same variables, there is a defined relationship between the two metrics, assuming for simplicity that standard deviation of the positive and negative controls are equal:

$$Z' = 1 - \frac{3\sigma_p + 3\sigma_n}{|\mu_p - \mu_n|} \qquad Z' = 1 - \frac{4.24}{S:N}$$

where
S:N = signal to noise (method B)
μ = mean signal
σ = standard deviation
p = positive control
n = negative control

Although the assumption that standard deviations of positive and negative controls are equal is not always true, we have observed that actual data correspond fairly well to this model relationship. A plot of the relationship between S:N (method B) and Z' factor is shown in Figure 4 together with the actual data from an EGFP-NFATc1 translocation assay we have developed. The actual data show a very good correlation to the theoretical curve.

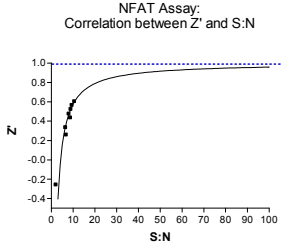


Figure 4: *Correlation between Z' factor and S:N. The points are actual values from a performance trial of the EGFP-NFATc1 assay (code 25-8010-42) read on the IN Cell Analyzer 3000. The curve is the theoretical curve for the predicted relationship.*

As the results in Figure 5 demonstrate, this relationship holds true for a variety of cell-based assays, including FYVE, AKT-1, PLCδ-PH, Rac-1, MAPKAP-k2, SMAD2 and NFATc1 (all GFP-based assays performed in live cells).

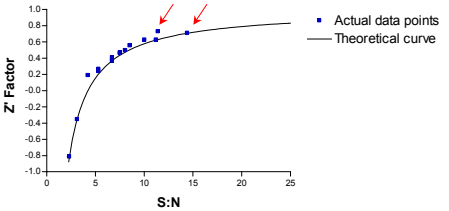


Figure 5: *Correlation between Z' factor and S:N. The points are actual values from several GFP translocation assays read on the IN Cell Analyzer 3000. The curve is the theoretical curve for the predicted relationship. Arrows show two assays whose performance is indistinguishable by Z' factor, but distinctly different as assessed by S:N.*

It can be seen from Figures 4 and 5 that, while Z' factor may be a sensitive indicator of assay performance at the lower end of the performance scale (i.e. Z' < 0.5, S:N < 8), S:N may be a more sensitive indicator at the higher end of the performance scale. As S:N increases, Z' factor approaches 1 asymptotically, making it an increasingly less sensitive measure of performance improvement. For example, the arrows in Figure 5 indicate two assays whose performance could not confidently be distinguished on the basis of Z' factor, but which have distinctly different S:N values. Both metrics of assay performance (Z' factor and S:N) therefore may be useful in optimizing assay performance.

Ref: Zhang et al J. Biomol. Screening 4, 67-73, 1999.

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CONCLUSION

High information-content image-based cellular assays have properties distinct from more traditional screening assays, and their evaluation may therefore require adoption of additional statistical metrics.

S:N calculations that take into account standard deviation of both positive and negative controls are less prone to error than those derived from the standard deviation of negative controls only.

Both S:N and Z' factor may provide valuable information during assay and image analysis optimization.

The theoretical relationship between Z' factor and S:N (method B) has been shown to be well supported by the results of several cell-based assays performed using IN Cell Analyzer 3000 and IN Cell Analyzer 1000.

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