Appendix B: Assessing the Statistical Significance of Shifts in Cell EC_{50} Data with Varying Coefficients of Variation

B.1 Introduction

Because of the high levels of variability in cell EC_{50} (cEC_{50}) data observed in M_2AChR experiments, we wondered what magnitude of shifts in mean cEC_{50} between wild-type and mutant experiments could be interpreted as significant. This appendix describes our attempt to address this concern. We reformatted the student's *t*-test in terms of cell-to-cell variability and mean cEC_{50} shift magnitude. With this equation, we then assessed our ability to differentiate cEC_{50} data sets with high levels of variability.

B.2 Methodology

B.2.1 Definitions and Assumptions

We first assumed that the sets of cEC_{50} data being analyzed do not have significant batch-to-batch variability in mean or standard deviation. Because the purpose of this exercise was to assess how increasing cell-to-cell variability affects our ability to differentiate shifts in mean cEC_{50}, we wished to avoid considering data that fluctuates in multiple ways. Also, we assumed that both wild-type and mutant data sets have the same level of variability. Through our past work with LGICs and our observations of GPCR

data, we have observed that the variations of cEC_{50} data for wild-type and mutant conditions are often similar (as examples see Figure 3.12 and Figure 3.20).

The student's *t*-test assumes that the data sets being compared are both normally distributed. We have found that cEC_{50} data are not normally distributed, but fit a log-normal distribution. A data set, X_i, is said to be log-normally distributed if the transformation of the data set, $\ln X_i$, is normally distributed. For example, the Shapiro-Wilk test for normality rejects the conventional wild-type M_2AChR data set as being normal ($W = 0.91$ and $p = 0.001$), but fails to reject the ln transformed data ($W = 0.96$ and $p = 0.1$). (Some data sets, such as the 10.10.0 W7.40F3Trp data set, fit both normal and log-normal distributions.) Type I error (false positive) rates—the probability of concluding two data sets are different statistically when, in reality, they are not—deviate above the standard $\alpha = 0.05$ level for log-normally distributed data sets that have unequal variances in the ln transformed data set. But if ln transformed data sets have equal variances, type I error rates return to acceptable levels[1]. We assume that because our hypothetical data sets have equal variation, they will also have equal variation when transformed into $\ln X_i$ data. A rigorous proof of this assumption has not been performed.

We have chosen to define the cell-to-cell variability of a given data set of cEC_{50} values as the ratio of the population standard deviation to the population mean, or the coefficient of variation (CV). The CV allowed us to compare the variability of different data sets with substantially different means and, thus, different magnitudes of absolute standard deviation. In our derivation, we expressed differences in mean cEC_{50} between mutant and wild-type data sets as a z-fold shift, where the mutant mean cEC_{50} is z times

the wild-type mean cEC_{50}. What follows is the derivation of the independent two-sample *t*-test in terms of CV, z, and the number of wild-type and mutant cells.

B.2.2 Derivation

Let $\mu_{mut} = \frac{\sum_i^n x_i}{n}$ be the mean cEC_{50} for a mutant cell population, where x_i is the cEC_{50} from mutant cell, i, and n is the number of mutant cells.

Let $\mu_{wt} = \frac{\sum_i^n y_i}{m}$ be the mean cEC_{50} for a wild-type cell population, where y_i is the cEC_{50} from wild-type cell, i, and m is the number of wild-type cells.

Let s_{mut} be the standard deviation of cEC_{50}s for a mutant cell population.

Let s_{wt} be the standard deviation of cEC_{50}s for a wild-type cell population.

$CV = \frac{s_{mut}}{\mu_{mut}} = \frac{s_{wt}}{\mu_{wt}}$ is the coefficient of variance for both mutant and wild-type cell populations.

$s_{mut}^2 = (s_{mut})^2$; $s_{wt}^2 = (s_{wt})^2$ are the variances for the mutant and wild-type cell populations, respectively.

$df = n + m - 2$ is the degrees of freedom.

For independent two-sample *t*-tests,

$t = \frac{\mu_{mut} - \mu_{wt}}{s_{\mu_{mut} - \mu_{wt}}}$, where $s_{\mu_{mut} - \mu_{wt}} = \sqrt{\left(\frac{(n-1)s_{mut}^2 + (m-1)s_{wt}^2}{n+m-2}\right)\left(\frac{1}{n} + \frac{1}{m}\right)}$.

By substituting $s_{mut}^2 = (s_{mut})^2 = (\mu_{mut}CV)^2$ and $s_{wt}^2 = (s_{wt})^2 = (\mu_{wt}CV)^2$,

$$t = \frac{\mu_{mut}-\mu_{wt}}{\sqrt{\left(\frac{(n-1)(\mu_{mut}CV)^2+(m-1)(\mu_{wt}CV)^2}{n+m-2}\right)\left(\frac{1}{n}+\frac{1}{m}\right)}} = \frac{\mu_{mut}-\mu_{wt}}{CV\sqrt{\left(\frac{(n-1)\mu_{mut}{}^2+(m-1)\mu_{wt}{}^2}{n+m-2}\right)\left(\frac{1}{n}+\frac{1}{m}\right)}}.$$

For a z-fold shift, let $\mu_{mut} = z\mu_{wt}$,

$$t = \frac{z\mu_{wt}-\mu_{wt}}{CV\sqrt{\left(\frac{(n-1)(z\mu_{wt})^2+(m-1)\mu_{wt}{}^2}{n+m-2}\right)\left(\frac{1}{n}+\frac{1}{m}\right)}} = \frac{(z-1)\mu_{wt}}{CV\mu_{wt}\sqrt{\left(\frac{(n-1)z^2+(m-1)}{n+m-2}\right)\left(\frac{1}{n}+\frac{1}{m}\right)}} =$$

$$\frac{(z-1)}{CV\sqrt{\left(\frac{(n-1)z^2+(m-1)}{n+m-2}\right)\left(\frac{1}{n}+\frac{1}{m}\right)}} .$$

B.3 Results and Discussion

To understand the effect of CV on the statistical significance of z-fold shifts in mean cEC_{50} data, we utilized the above-derived equation for *t* and performed two numeric analyses. The derived equation was coded into an Excel (Microsoft) worksheet and the *t*-distribution *p* values were calculated using the TDIST function of the software. Confidence levels described below are calculated by the equation, $CL = 100(1-p)$.

In the first analysis, we considered the *t*-test confidence level of z-fold shifts in mean cEC_{50} for an experiment with 10 wild-type and 5 mutant cells. These quantities are typical for a LGIC experiment. We examined CV values of 0.25, 0.5, and 1.0 because these levels of variation were observed in our LGIC and GPCR data sets (see Figure 3.12 and Figure 3.17). Figure B.1 shows the relationship between z-fold shift and confidence level in differentiating the two cEC_{50} means by the *t*-test. Confidence levels exceeded the standard 95% threshold for CV = 0.25 at z = 1.33, CV = 0.5 at z = 1.76, and CV = 1.0 at

z = 3.5. Traditionally, we do not try to physically interpret z < 3 shifts. Therefore, even in the most variable data sets we have observed, interpreting physically relevant EC_{50} shifts would not be problematic for data sets containing 10 wild-type and 5 mutant cells.

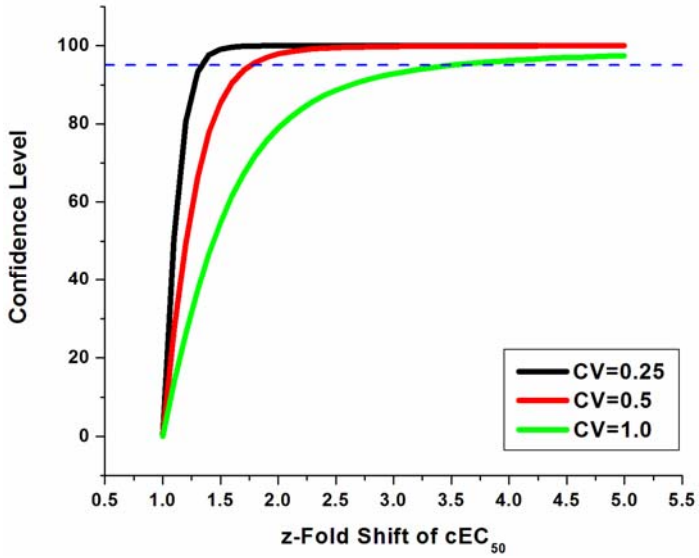

Figure B.1. The confidence levels of *t*-tests comparing cEC_{50} means with z-fold shifts. Three CV values were considered: 0.25, 0.5, and 1.0. The dotted blue line denotes the standard 95% confidence level

The goal of our second analysis was to understand how increasing the number of cells in the data sets affected *t*-test confidence levels. We considered 3-fold mean cEC_{50} shifts, the minimal shift we would want to interpret physically. Relationships between CV and confidence levels were determined for experiments with 10 wild-type and 5 mutant cells, 12 wild-type and 12 mutant cells, and 20 wild-type and 10 mutant cells (Figure B.2). The highest possible CV at which the confidence level of differentiating a 3-fold shift exceeded the 95% level increased with the number of cells in each data set. CVs below 0.91, 1.06, and 1.33 were sufficient to pass the 95% confidence level for the 10 wild-type / 5 mutant, 12 wild-type / 12 mutant, and 20 wild-type / 10 mutant cell cases, respectively. We therefore concluded that the ability to differentiate small EC_{50}

shifts between data sets with higher than normal cell-to-cell variability could be improved by collecting additional data.

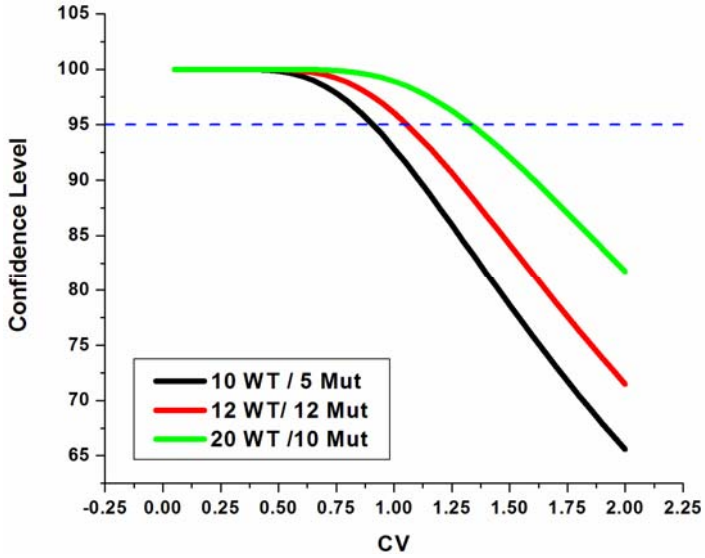

Figure B.2. The effect of sample sizes on discerning 3-fold shifts in mean cEC_{50} using the *t*-test. The dotted blue line denotes the standard 95% confidence level.

As discussed in Section 3.2.6, we assume that significant shifts in cEC_{50}s produce significant shifts in EC_{50}. Therefore, these exercises suggest that cell-to-cell variability at levels we observed in M_2AChR data will not hinder our ability to interpret EC_{50} shifts that we have been accustomed to in LGIC experiments. Collecting more cells per condition will allow us to strengthen the statistical significance of small EC_{50} shifts. We therefore concluded that batch-to-batch variability was the real concern in GPCR data, not cell-to-cell variability.

B.4 References

(1) Zhou, X.-H.; Gao, S.; Hui, S. L. *Biometrics* **1997**, *53*, 1129–1135.