1. **What is a probability density function (PDF) vs. a cumulative distribution function (CDF)? Describe at a conceptual level how you can obtain p-values using the density functions.**

A PDF describes the point probability for any particular value of X. For a discrete distribution the height of the curve is equivalent to the probability that a random variable takes on a specific discrete value. This is not true for a continuous variable since getting the probability, or area under the curve, requires integration.

A CDF gives the cumulative probability that a random variable x is less than or equal to a particular value of X. An upper-tail probability can be computed by subtracting the lower-tail probability for a particular X from 1 (the total probability).

Both of these correspond to a p-value, i.e. the probability of observing a value at least as extreme as the one observed, under the null hypothesis.

1. **Why is the negative binomial a better model than the Poisson for RNA-seq data?**

It is appropriate to model data with a Poisson distribution, where the mean and the variance are given by the same parameter, λ and the variance is proportional to the mean. This is true for count data for technical replicates. When considering biological replicates, however, it turns out that the variation in RNA-Seq counts increases with the number of counts per feature (expression level) and is therefore ‘overdispersed’. In such cases, the Poisson is no longer the best model for the data.

Instead, the NB is used to model the uncertainty in the variance as the variation is proportional to the mean, with an added term to account for the dispersion:

σ2 =μ+αμ2, where α is the dispersion parameter. For α > 1, the dispersion is greater than the mean; as α goes to 0, the NB converges on a Poisson distribution.

1. **Hypothesis testing**
2. **What kinds of errors can arise in comparing two samples?**
3. **What’s the tradeoff between error and power? Be sure to define error types and power in your answer.** 
   1. Type I error: False Positive (alpha) – erroneously accept HA when H0 is true

Type II error: False Negative (beta) – erroneously reject HA when it is true

* 1. Power is the probability that a test will correctly reject the null hypothesis when the alternative hypothesis is true — i.e., the probability of avoiding a type II error. It can therefore also be thought of as the ‘True positive rate’ or (1 – beta). Power depends on the significance threshold (alpha), the effect size (E), the sample size (n), and the population variance (sigma2). Power is proportional to alpha, so increasing alpha gives more power to detect true positives but also produces more false positives. Decreasing alpha on the other hand decreases false positives at the expense of false negatives.

Power = True positive rate = 1 – beta = E\*alpha/(sigma/sqrt(n))

**4) Confidence intervals and p-values**

**(a) Define a confidence interval. What, specifically, does a 95% CI mean?**

**(b) Why and how are confidence intervals useful?**

**(c) Why do confidence intervals provide an important complement to p-values?**

a. A confidence interval provides a range estimate for a population parameter. A 95% CI indicates that 95% of the time, when taking samples of the same size, the true statistic (mean) is expected to be contained in the interval (95/100 samples will contain the expected value).

b. The CI represents the uncertainty in the estimate (precision): a smaller CI represents higher confidence in the parameter estimate. In addition, the CI also provides an estimate for the magnitude and direction of the effect.

c. Since p-value can vary a lot from experiment to experiment (particularly if the sample sizes are small), they may not be representative of the true population parameters. P-values also use arbitrary cutoffs. The CI gives an estimate for the direction and strength of an effect, an idea of how reliable we think our estimate would be in repeated experiments (precision).

1. **T-tests**

**(a) What is a two-sample t-test used for?**

**(b) What assumptions need to be met in order to apply a t-test?**

**(c) Define in words how the test statistic is calculated.**

**(d) How is the confidence interval related to the t-distribution?**

a. To test if two different samples are obtained from the same distribution, in which case the difference in the means of the two samples should be close to 0.

b. The main assumptions are that the:

* Sample data are normally distributed.
* Sample data are random and independent.
* Parent populations have equal variances.

c. The t-statistic is the ratio of the departure of the estimated value of a parameter from its hypothesized value to its standard error ( where SE = SD / sqrt(sample size) ). If the t-statistic is greater in magnitude than the critical value, then the difference is considered to be significant.

d. The confidence interval is determined using the standard error and the critical value for the t-distribution for the available degrees of freedom (for two-sample tests, this is the distribution of mean differences). 95%CI = mean estimate +/- t(crit)\*SE(y). For alpha=0.05, t(crit) ~ 2.78.

1. **Resampling**
   * + 1. **Why might you choose resampling to test for significant differences instead of a standard statistical test?**
       2. **How is significance determined using resampling methods? Describe the steps you would use to obtain a bootstrap p-value and CI for the mean difference between two samples.**
          1. When you have reason to believe that data may not be normally distributed, or you just want to get empirical estimates using the data itself rather than making any assumptions about them.
          2. Resampling methods empirically determine significance and confidence intervals by sampling different subsets of the data many times. Significance is determined as the proportion of data that fall at the extremes of the sampled data (e.g. 5% of the data).

For example, let’s say we have 10 measurements for each of two different samples, groupA and groupB. We can sample 10 values from each group ( with replacement ) and record the difference of their means. If we do this 1000 times, we will be able to determine the distributions of the means, which will provide the standard error and confidence intervals. This can be used to calculate a p-value for the difference between the groups.

1. **Multiple Hypothesis Testing**

**(a) Why is multiple hypothesis testing important for high-dimensional data?**

**(b) What are two popular methods of p-value adjustment, and what is the essential difference between them?**

a. For studies like genome-wide gene expression, where we are performing tens of thousands of tests in one dataset, the likelihood of obtaining false positives by chance is greatly increased. For example, for 20,000 t-tests of differential expression at a significance threshold of 5%, 1000 genes will always be considered as “differentially expressed” whether or not this is really the case.

b. Bonferroni = p-value / total # of tests. It controls the FWER, i.e. the probability that the null hypothesis is false for at least one test.

The FDR is the false discovery rate, a.k.a. Benjamini-Hochberg correction. It specifies the rate of false positives you are willing to accept within a set of statistically significant results. In genomics, typical FDR values are 5% or 10%. The FDR uses the q-value as a cutoff rather than the p-value. To compute the FDR, the p-values are sorted form smallest to largest and compared with the BH q-value (rank/number of samples). All the p-values that are less than the q-value are significant.

**8) Tabular Data**

**(a) What kind of data would you use a contingency table for? Give an example.**

**(b) What is a Chi-square test? How is the test statistic calculated, and what distribution does the test statistic follow?**

**(c) Under what conditions is a Chi-squared test not recommended?**

* 1. When you have two groups in which some proportion of each displays a certain characteristic, and you want to determine whether the proportions are the same or different between the two groups. An example from class asked whether there is an association between the incidence of breast cancer among women who first gave birth below or above the age of 30.
  2. Fisher’s exact test is a special case of the hypergeometric distribution, which gives the probability of x successes when sampling without replacement from a finite population. Fisher’s test follows this model because, with fixed row margins, changing the value of one cell in a contingency table necessarily changes the values in the others. The p-value of the Fisher’s Exact test is calculated by summing the probabilities of all possible contingency tables that represent equal or greater deviation from independence (neutral expectation) than the observed table.

The Chi-square test uses a normal (continuous) approximation of a binomial (discrete) distribution to compute significance (p-value). The Chi-squared test statistic compares individual proportions in each group to the expected proportion based on the population mean estimate and is calculated as the sum of squared differences between observed and expected values over the expected value across all cells in the table.

Text, letter

Description automatically generated

* 1. For a 2x2 contingency table, a Fisher’s Exact Test is preferred when the number of sampled items is small (typically, when any of the cells has a value less than 5). Since it is based on the hypergeometric distribution, its calculation requires factorials and so is computationally more intensive. For larger tables, R can compute Fisher’s when total counts are not too large; otherwise, Chi-squared is preferred.

For a Chi-squared test, the sampling distribution of the test statistic is well approximated by an ideal χ2 distribution when the measurements are independent and the number of items in each sample is sufficiently large. *[NOTE: also accepting “normal” distribution]* When these conditions are not met, the test is not reliable and it is recommended to use Fisher’s exact test instead. General rules of thumb are:

* Expected frequencies for all categories are at least 1 or greater.
* Expected frequencies should be <5 for no more than 1/5 of categories.

**9) Experimental Design**

**(a) What is the difference between planned and unplanned experimental designs?**

**(b) Give an example of each type of experiment.**

**(c) What test is used to determine the effect size in each type of experiment?**

a. A planned experiment has a single reference, or control, group. In an unplanned experiment, we are interested in comparing everything to everything.

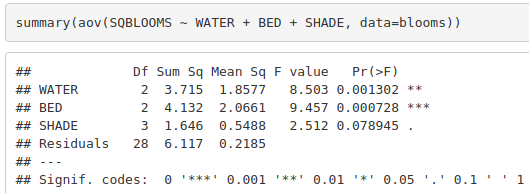
b. …

c. Planned experiment: apply Dunnett’s Test to an aov() model to look at the differences between each group and the control.

Unplanned experiment: apply Tukey’s Honest Significant Differences (Tukey’s HSD) Test to an aov() model to look at all pairwise differences between the groups.

**10) ANOVA models**

* 1. **What does ANOVA test, and what types of values (continuous, discrete, or categorical) are the Response and Predictor variables?**
  2. **For the image of an ANOVA result below:**
     1. **What is the Df column describing?**
     2. **What is the Sum Sq column describing?**
     3. **How is the F distribution created? How is the F-statistic calculated? Either write a formula or describe the general idea behind it.**
     4. **What is the null hypothesis of the F-test?**
     5. **What factor(s) are significant in this model?**



a. ANOVA tests whether there is a significant difference between more than two groups. Response is continuous, predictor is categorical (though, can run ANOVA on linear models with continuous predictors).

b. i. The Df column displays the degrees of freedom for the groups and the residuals

ii. The Sum Sq column displays the sum of squared deviations for the groups i.e., the total variation between the group means and the overall mean and the sum of squared deviations for the residuals i.e., the variation between the group means and the data.

iii. The F-distribution is created by generating all possible values of the F-statistic. The F-statistic is calculated by first computing the sum of squared deviations of each group around the grand mean and the sum of squared deviations of the data around group means. These values are then divided by the appropriate degrees of freedom to calculate the mean square groups and mean square error. The F-statistic is the ratio of the mean square groups and the mean square error.

iv. The null hypothesis of the F-test is that the ratio of the mean square groups and the mean square error is close to 1 i.e., the variance of the groups being compared is equal.

v. WATER and BED are significant.

**11) GLM**

**(a) What are the main differences between ANOVA, linear, and logistic regression?**

**(b) In looking at the results of a linear model, what does R2 represent? What kind of information is used to calculate it?**

**(c) What is the null hypothesis of the test that provides the p-value for the predictor in a regression model?**

**(d) In logistic regression, what function is used as the Response variable in the linear model?**

ANOVA tests whether there is a significant difference between groups.

Linear regression models the magnitude of response between independent and dependent variables and provides predictions for new data.

Logistic models qualitative outcomes using quantitative predictors. It is used when the outcome to be modeled is the probability of two alternative outcomes, the predictors are quantitative (or can be encoded as binary), and there is some correlation between the predictors and response variables.

|  |  |  |
| --- | --- | --- |
| **Method** | **Predictor Variables** | **Response Variables** |
| **ANOVA** | quantitative / categorical | quantitative |
| **Linear regression** | quantitative / categorical (ordinal / interval) | quantitative |
| **Logistic regression** | quantitative or discrete (categorical can be encoded if binary) | qualitative (binary, mutually exclusive) |

* 1. R2 tells us how much of the variance can be explained by the model. It is essentially the Sum of Squares of the model (SS\_regression) divided by the total sum of squares (SS\_total). The total sum of squares is the difference between the points and the mean of all points squared. The Sum of Squares of the linear model is the difference between the points and the linear model squared.

*Another definition of R2 is the ratio of the covariance between two variables divided by the product of their individual variances. (Also square of the Pearson correlation coefficient, when only two variables.)*

* 1. There is no association between the predictor and the response variable (slope, coefficients = 0).
  2. Log odds (logit)

**12) Bayesian Statistics**

**(a) Describe the difference between the “frequentist” and Bayesian worldviews.**

**(b) What are the three main components of a Bayesian model called, and what do they mean?**

**(c) Using an example from class, outline the basic framework for a Bayesian analysis and describe why this is a useful paradigm.**

a. Frequentist statistics views the population parameters as a ground truth that is being estimated by the data measured. In the Bayesian worldview, the ground truth is considered as a distribution of probabilities itself. Thus, the values being estimated by the response variables do not represent single, fixed outcomes but a family of possibilities, each with some degree of probability.

b. A typical Bayesian analysis uses priors (known probabilities or beliefs based on past experiences e.g., the known rate of a disease in a population) and likelihoods (known conditional probabilities e.g., the false positive/false negative or true positive/true negative rates of a diagnostic test) to compute posteriors or unknown conditional probabilities using the Bayes theorem (such as the probability of a test correctly diagnosing a patient with the disease).

c. An example discussed in class was estimating the chances that a random Down’s syndrome test would produce a positive result in a diagnostic (sequence-based) test given that the fetus actually has the disease – i.e. P(Downs | POS). The data used for this are data on whether known Down’s syndrome or normal cases gave negative or positive results – e.g. P(POS | Downs), etc. As more data is collected, the prior probabilities may be updated and therefore produce different results.

1. **Dimensionality Reduction**
   1. **Why is it useful to use dimensional reduction methods like PCA, t-SNE, and UMAP?**
   2. **What's the basic idea behind PCA, and how are principal components identified?**
   3. **What is the biological meaning of principal components and t-SNE/UMAP dimensions?**
   4. **How many principal components can be calculated for FACS data that has 2500 observed cells and eight features (six fluorescent data channels, side scatter, and forward scatter)? Why?**
   5. **How many t-SNE/UMAP dimensions would you typically use for the same data?**
      * + 1. Sometimes there are many measured variables that can be used as predictors, but some of them may be correlated and thus do not offer much new / independent information for prediction. Dimensional reduction allows identification of a smaller number of predictive variables, resulting in a simpler model.
          2. Principal components analysis (PCA) uses linear combinations of predictors to identify a new coordinate system that explains most of the variation in the original data. The first PC explains the largest proportion of variation, the 2nd PC explains the second most variation, etc. It is then possible to identify the minimal number of dimensions required to explain most of the variation in the data. This enables visualization and analysis of major factors contributing to observed results.
          3. Strictly speaking, the axes of PCA, t-SNE or UMAP have no biological meaning. However, they may correlate with certain biological features, which can be useful for the interpretation of the plots.
2. Since there are 8 variables, there are 8 principal components, which are linear combinations of the 8 variables that together can explain all the variation among the 2500 cells.
3. UMAP can embed higher-dimensional spaces into any arbitrary number of dimensions (and probably t-SNE too, but I don’t remember how it works exactly). For both t-SNE and UMAP, you would normally project the data onto only 2 or 3 dimensions for the purpose of visualization.

PCA and UMAP are commonly used sequentially – PCA is first used to find the most highly varying datapoints, which are then used for visualization with UMAP in 2 or 3 dimensions.

**14) Clustering**

**(a) What is the difference between Euclidean vs. Correlation Distance? When might you prefer one over the other?**

1. **What are three major differences between the mechanics of Hierarchical and K-means clustering?**
2. **What are advantages and disadvantages of these two methods?** 
   1. Euclidean distance is the geometric distance between two points and is calculated using the Pythagorean theorem. Pearson correlation normalizes the covariance to produce correlation values ranging from -1 to 1. The correlation is independent of scale because it measures changes relative to the total variation of each variable.

Whether Euclidean distance or correlation are more appropriate for clustering depends on whether the magnitude or patterns of expression changes are more important for the question being investigated. Euclidean distance will cluster together genes that show similar magnitude of expression, while correlation will cluster together genes that exhibit the same pattern of expression across different samples or timepoints.

Correlation is more often used for hierarchical clustering, whereas k-means clustering uses average Euclidian distance to compute squared deviations of data points from the centroids. This operation may be performed on data that has already been standardized.

* + - * 1. Hierarchical clustering is a bottom-up approach where a pairwise distance matrix is calculated between all genes (or samples). The pair(s) that are closest to each other are then grouped, and all the distances from each group to the other genes and groups of genes is computed using some linkage method (single, complete, average, centroid). This process is repeated until all the genes are linked.

K-means is a top-down approach where we first decided the number of groups we want and then we start by randomly assigning the centroids of these k groups. We then assign genes to the cluster whose centroid is closest to them and recalculate the centroid (middle point) for each group of genes. This process is repeated until the centroids are no longer moving.

* 1. An advantage of Hierarchical clustering is that it if you repeat the analysis you will get the same results. In contrast, since the initial centroids for k-means clustering are chosen at random, the results will vary between runs. In addition, the number of clusters must be chosen in advance for k-means clustering, whereas for Hierarchical clustering the number of groups can be chosen after the analysis is done using some evaluation criteria (such as silhouette widths). Another disadvantage of k-means clustering is that it uses Euclidean distance, whereas Hierarchical clustering can use a variety of distance measures.