Instructor's Manual – Journal Article Reading Assignments and In-Class Activities

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General Notes

These assignments are designed to be capstone activities at the end of units on figures of merit (such as sensitivity and LOD), acid-base equilibria, separations, spectroscopy, mass spectrometry, and electrochemistry. Each assignment consists of an out-of-class reading assignment accompanied by objective questions and an in-class discussion of more open-ended questions. Each reading assignment is drawn from the peer-reviewed literature. The assignments are designed to require just one class period, and I commonly use them just before an exam as an opportunity for students to review important concepts, examine them from new angles, and apply them to new situations.

The reading and out-of-class questions should be completed before the in-class discussion and are designed to encourage close reading, article comprehension, and application of concepts from class. For example, the out-of-class questions for the fast-scan cyclic voltammetry article ask students to identify the oxidation and reduction half-waves of a representative cyclic voltammogram in the manuscript and to calculate the signal-to-noise for one of the dopamine release peaks. These out-of-class questions can be answered by students individually or in groups, depending on the arrangement that is most effective and feasible at your institution. All of the out-of-class questions are designed to elicit specific objective answers based on the article or material from class or the textbook. A few questions require some additional thought or extra care, and notes on these questions are included in this instructor manual.

The second part of the assignment consists of several open-ended, more conceptually challenging questions designed for in-class discussion. At the start of class, I find it helpful to solicit questions from the students and clarify any confusing points in the paper. In particular, it is useful to make sure that the students understand the objective out-of-class questions before they begin their discussions. Students should then break into small groups of 3-5 students to discuss the in-class questions. (For a discussion of effective methods for creating small groups, see the Instructor's Guide for the In-Class Module on Separations.) During the discussions, I circulate to answer questions, highlight important points, or keep groups on-task as needed. Discussions for a question typically last from 5-15 minutes. When most of the groups have had time to develop their ideas, it is helpful to have each group report out to the class. If you are unable to check in with each group during their discussion, this is a useful opportunity to check for misconceptions or incomplete responses. Additionally, if you would like a written record of the students' discussion to be turned in for evaluation or assessment, you may find it helpful to have one student to take notes for each group.

Note: If you do not provide the students with a color copy of the article, be sure to remind them to print the article in color or access it in color online while answering the questions because several of the articles include figures that are uninterpretable in grayscale.

Comparing Analytical Methods: Cocaine Determination in Urine

Article: D.L. Phillips, I.R. Tebbett, and R.L. Bertholf, "Comparison of HPLC and GC-MS for measurement of cocaine and metabolites in human urine" *J. Anal. Toxicol.* **1996**, *20*, 305-308.

This article compares two different methodologies, HPLC with UV absorbance detection and GC-MS for the quantification of cocaine and its metabolites in urine. The assignment is designed so that no prior knowledge of the separation mechanisms of HPLC and GC is needed. Similarly, minimal background knowledge about MS is needed. Before the students start their discussions, I check to make sure they have understood that MS detection provides much more detailed structural information about the analyte compared to UV absorbance. The assignment is designed to highlight the figures of merit used to compare two techniques: measures of sensitivity, precision, LOD and LOQ, etc. The methods used also provide an opportunity to discuss matrix effects, use of an internal standard, and statistical analysis of data. Additionally, because these topics are typically covered early in the analytical curriculum, when students might first be encountering the primary literature, some questions are included to draw students' attention to certain conventions of writing in the scientific literature.

Out-of-Class Questions

Several questions in this assignment (**Q1, Q3, Q4**) are designed to draw students' attention to the structure of a scientific article and important conventions in scientific writing for the literature. The remaining questions review important concepts in method calibration and statistics or ensure that students have the necessary background for in-class discussion.

Q1. Using only the information in the abstract, answer the following questions about this work.

(a) What did the authors do?

The authors compared HPLC to GC-MS for detection and determination of cocaine and its metabolites.

(b) Why did they do it?

The aim of the study was to evaluate whether HPLC is a useful alternative to GC-MS for this application.

(c) How did they do it?

The authors used solid phase extraction followed by either HPLC or GC-MS on samples of urine with bupivacaine added as an internal standard. The methods were compared based on sensitivity, precision, and dynamic range.

(d) What did they find?

They found that HPLC is a useful alternative to GC-MS.

Q2. Fill in the table below using the information from the introduction.
(This question prepares students for in-class discussion questions **Q4** and **Q5**.)

	GC-MS	HPLC-UV
Advantages	High specificity Currently the accepted method	Very sensitive methods available DAD gives spectral information Low cost Technology is improving
Disadvantages	Analytes must be volatile and therefore often require derivatization	No UV detection of the metabolite ecgonine methyl ester available, more susceptible to matrix interferents

Q3. Toward the end of the introduction, often in the last paragraph, it is common to transition from background information to a brief summary of the specific question addressed in the manuscript. What question did these authors want to answer?

From p. 305, "The purpose of this work was to determine whether HPLC analysis of urine samples containing cocaine and metabolites produced comparable precision, sensitivity, and reproducibility to GC-MS analysis of the same samples.

Q4. Skim the Materials and Methods section with a focus on what types of details are included. Check the boxes to indicate which pieces of information below are in the methods section, and be sure to note the level of detail.

Discussing this question in class is a useful opportunity to clarify the differences in format and detail between lab manual protocols, procedures recorded in lab notebooks, and the methods sections of journal articles.

■ chemicals used

□ glassware used

■ concentrations of solutions

■ instruments used

■ operating parameters for instruments

■ manufacturers of supplies

□ sample calculations for how to make stock solutions or dilutions

■ description of data analysis procedures

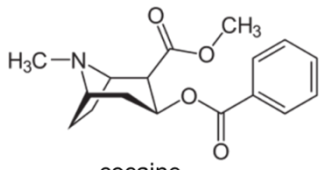
□ results of the experiment

Q5. Why do the authors spike the standards into a urine sample instead of diluting them in water or buffer?

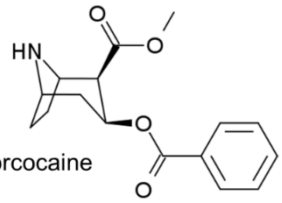
Spiking the standards into a urine sample helps to keep matrix effects constant between samples and calibration standards.

Q6. Look up the structures of the analytes and bupivacaine and record them below.

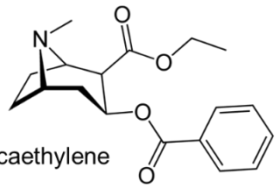
(This question ensures that students have information required to evaluate the suitability of the internal standard in class.)



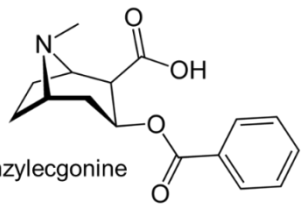
cocaine



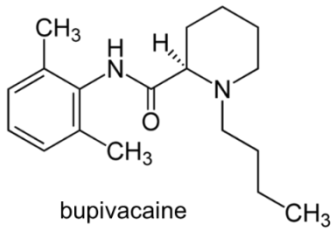
norcocaine



cocaethylene



benzylecgonine



bupivacaine

Q7. For their statistical analysis, why did the authors use paired *t*-tests?

As noted on p. 306, the same samples were used for each method after being split in half after the SPE step. This is a common experimental design for comparing two methods and means that a paired *t*-test is appropriate.

Q8. **How did the authors define the limit of quantitation (LOQ)? What is the typical way to define LOQ?**

(This question asks students to recognize that the LOQ determination in this paper may not match the methodology described in previous classes. If desired this question can be moved to the in-class assignment and used to prompt discussion about practical considerations of false positive and false negatives in determining the best way to assess LOD and LOQ for a specific application.)

On p. 307, the authors defined the LOQ as five times the LOD. [The LOD was determined empirically by running samples of progressively lower concentration until the signal was not consistently detected.] A typical textbook definition of the signal LOQ is 10 standard deviations above the blank. The concentration LOQ is then $10s_{bl}/m$. With the standard definition of LOD as $3s_{bl}/m$, the LOQ is typically defined as 3.33 times the LOD.

Q9. What metric was used to compare the precision of the data? What was used to evaluate sensitivity?

(This question prompts students to identify the interday variation in the slope as a metric for precision and the slope itself as the metric for sensitivity. As students discuss in-class **Q2**, I encourage them to consider their responses to this question. Note that the authors use the term "analytical sensitivity" in referring to the interday variation of the slope (calibration sensitivity), which may confuse students who have learned that analytical sensitivity, γ, refers to the calibration sensitivity, m, divided by the standard deviation of the signal for a given calibrator.)

As noted on p. 307, the authors use the interday variability of the average coefficient of variability for all calibrators to evaluate precision. [The coefficient of variation, CV, is the relative standard deviation expressed as a percent.] These values for each calibrator were averaged, and then the average value and standard deviation were used to perform a paired *t*-test to determine whether there was any difference in precision between the methods. This measure of precision emphasizes day-to-day reproducibility.

Also on p. 307, the authors state that the average slope for each analyte was used to evaluate sensitivity. Again, a *t*-test was used to determine whether there was a significant difference in sensitivity for each method.

In-Class Questions

Q1. Consider the structures of the analytes and bupivacaine that you looked up outside of class. What was the purpose of the bupivacaine in this analysis? Do you think this compound was a good choice for this purpose? Explain.

Students should consider the presence of acidic and basic groups, hydrophobicity, molecular weight, etc. Because the internal standard is expected to behave similarly to the analytes, students should also consider relative retention times of the analytes and internal standard in Figures 1 and 2. Students may also consider the possibility of including a second internal standard that can be derivatized like BZE and NC for GC analysis to account for incomplete derivatization of these analytes.

If students conclude that bupivacaine is not sufficiently similar to the analytes, it may be helpful to prompt students to consider challenges to identifying an internal standard that is more structurally similar to the analytes. For example, one must be certain that the internal standard cannot possibly occur in the samples due to cocaine metabolism. Time-permitting, you may give the students an opportunity to search the literature for internal standards used in other studies of cocaine metabolites. (They may be surprised to find that for GC-FID studies the most commonly used internal standards may be even less structurally similar to cocaine than bupivacaine.)

Q2. The authors state that "Overall, GC-MS demonstrated better precision than HPLC, but the methods had generally equivalent sensitivities." Consider the data in Tables 1 and 2. Do you agree with the authors' statement? Why or why not? What additional data might be useful in comparing the two methods' sensitivity and precision? What other information might you want to know when choosing a method for your application? Is this information provided in the manuscript?

This question asks students to make a global assessment of the data in Tables 1 and 2. It is important to ensure that the students understand their answers to out-of-class **Q9** before this discussion gets underway. The authors provide their interpretation in the text, but this question is actually fairly complex when one considers the standard deviations of the slopes reported in Table 1. Students should be encouraged to consider all the data rather than relying on the authors' conclusions. Based on Table 2, GC-MS is more precise, as the individual calibrators appear to agree well from day-to-day based on the average CV values reported. However, the slopes for the GC-MS calibration curves had much larger variation than those for HPLC, as seen in Table 1. Students may consider whether reporting the analytical sensitivity, γ, in addition to

the slope would be useful, as this metric considers both sensitivity and precision. Some students may bring up the LOD values presented at the end of the paper, giving an opportunity to underscore the distinction made by analytical chemists in discussing sensitivity and LOD.

Q3. Do you agree with the authors' conclusion that HPLC with UV detection is a suitable alternative to GC-MS for analysis of cocaine in urine? Consider this conclusion generally and in the context of the following scenarios. Decide whether you would recommend HPLC-UV or GC-MS for each analysis, and justify your choices.

a. You manage a small business. The owner of the company decides to require drug testing for cocaine for all new hires.

b. You are working on a research project to study how genetic variation in rats affects the metabolism of cocaine.

This question asks students whether they agree with the paper's conclusion, which is that HPLC is a useful alternative to GC-MS. Rather than treating this as a question with "correct" answers, students should be encouraged to consider this conclusion critically and use data and experience to support their answer. For example, students who are familiar with derivatization as a sample preparation step may bring up issues of cost and time associated with this technique. I often find it useful to direct students to both the text and the tables and to instruct them to consider all the available data in their decision. The first paragraph of the Results on p. 307 of the article discusses the specificity of each method. The last paragraph of the Results on p. 307 discusses the agreement between the tested methods and the results of an established drug lab. The LOD data are at the end of the Discussion. Students should also be encouraged to consider the ramifications of a false positive in each situation. In summarizing the class discussions, I explain that GC-MS is commonly used as a confirmatory tool in drug testing because of the structural information provided by MS. For this reason, GC-MS is likely the best of the two options for scenario (a), while HPLC-UV may be acceptable or even preferable for scenario (b).

Q4. Since this article was published, a method that readily couples HPLC to MS, called electrospray ionization, has become much more widely available. What would be the advantages of this method for this application?

If needed, students can be referred back to **Q2** from the out-of-class questions, which highlights the advantages and disadvantages of each technique. Depending on students' familiarity with HPLC, GC, and MS, it might be helpful to briefly discuss the difference between a separation technique and a detection method. This should lead into a discussion of how HPLC-MS combines the minimal sample preparation of HPLC with the structural information available by MS.

Acid-Base Equilibria: Titrations of Bacterial Cell Surfaces

Article: M. Dittrich and S. Sibler, "Cell surface groups of two picocyanobacteria strains studied by zeta potential investigations, potentiometric titration, and infrared spectroscopy," *J. Coll. Int. Sci.* **2005**, *286*, 487-495.

This article presents an investigation of the surface chemistry of picocyanobacteria, which contribute to calcite precipitation in lakes. The article highlights a unique application of acid-base measurements while also encouraging students to think of the challenges presented by chemical investigations of live cells. Potentiometric titration is used to determine the pK_a values of functional groups on the cells' surface, and infrared spectroscopy is used to confirm identification of these groups. While the potentiometric titration plots look quite different from the titration curves students have seen in class, the same acid-base principles are used to interpret the data. When I use this paper in analytical chemistry, we skip the IR data so no questions on those sections are included; however, if the paper is read in a class that covers both techniques, questions on the second part of the paper could be added.

Out-of-Class Questions

Q1. Because this article deals with a microbiological and geochemical application, you need to familiarize yourself with a few terms. Match the following terms with their definitions/descriptions.

Students should have no trouble finding the definitions of these terms online:

__A__ autotropic	__F__ peptidoglycan
__J___ oligotrophic	__H__ picocyanobacteria
__C__ pelagic	__G__ picoplankton
__B__ calcite	__I__ *Synechococcus*
__L__ electrophoretic mobility	__D__ phycocyanin
__K__ zeta potential	__E__ phycoerythrin

Q2. In the abstract of the article, mark which sentences correspond to (1) background information and significance, (2) methodological details, (3) results, and (4) conclusions.

There is some flexibility in the correct answer to this question, but a suggested mark-up is shown below: background and significance, methodological details, results, conclusions.

Abstract

In order to clarify the role of picocyanobacteria in aquatic biogeochemical processes (e.g., calcite precipitation), cell surface properties need to be investigated. An experimental study of the cell surface characteristics of two *Synechococcus*-type unicellular autotrophic picocyanobacterial strains was carried out. One strain was isolated from Lake Plön and contained phycocyanin, the other strain came from Lago Maggiore and was rich in phycoerythrin. Potentiometric titrations were conducted to determine the different types of sites present on the bacteria cell walls. Infrared spectroscopy allowed characterization of the various functional groups (R–NH_2, R–COOH, R–OH, R–PO_3) and investigations of zeta potential provided insight into the isoelectrical points of the strains. Titrations reveal three distinct sites on the bacterial surfaces of phycocyanin- and phycoerythrin-rich strains with pK values of $4.8 \pm 0.3/5.0 \pm 0.2$, $6.6 \pm 0.2/6.7 \pm 0.4$, and $8.8 \pm 0.1/8.7 \pm 0.2$, corresponding to carboxyl, phosphate, and amine groups with surface densities of $2.6 \pm 0.4/7.4 \pm 1.6 \times 10^{-4}$, $1.9 \pm 0.5/4.4 \pm 0.8 \times 10^{-4}$, and $2.5 \pm 0.4/4.8 \pm 0.7 \times 10^{-4}$ mol/g of dry bacteria. The deprotonation constants are similar to those of bacterial strains and site densities are also within an order of magnitude of other strains. The phycoerythrin-rich strain had a higher number of binding sites than the phycocyanin-rich strain. The results showed that picocyanobacteria may adsorb either calcium cations or carbonate anions and therefore strongly influence the biogeochemical cycling of calcite in pelagic systems.

Keywords: Cyanobacteria; Cell surface; *Synechococcus*

Q3. In your own words, describe why the surface chemistry of picocyanobacteria is an important area of research.

I typically look for some form of the underlined concepts in student responses:

The surface chemistry of picocyanobacteria is an ecologically significant area of research since these organisms play a role in biogeochemistry of lakes by precipitating calcite, potentially through Ca^{2+} binding to the cell surface followed by hydroxide diffusion through the membrane.

Q4. Research and summarize the major difference between Gram negative and Gram positive bacteria. How might this difference affect the surface properties of these two types of cells?

As student should readily be able to find online or in a biology text, Gram positive bacteria have a simple peptidoglycan cell wall, while Gram negative bacteria have a bilayer membrane around their cell wall.

According to the article, published results show no clear trend in the relative density of reactive sites between Gram positive and Gram negative bacteria; however, one can reasonably hypothesize that a peptidoglycan layer, which is composed of amino acid chains and sugars, would potentially have different acid-base chemistry than a lipid bilayer.

Q5. Re-read the final paragraphs of the introduction. What specific question/problem is being addressed in this article?

The authors want to characterize the surface chemistry of Gram negative picocyanobacteria with respect to "the identity, abundance, and acid-base properties of binding sites" on two strains of *Synechoccocus* (p. 488).

Q6. In Section 2.5, the authors state that the titration data will be plotted with –log[H+] on the x-axis and CA-CB-[H+]+Kw/[H+] on the y-axis. Assuming that γ =1 (i.e., that activity is equal to concentration), fill in the blanks below.

Assuming that concentration is roughly equal to activity ($\gamma \approx 1$)

a. $-\log[H^+] = pH$

b. $K_w/[H^+] = [OH^-]$

Q7. In their modeling of the data, what assumption do the authors make about surface charge on the bacteria?

The authors assume that the surface charges are homogeneously distributed across the bacterial surface. While this simplifies the modeling, it may not be entirely justified based on prior published research about bacterial surface chemistry.

Q8. On Figure 1, circle the isoelectric point of the bacteria. How do you know that this is the isoelectric point?

[This question prepares students to discuss in-class question 3.)

The isoelectric point is the pH at which a molecule (or surface in this case) has a net charge of zero. Because the zeta potential arises from a net charge at a surface, the zeta potential will also be zero at the isoelectric point.

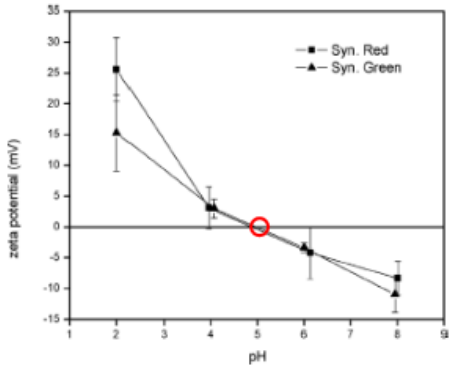


Fig. 1. Zeta potentials of two picocyanobacterial strains in 0.1 M $NaNO_3$ as a function of pH.

Q9. Based on their modeling results, the authors identify three separate pK_a values associated with the picocyanobacteria surface. Fill in the table below to match the range of fitted pK_a values with the most likely corresponding functional group.

Approximate Fitted pK_a Value	Corresponding Functional Group
~5	carboxyl
~6.5	phosphate
~8.8	amine

Q10. The inflection points at the three pK_a values are very weak, making them impossible to identify accurately without modeling. What explanation do the authors give for the weakness of these inflection points?

The bacterial surface includes several functional groups with similar pK_a values (Section 3.2, p. 490). If the students have looked at titration curves for diprotic weak acids, this is a helpful

analogy since as the pK_a values of the two acidic groups get closer together the two distinct inflection points are harder to distinguish.

Q11. Note that generally the conclusion section of an article should not just summarize the paper. Instead, the conclusion might address (1) questions that remain to be answered about the data, (2) potential future experiments, (3) limitations of the work, and/or (4) the broader significance of the results. Give an example of one of these from the conclusion of this paper.

Although the section is labeled "Summary," the authors do extend their comments beyond a mere summary of their results. For example, they address the broader significance of the results to calcite formation by discussing how the functional groups identified could interact with calcium and carbonate ions. They also describe an additional potential application in remediation of metal pollution that would require further study of bacterial surface chemistry as well as studies to identify conditions for culturing picocyanobacteria in the lab.

In-Class Questions

Q1. Prior to the titrations, the authors washed the cells in a solution of 1 mM EDTA and then resuspended them in $NaNO_3$. Both the $NaNO_3$ and the NaOH used in the titration experiments were degassed with N_2 before use. What was the purpose of each of these steps, and why were they necessary?

The composition of the cell culture medium is described in Section 2.1 (p.488) as containing numerous divalent metal ions, including Ca^{2+}, Mg^{2+}, Fe^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, and Cu^{2+}. These divalent metal ions disproportionately influence ionic strength and can interact with negatively charged groups on the cell surface, influencing measurements of zeta potential. While students may or may not realize that, classes which have covered complexometric titrations should recognize that the EDTA will chelate these metal ions (with varying affinity). The EDTA wash removes most of these ions, minimizing their effect on measurements of bacterial surface charge. The cell culture media is also buffered by dibasic potassium phosphate and boric acid. To titrate functional groups on the cell surface, it is necessary to eliminate the these weakly basic and weakly acidic components of the culture medium. Re-suspending the cells in sodium nitrate, which is a neutral salt, eliminates buffering from the solution so that acid-base behavior is coming only from the cells. Finally, the solutions were degassed with N_2 to displace dissolved CO_2, which forms carbonic acid in water and affects pH.

Q2. When interpreting Figures 2-4, it will be helpful to consider how these plots differ from typical plots of titrations.

(a) Have we typically plotted pH on the x- or y-axis? How does that compare to these plots?

Most potentiometric titration plots have pH on the *y*-axis. In these plots, pH is on the *x*-axis.

(b) What have we plotted on the other axis? How is that different from what is plotted here?

Most titration plots give mL of titrant on the *x*-axis. In this case, pH is on the *x*-axis and an expression representing "charge excess" (C_A-C_B-[H^+]+K_w/[H^+]) is on the *y*-axis.

(c) We know that ultimately the charges must balance, so where is this "excess" [H+] coming from? What is being deprotonated to release these hydrogen ions?

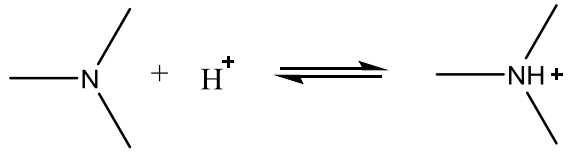
As the researchers add NaOH to the bacterial cell suspension to increase pH (on the *x*-axis), the change in pH does not directly correlate with the moles of base added. This occurs because as the NaOH is added, additional H^+ is released into the solution as functional groups on the bacteria are deprotonated.

(d) How do the bacteria contribute to the buffering of the system, i.e. how is this possible?

Because the functional groups found in biological molecules, such as amines, carboxylic acids, hydroxyl groups, and phosphate groups act as weak acids or bases, biological molecules, including those at the surface of the cells act as buffering agents and influence the pH of their environment.

Q3. For the third pK_a value (pK_3 in the manuscript), the authors state that either amine or hydroxyl functionality could give rise to the observed pK_a value, but they conclude based on the zeta potential measurements that this pK_a corresponds to amine groups on the cell surface. Sketch the protonation reactions for a generic amine and a generic hydroxyl group in aqueous solution. Use your sketches to explain the authors' reasoning.

In the manuscript, pK_3 has a value of ~8.8, which could correspond to a hydroxyl or to an amine. If the functional group giving rise to pK_3 were an amine, then below pH 5 the functional group would be protonated (because pH < pK_a) and positively charged. If this functional group were a hydroxyl, then the protonated form predominant below pH 5 would be neutral. We know that the other two functional groups present, carboxylic acid and phosphate groups, have pK_a values of ~5 and ~6.5, respectively. Both of these groups will be negatively charged above pH 5 and neutral below pH 5. If pK_3 corresponded to the hydroxyl, all functional groups would be neutral below pH 5, resulting in a zeta potential of zero. However, the data indicate that below pH 5 the zeta potential is positive, corresponding to the presence of amines.



Q4. A related article (*Aquat. Sci.*, **2004**, *66*, 19-26) **critiques studies like this one, in which a cell suspension is titrated. In the related article, Claessens *et al.* argue that because cells are dynamic, living systems, they respond differently to titrant than a chemical solution of weak acid or weak base would respond. For example, in addition to the chemical process of protonation or deprotonation, cells may also respond with metabolic activity or biochemical reactions, including pumping of protons across the cell membrane, unfolding**

of cell wall proteins, cell lysis, etc. As a result, Claessens *et al*. suggest that titration data does not necessarily just reflect the acid-base surface chemistry of bacterial assemblies. If you were a program officer at a funding agency, would you provide financial support for further studies like the one you read? Consider the authors' purpose, as you described it in out-of-class questions 3 and 5, and justify your answer.

The authors' goal was to evaluate the surface chemistry of the bacteria to see how it might be involved in calcite precipitation. If the available proton donors are changing during the measurement because of biological effects of the titration then this is a problem for this application. Interestingly, the authors acknowledge that they could not measure the acid-base behavior of the cell surface as a function of ionic strength because the cells may lyse (Section 3.2, p. 491), but they do not consider the possibility of physiological responses of the cells to pH values tested during the titration. Students who have taken a cell biology course may make suggestions to fix the cells (which stops metabolic activity but also causes many of the acidic and basic groups on the cell surface to react during fixation) or to isolate just the membrane components by centrifugation for titration (which could be done, but assumes that the inner and outer membrane leaflets are the same and that all embedded proteins and other biomolecules remain intact, which may not be the case). If students immediately suggest that they would not fund these studies, consider asking if they can suggest other methods to obtain the necessary data. Classes that discuss the FT-IR data may also note that these data were obtained in the absence of any titration, allowing the authors to make some functional group assignments without dramatically perturbing the cells (although there were still suspended in $NaNO_3$). This is a challenging problem, in that the titration of live cells is not an ideal method, but no perfect alternative currently exists, so some useful information may still be obtained by this technique.

Separations: Colloidal Particle Column Packings

Article: B.Wei, D.S. Malkin, and M.J. Wirth, "Plate heights below 50 nm for protein electrochromatography using silica colloidal crystals," *Anal. Chem.* **2010**, *82*, 10216-10221.

This article describes the use of colloidal particles just 330 nm in diameter as a packing material for electrochromatography. Students do not need to have been previously introduced to electrochromatography specifically, but they should have a background in reverse phase HPLC and capillary electrophoresis. The article provides an opportunity for students to apply their conceptual understanding of band broadening and the van Deemter equation to a novel experimental system. To answer several of the questions, students will need to have a detailed understanding of band broadening in separations, such as that provided by the ASDLIB Active Learning In-Class Activity on Separation Science written by Prof. Tom Wenzel. Also of interest: the small size of the particles means that when crystalline packing is achieved Bragg diffraction results in an artificial opal and the column appears blue. This can be seen in images on the website of the corresponding author, Mary Wirth.

Out-of-Class Questions

Q1. This abstract is a good example of how to present a large amount of quantitative data concisely in an abstract. Using the abstract and the body of the article as needed, report the following values for these experiments, and compare them to typical values for HPLC and CE from your text, class notes, or lab data. Don't forget units where needed!

Parameter	This Work	Typical HPLC	Typical CE
d_p	330 nm	1-5 µm	no packing
L	2 cm	15-25 cm	20-50 cm (to detector)
i.d.	75 µm	4.6 mm	50-100 µm
H	< 50 nm	~10 µm	0.5-5 µm
N	10^6	10^4	10^5-10^6

The length of the capillaries in this work is found in the Experimental section on p. 10217. Typical data for HPLC and CE should be provided previously during class lectures or laboratories, though some of the values are also provided in commonly used textbooks.

Q2. Using a form of the resolution equation, the authors remind the reader that there are two contributors to peak resolution.

(a) What are they?

Efficiency (peak sharpness/peak width) and selectivity are the two overall contributors to peak resolution. Many experimental variables can be changed to affect these two terms.

(b) Copy equation (1) below, then circle and label the terms that correspond to each of the contributors to Rs that you listed above.

$$R_s = \frac{\sqrt{L/H}}{4}\frac{\Delta t}{< t >}$$

The $\sqrt{L/H}$ term is equal to $\sqrt{N}$ and corresponds to the efficiency of the separation. The $\Delta t/< t >$ term corresponds to selectivity.

Q3. Why do the authors choose to target plate height as a means to improve resolution? What other parameters could they have targeted?

The authors choose to target efficiency by improving the plate height because the experimental factors that increase plate height are determined primarily by materials science. Advances in materials science, such as formation of colloidal crystals from nanoparticles, can improve plate height. Improvements to efficiency only increase resolution by the square root, so the authors might have chosen to target selectivity instead. This requires differentially affecting the partition coefficient of each analyte between the mobile and stationary phases. This can be achieved by varying the temperature or by varying the chemical composition of either the mobile phase or the stationary phase.

Q4. Based on the final paragraph of the introduction, what was the objective of this work?

The authors purpose was to explore theoretical possibilities for this new packing, not to develop a practical method. The goal was "to characterize the contributions of [a silica colloidal crystal packing] to plate height for proteins." (p. 10217)

Q5. Why do the authors use an electric field, rather than pressure, to drive these separations?

The pressure required to drive fluid flow through a column which such a packing would be immense. [This is the extent of the answer provided by my students; however, for those interested, a more detailed discussion is provided below.]

For a given flow rate, u, the pressure difference across the column required, ΔP, is given by

$$\Delta P = \frac{\phi \eta L u}{d_p^2}$$

where Φ is a flow resistance factor, η is the viscosity of the mobile phase, L is the length of the column, and d_p is the diameter of the packing particles (see, e.g., J.E. McNair, *et al. Anal Chem.* **1999**, *71*, 700). Because ΔP increases as the inverse square of the packing particle diameter, much higher pressure are required as diameter decreases. In contrast, electroosmosis is a surface-driven phenomenon that does not depend on particle diameter.

Q6. The authors use silanes to polymerize the packing material and to form what is likely to be a very thin layer of short carbon chains on the surface (i.e., d_f is extremely low). If the stationary phase is so thin, how do the authors know that chromatography, rather than just electrophoresis, is occurring?

On p. 10218, the authors note that "[t]he retention order (Lyz, RnaseA, CytC) is different from what we observe in a CE separation (Lyz, CytC, RnaseA) by the same as what we observe in a reversed phase separation." Because these two separation methods resolve analytes based on different chemical characteristics, they result in different retention orders.

Q7. Estimate the value of H for the lysozyme peak in Figure 2. Show your work to receive credit. Does your estimate match the authors'?

To calculate the plate height, *H*, from experimental data, it is easiest to first calculate the plate number, *N*, using

$$N = 16\left(\frac{t_r}{w}\right)^2 = 5.54\left(\frac{t_r}{w_{1/2}}\right)^2$$

where t_r is the retention time, *w* is the width, and $w_{1/2}$ is the full-width at half-maximum, which is often easier to estimate accurate than the width of the peak at its base. For lysozyme in the Figure 2 inset, t_r appears to be approximately 107 s and $w_{1/2}$ appear to be ~1 s:

$$N = 5.54\left(\frac{107\ s}{1\ s}\right)^2 = 63{,}000$$

H is related to *N* by *N = L/H.* The caption of Figure 2 states that the separation distance for the data shown is 0.91, so

$$H = \frac{L}{N} = \frac{0.91\ cm}{63000} = 1.4\ \times 10^{-5} cm = 140\ nm$$

The authors state that the plate heights for the peaks in Figure 2 are all less than 100 nm. Considering that we do not have the raw data, this calculation is in fairly good agreement.

(Note: students may estimate the values of t_r and *w* or $w_{1/2}$ slightly differently, resulting in varying degrees of agreement between their value and the authors'.)

Q8. Why is the plate height lower for these separations than for previous separations of dyes using the same packing material?

The authors state that the lower plate heights occur "presumably because the injected widths and diffusion coefficients are lower" (p. 10218). Students can be reminded that although we most often consider how to minimize the contribution of the column to peak width, the injection has a finite width that may also appreciably increase plate height. Additionally, proteins have much lower diffusion coefficients than dyes due to their larger size. As a result, the longitudinal diffusion term, *B*, in the van Deemter equation is smaller, leading to lower plate heights.

Q9. Why does heterogeneous packing increase the plate height? What term(s) in the van Deemter equation is/are affected by packing inhomogeneity like that seen in Figure 3?

Heterogeneous packing can lead to channels in which the packing particles are not well-packed, resulting in larger interstitial spaces in some parts of the column. This means that analyte molecules that pass through the void take a much less tortuous path than molecules that go through a well-packed section of column, a process called channeling. This primarily contributes to band broadening by increasing the *A* term of the van Deemter equation. Uneven packing also

means that some molecules will travel further through the column without encountering the stationary phase than others. This results in increased band broadening due to the mass transport term, *C* (specifically mass transport in the mobile phase, C_m).

Q10. In Figure 4C, why does peak variance increase linearly with time? In other words, what process causes this peak broadening?

Diffusion causes the peak variance to increase linearly with time. As seen in Figure 4C, the relationship between peak variance and time is given by $\sigma^2 = 2Dt$. The longer the plug spends on the column, the more diffusion contributes to band broadening through the *B* and *C* terms. [Note to instructors: In well-packed columns in this paper, the *C* term is negligible, but in typical HPLC columns this is not the case.]

In-Class Questions

Q1. When characterizing plate height for lysozyme in Figures 4 and 5, the authors determine the width of the peak in space rather than in time. They are able to do this because they are using a camera as a detector, but why do they need to do this? How does the width of the peak in space relate to the width of the peak in time? How does the detector contribute to plate height in these experiments?

To investigate band broadening in their columns, the authors needed to determine the variance, σ^2, of their peaks. The variance is found by fitting the peak to the equation for a Gaussian; however, the authors could not fit the peaks as a function of time because they were only a few points wide. This occurred because the camera needed to use a relatively long exposure time (0.2 s, given as the acquisition time on p. 10218) to capture the image of each peak because the channels are small and the analytes are dim. As a result, by the time the 0.2 s exposure time has passed, the peak is almost passed the detector. The authors can only capture a couple images of the peak as it passes.

However, each picture of the peak is many pixels wide on the camera sensor. This means the authors can fit the Gaussian peak in space very well, as seen in Figure 5. Because the width of the peaks in space is related to their width in time by the velocity of the peaks, the authors can then convert between these two data domains.

[Students can be prompted to sketch the peak as a function of time and as a function of space to help bring them to the conclusion described above.]

One caveat is that the authors must account for the fact that the detector is contributing some variance, $\sigma^2_{detector}$. This occurs because the peak is moving as the image is acquired. As a result, the peaks look a bit wider than they really are. (Image using a camera with a slow shutter speed to take an image of a sprinter. The picture of the runner looks smeared out wider than it should because the runner is moving as the image is exposed.) The authors account for this using Equation 3 on p. 10219 which relates the variance to the exposure time, τ, and the velocity of the peak, *v*.

Q2. In discussing Figure 6, the authors assert that the *A* and *C* terms of the van Deemter equation are negligible for their separations. In Figure 7 and the latter part of the Results & Discussion, the authors address whether the extremely low plate heights observed could

be due to focusing rather than to the achievement of a diffusion-limited separation. Why would the *A* and *C* terms be negligible under the conditions used in this work? What evidence supports the authors' assertion that the efficiency of their separations is limited only by diffusion?

The *A* term, Eddy diffusion, is negligible because the particle diameter is so small. Recall that *A* decreases with d_p. The *C* term is negligible because C_s is minimized by the very thin stationary phase since it is inversely related to d_f and because the C_m term is minimized by the small packing particles which result in very small interstitial spaces for analyte to diffuse across before encountering the stationary phase.

The authors present quite a bit of evidence that their peak widths are diffusion-limited. One of the easiest pieces of evidence for students to appreciate is the shape of the curve in Figure 6. This is a van Deemter curve, but rather than having the characteristic "Nike Swoosh" shape of a curve that includes a *C* term, it has the shape of the *B/u* term, which depends only on longitudinal diffusion. The authors also check to make sure that the diffusion coefficient obtained from the data in Figure 6 matches the diffusion coefficient in the absence of an electric field (Figure 7). This rules out the possibility of focusing that could occur if aqueous solution entered during the injection, increasing the retention of the front of the sample plug (since this is more polar than the mobile phase, which contained some acetonitrile). Finally, the authors calculated the expected diffusion-limited peak width based on the diffusion coefficient of lysozyme and the velocity of the peaks (Equation 5) and found good agreement with their experimental data.*

*This calculation is fairly hard to follow. I do not usually go through it in detail in class. We simply discuss how it is related to the authors' assertion that their separation is diffusion-limited.

Q3. The authors specifically state that their goal for this work was not to achieve a practical method for protein separations. That would have been outside the scope of this paper because many practical considerations would need to be addressed before this type of packing could be made available in commercial columns. Imagine that an instrument manufacturer wants to use columns like these in a commercial HPLC instrument. What changes to the instrument and practical improvements in the column would be needed?

The most important improvement to the column would be to eliminate the channeling issue by improving the packing process. On p. 10218, the authors state that about two-thirds of the columns they packed had gaps in packing at the walls. This would be unacceptable for commercial preparation of columns.

A traditional HPLC instrument would need to be significantly modified to accept these columns. The pressure system would need to be replaced with a high voltage power supply to produce electroosmotic flow. The injection system would also need to be modified for electrically-driven injections. The detector would need to be replaced with a highly sensitive camera, and the software would need to be re-written to accept the output of this detector and convert it into a chromatogram. Ultimately it would probably make more sense to have a dedicated instrument for electrochromatography rather than to retrofit these columns into an existing HPLC design.

Spectroscopy: Infochemistry using Atomic Emission Beacons

Article: C.N. LaFratta, I. Pelse, J.L. Falla, M.A. Palacios, M. Manesse, G.M. Whitesides, and D.R. Walt, "Measuring atomic emission from beacons for long-distance chemical signaling," *Anal Chem.* **2013**, *85*, 8933-8936.

This paper describes a method of sending information via atomic emission signals from emergency beacons consisting of alkali-metal doped string soaked in methanol. The authors describe a telescope for reliably quantifying atomic emission from three alkali metals at a distance of up to 1.7 km from the flame. This novel application is engaging to students while also addressing instrument design and fundamentals of atomic emission. Consequently, the assignment is designed to be used after previous in-class discussions of AES theory and instrumentation. Students who have taken linear algebra may appreciate the mathematics used to determine the instrument response matrix on p. 8935; however, the questions are designed so that it is not critical to understand these mathematical sections of the paper as long as students reach some conceptual understanding of why the response matrix is used (in-class question **Q2)**.

Out-of-Class Questions

The out-of-class questions ensure students understand new terms (**Q1**, **Q5**) and review concepts from previous class periods on atomic emission (**Q2**, **Q3**, **Q6**).

Q1. Define "infochemistry".

According to the abstract, infochemistry is "encod[ing] and transmit[ting] information using chemistry instead of electronics."

Q2. In this paper, the salts are atomized by combustion (i.e., a flame). What other methods for atomization for atomic emission are available?

Individual instructors may vary in their coverage of atomization methods for emission, but common methods presented in textbooks include arcs, sparks, lasers, and of course, inductively coupled plasma (ICP).

Q3. A methanol and air flame like the one used here typically obtains a maximum temperature of 2000 °C. What is the maximum temperature of an ICP? Why are high temperatures necessary for AES?

The maximum temperature of an ICP is approximately 10,000 °C. The high temperature is needed to excite atomic electrons (since atomic emission, unlike atomic fluorescence, does not use light to do this). The high temperatures of an ICP also reduce the formation of complexes, minimizing interference from broad molecular absorption bands.

Q4. Read section 10C-1 on p. 273 of the textbook. Why did the authors use alkali metals, instead of other elements, for this application?

The section and page number reference given are for Skoog, Holler, and Crouch, *Principles of Instrumental Analysis*, 6th ed., Brooks/Cole, © 2007. Alternatively, students can be referred to p. 628-629 in Chapter 10 of David Harvey's freely available text *Analytical Chemistry 2.0* on the

ASDL website. Both texts address the fact that most other elements are not excited by the low temperatures of a methanol flame.

Q5. What do the authors mean when they say the signal is "isotropic"? How is isotropic emission an advantage for this application?

Isotropic processes occur in all directions equally. In this application, isotropic emission means that light will be emitted in all directions, so that the person transmitting the signal does not need to consider in which direction the receiver might be located.

Q6. In the custom telescope in Figure 2, what components act as wavelength selectors? What wavelength selector is typically used in AES instrumentation? Explain the advantages of these two types of wavelength selectors for each application.

The telescope uses band pass filters (BP) and dichroic mirrors (DC) as wavelength selectors. Band pass filters pass a range of wavelengths above and below specific thresholds. Dichroic mirrors typically reflect light with a wavelength lower than a threshold and transmit light of longer wavelengths. More conventional atomic emission instrumentation uses a monochromator to disperse all wavelengths so that many different elemental lines can be measured. Filters are good for applications where a small number of specific wavelengths will be measured but tend to be impractical for applications that scan across a wide wavelength range.

[In my course, we discuss both filters and monochromators as wavelength selectors. For students who have not previously discussed the advantages and disadvantages of these options, this question could be moved to the in-class portion of the assignment.]

Q7. What signal processing method was used on the data in Figure 3? What are the advantages of this method for this application?

The signal is processed using boxcar averaging. [This question can be omitted from classes that do not discuss signal processing.] Students who are familiar with these techniques, however, should note that this method can be done in real-time without intensive computational resources; it does not require a repeatable signal (as would ensemble averaging); and although it can result in loss of temporal resolution, this is not an issue for this application since the beacons should burn steadily, and no useful information is encoded in their variation in time.

Q8. Estimate the signal-to-noise ratio of the processed cesium signal in the top right panel of Figure 6.

[This question is a review of signal-to-noise calculations and can be a useful reference for the in-class discussions of the distance LOD (in-class question **Q1**).]

The average signal, *S*, is approximately 0.8 V. The peak-to-peak noise is approximately 1.3-0.6 V = 0.7 V. Assuming that the root mean square noise, *N*, is approximately one fifth the peak-to-peak noise gives $N \approx 0.7\ V/5 = 0.14\ V$.

$$\frac{S}{N} = \frac{0.8\ V}{0.14\ V} = 6$$

Q9. Go to the *NIST Handbook of Basic Atomic Spectroscopic Data* and look up the most intense persistent strong line(s) between 700-900 nm for Na, Li, and Ca in air. Lines indicated "P" next to the intensity refer to persistent lines, which are detectable even for low concentrations of the element in the presence of other species.
http://www.nist.gov/pml/data/handbook/index.cfm#

[Along with out-of-class **Q10**, this question ensures that students have the information needed to answer in-class **Q4**.]

Sodium: 819.4824 nm

Lithium: 812.6453 nm

Calcium: 854.2089 and 866.2140 nm

Q10. Peruse the bandpass filters available from Spectrofilm.com (the vendor used for parts in this manuscript). What is the narrowest range of wavelengths that can be passed by these commercial filters? How does this compare to the width of an atomic emission line?

In addition to the lines above, the elements used in the paper were detected at 766, 852, 780 nm for K, Cs, and Rb, respectively. The closest lines are Cs at 852 nm and Ca at 854 nm. The Spectrofilm bandpass filters are available with bandwidths as narrow as 1 nm. The width of the atomic emission lines is 1.5-5 pm (even after Doppler broadening and other contributions beyond the natural line width), which means the two wavelengths will be well-separated.

In-Class Questions

Q1. Consider Figure 4.

This question addresses a fundamental property of light, LOD calculations for non-linear relationships, and the practical considerations needed in designing beacons based on emission.

(a) Show the calculation used to determine the distance limit of detection of 1.7 km.

From Figure 4, the equation for the calibration plot is $y = 50760x^{-1.94}$, an inverse square relationship as expected based on the decrease in intensity of light with distance. The standard deviation of the blank, s_{bl} = 9 mV, which means the signal LOD = 3*9 mV = 27 mV. Plugging this value in for *y* in the calibration plot gives us the distance at which the signal will drop to LOD:

$$0.027 = 50760x^{-1.94}$$

$$x = \left(\frac{0.027}{50760}\right)^{1/-1.94}$$

Solving for *x* gives 1700 m, the reported LOD.

(b) If the authors wanted to double the distance limit of detection, by what factor would the intensity of the atomic emission need to be increased?

The intensity is related to distance by an inverse square relationship (rounding from -1.94 to -2 for the exponent, as predicted by theory). As a result, increasing the distance by a factor of 2 means that the intensity of the signal must be increased by a factor of 4.

[Students may initially try to solve this problem using the calibration curve equation from (a). It is helpful to explain that an increase in the signal means that this calibration curve will change since the same distance will give a higher signal value.]

Some students with good mathematical intuition will readily appreciate the explanation above. Students who value a more concrete mathematical explanation may find the following helpful:

$$2x = 2 * 1700\ m = 3400\ m$$

We need the signal at 3400 m to be 27 mV, assuming that the LOD is improved by increasing the signal rather than decreasing the noise. The current signal at 3400 m is 7 mV, as shown below:

$$y = 50760(3400)^{-1.94} = 0.007$$

If the signal LOD is 27 mV, then this means that the signal must increase by a factor of 27/7, or 3.9. (The discrepancy between the 3.9-fold increase in intensity calculated here and the 4-fold increase estimated above arises because the exponent used in these explicit calculations is -1.94 rather than -2.)

(c) Is it reasonable to expect that this increase in intensity (needed to double the distance limit of detection) could be achieved by increasing the concentration of the metal salts? Explain your answer.

For this question, I encourage the students to visualize the process of preparing the flares, burning them, and detecting the light. Possible complications could involve solubility limits of the salts being used and self-absorption of the emission at high concentrations.

If desired, students can investigate this question more quantitatively. The supporting information states that between 15-375 µL of 1 M solutions of the nitrate salts were used to prepare the beacons. Students can research the solubility limits for the three salts, which are very near 1 M. At 20 °C, the limits for nitrate salts of cesium, potassium, and rubidium are 23 g, 33 g, and 53 g per 100 g of water respectively. Ignoring changes to solvent volume, these concentrations correspond roughly to 1.2 M, 3.3 M, and 3.6 M. These values mean that increasing the concentration of each salt in the beacon by a factor of 4 would be impossible to do simply by increasing the concentrations of the solutions used to prepare the beacons. Depending on the time allotted for this question, the supporting information and solubility limits can be given to the students so that they can do these calculations themselves, or this information can be presented by the instructor once all the groups have reported on their discussions.

Q2. In the top panels of Figure 6, why is it that the cesium signal can be the highest magnitude in the raw data, but is at the "low" level after decoding in the processed data?

This question is meant to lead into **Q3**, which asks students to explain the instrument response matrix. The emission of Cs is higher at all concentrations than the emission of the other

elements. The high and low levels of Cs relative to the other elements are only apparent after normalization using the instrument response matrix. The higher intensity of the Cs emission compared to the K and Rb emission at equal concentrations (as seen in Figure 3) is one of the more easily grasped difference between the three "channels" used to encode information in the beacons.

Q3. What is the purpose of the instrument response matrix and what factors affect it?

While the mathematics on p. 8935 of the article may not be accessible to students who have not taken linear algebra, the text does provide some guidance here, noting on the same page that "The 3 × 3 instrument response matrix, **R**, corrects for differences between the three channels of the detector and any crosstalk." The key is for students to consider what these two items mean and explain them in their own words. Difference between the three channels include differences in the intensity of the emission from the three metals, as highlighted in the previous question, **Q2**. Other differences might include the sensitivity of the PMTs to specific wavelengths and other imperfections in the instrumentation. In my experience, this may be the first time that students consider, for example, that filters may not be perfect. If students have not looked up the term "crosstalk" or learned about it in a previous class, a short conversation about what this term means is helpful. In particular, students will probably need to be told that crosstalk can be spectral as well as electrical.

Q4. The authors suggest that greater information density could be achieved by adding Na, Li, and Ca to the signal. Do you think that this would be feasible? Support your answer using the information you looked up for out-of-class questions 9 and 10.

See notes for out-of-class **Q10** above. Make sure that the students consider both the width of the bandpass filters and the width of the emission lines. Encourage students to consider the added complexity of the instrument with the addition of three more channels. For students who have not taken a physics class with an optics component, it may be helpful to tell them that light is lost at each interface (mirror, filter, etc.) and have them consider the effect of additional signal channels on LOD. Some students may suggest redesigning the telescope to include a monochromator, leading to a discussion of cost and the increased complexity of instruments that involve moving parts.

Q5. The authors suggest that this beacon could be used to transmit data in resource-poor environments, such as natural disaster sites. Evaluate the feasibility of this application given the information in the manuscript.

Students should consider the distance LOD, the information density, the susceptibility of the technique to sunlight and adverse weather conditions. When students have concluded fairly quickly that the telescope is not practical for emergency situations, I often start a conversation about whether this research and the idea of "infochemistry" in general might have other applications or value.

Mass Spectrometry: Fruit Fry Proteomics and Aging

Article: R.A. Sowell, K.E. Hersberger, T.C. Kaufman, and D.E. Clemmer, "Examining the proteome of *Drosophila* across organism lifespan," *J. Proteome Res.*, **2007**, *6*, 3637-3647.

This article describes an in-depth study of the changes in the identity and abundance of the proteins present in fruit fly heads as a function of fly age. Three methodologies, LC-MS/MS, SCX-LC-MS/MS, and LC-IMS-MS, were used, and the article discusses the relative sequence coverage obtained by each technique. The assignment assumes prior knowledge of ESI, common mass analyzers, and peptide fragmentation patterns (i.e., *b* and *y* ions). The article by Gary L. Glish and Richard W.Vachet, "The basics of mass spectrometry in the twenty first century," *Nature Rev. Drug Discov.* **2003**, *2*, 140-150 is a good resource. No previous knowledge of IMS is required, as briefly researching this technique is part of the assignment. The LC-IMS-MS is a home-built instrument, which gives students an opportunity to identify components in an instrument block diagram. Additionally, the 2D separations demonstrate the "Big Data" obtainable by shotgun proteomics and provide opportunities for students to practice interpreting complex data plots. The latter part of the discussion requires more biological background knowledge, and the assignment has been designed to focus more heavily on the earlier section of the paper describing the separations and MS instrumentation. However, questions could be added on the later sections in a class for biochemistry majors.

Out-of-Class Questions

Q1. In the paper, the authors focus solely on proteins expressed in the fly's head.

(a) Give one reason for this choice.

The authors give two reasons to focus on the flies' heads as opposed to the bodies (p. 3638). First, previous studies of the transcriptome demonstrated an observable difference between fly heads and bodies. Second, studying the head makes the work more applicable to studies of neurology. [On p. 3637, the authors list many reasons to study fruit flies, but none of these explanations are specific to studies of the head.]

(b) How were the fly heads separated from the bodies?

From p. 3638: "Briefly, heads are obtained as follows: animals were anesthetized by exposure to CO_2 gas, transferred to Nalgene tubes, and frozen by submerging the tube in liquid nitrogen; tubes were shaken by hand several times to separate body parts, and heads were collected over a bed of dry ice and stored at -80 °C until further use."

Q2. Based on the details in the sample preparation section, about how many flies were used in this study?

From p. 3638: The authors pooled 175 heads/time point for 8 time points. This pooling strategy was conducted twice, for a total of 2*175*8 = 2800 flies. The authors state that "time-point 9 includes only 100 heads due to the lack of living animals that remained at later ages." So the total number of flies can be estimated as 2900.

Q3. Review or research the chromatographic method SCX. What is SCX, and how does it work?

SCX stands for strong cation exchange chromatography, which is a separation method based on the affinity of the cations in the sample for an anionic resin (e.g., with sulfonic acid residues) as the stationary phase. Retention times depend on ionic interactions, and therefore on the charge and size of the ions.

If students have not covered ion exchange chromatography previously, they can be directed to p. 635 in the 8th edition Harris text, p. 839 in the 6th edition Skoog text, or p. 844 in Chapter 12 of the freely available Harvey text, Analytical Chemistry 2.0.

Q4. What ionization method was used for these experiments? What is one advantage of this ionization method for these experiments?

Electrospray ionization (ESI) was used for the LC-IMS-MS experiments (see Figure 1). Although unspecified, it is most likely that ESI was also used for the LC-MS/MS experiments and SCX-LC-MS/MS experiments.

Advantages of ESI for this application include its easy coupling to condensed phase effluent from LC, soft ionization with minimal fragmentation for easy database searching, and multiple charges for MS analysis of heavier analytes (although singly-charged tryptic peptides would typically be within the mass range of both a quadrupole and a TOF).

Q5. Look up ion mobility spectrometry (IMS). How does this technique work? How does it differ from TOF-MS in the experimental parameters and the separation of analytes?

In IMS, the drift tube is filled with a low pressure of buffer gases (~2.6 Torr He and 0.1 Torr N_2 in this work), and the ions are separated in a weak electric field (~11.67 V/cm in this work) based on ion mobility, which depends on mass, cross-sectional area, and charge. Cross-sectional area influences the drift time of the ions because ions with larger cross-sectional areas will experience more collisions with the buffer gas, resulting in longer drift times. The conditions in the drift tube are in contrast to those of a TOF flight tube, which consists of a field-free region kept at high vacuum (~10^{-6} Torr). As a result, TOF analyzers separate ions based on mass and charge. Cross-sectional area has a negligible influence on flight time because the probability of collisions is low.

Q6. What is the total pressure in the IMS drift tube? Calculate the mean free path of an ion in an IMS drift tube and compare it to the mean free path of an ion in the mass analyzer used for the MS analysis part of the IMS-MS experiments.

The total pressure in the IMS drift tube is 2.6 Torr + 0.1 Torr = 2.7 Torr or 2.7×10^3 mTorr.

Mean free path, λ, can be determined by the equation

$$\lambda = \frac{k_B T}{\sqrt{2}\pi d^2 P} \approx \frac{5\ cm}{P\ (mTorr)} \text{ for a small molecule at room temperature}$$

where k_B is Boltzmann's constant, T is the temperature, d is the diameter of the molecule, and P is the pressure. [I present this simplified version of the equation in class before this assignment is due.]

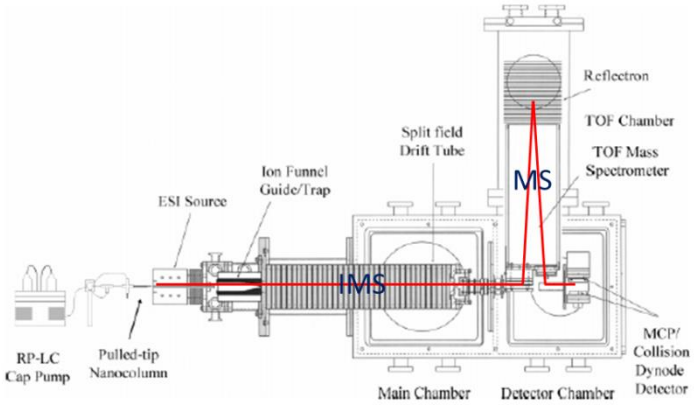
For the IMS, $\lambda \approx 5\ cm/(2.7 \times 10^3) = 2 \times 10^{-3} cm$

For the TOF, $\lambda \approx 5\ cm/(10^{-3}) = 5 \times 10^3 cm$

The much shorter mean free path in the IMS results in collisions which separate ions in drift time based on cross-sectional area.

Q7. Consider Figure 1.

(a) Sketch the path of ions from the source to the MS detector, and label the IMS and MS regions.



(b) What type of mass analyzer is used for the MS analysis on the IMS-MS instrument?

The mass analyzer on the IMS-MS instrument is a time-of-flight (see Figure 1).

(c) What type is on the LC-MS-MS instrument?

The mass analyzer on the LC-MS-MS is a quadrupole.

(d) Which has higher mass resolving power?

A TOF has higher mass resolving power than a quadrupole. A TOF typically has a value of *R* in the thousands or even above 10,000, while a quadrupole usually has unit resolution (Δm = 1 with $R = m/\Delta m$ depending on mass).

Q8. Briefly and *in your own words* describe how mass spectra were assigned (matched) to specific peptides.

In evaluating student responses, I look for correct application of the underlined concepts:

The spectra were matched against a database (the National Center for Biotechnology Information *Drosophila* protein database) using an algorithm (MASCOT). At least one unique peptide hit was required for assignment of a protein, and results from IMS-MS experiments were confirmed visually to reduce the false positive rate. (See p. 3639).

Q9. What "semi-quantitative" methods were used to measure changes in protein abundance with fly age in this work?

Changes in protein abundance were estimated based on (1) the number of unique peptide hits identified for each protein and (2) the integration of peaks from the LC-IMS-MS data sets. In

method (1), the number of hits was normalized to the number of hits at time point 2 (ages of 8-14 days). Method (2) was used to validate or further investigate trends identified by method (1), and the integration values were normalized to the total ion signal at each time point.

Q10. The bottom part of Figure 2 shows the base peak chromatograms for three separations. Define base peak. What is a base peak chromatogram?

The base peak is the most intense peak in the mass spectrum. (The other peak intensities are often normalized to this peak, which is assigned an intensity of 100.)

A base peak chromatogram plots the intensity of the base peak in each mass spectrum (*y*-axis) versus the separation time (*x*-axis).

Q11. How effective was the SCX-LC-MS/MS method at identifying peptides compared to the LC-MS/MS method? What do you think is the reason for this difference in performance when an SCX step is added?

The addition of the SCX step greatly increased the number of peptides (and therefore proteins) identified from 1102 peptides assigned to 367 proteins to 5430 peptides assigned to 1437 proteins.

The improved performance is likely due to the increased peak capacity with the two-dimensional separation. By further separating the samples before MS analysis the data are cleaner and mass spectra are easier to match.

Q12. Which proteins in Figure 6 change with the flies' age? What biological processes are associated with these proteins?

Prophenoloxidase and CG4784 both decrease with age. "[P]rophenoloxidase is an enzyme that catalyzes the synthesis of melanin as a defense response and part of wound healing" (p. 4643). CG4784 is a component of the cuticle (exoskeleton).

Q13. How do the authors' results compare to results obtained by more traditional methods used to study proteins?

[For this section, students who do not have a biology background will benefit from an explanation of the central dogma of biology: DNA → RNA → protein and the information that Western blotting is a traditional method of assessing protein expression in molecular biology.]

The authors' data for certain proteins agree with previous Western blotting results. For example, data on heat shock protein 22 and larval serum protein 2 are consistent with previous studies (p. 3644).

The authors' results show mixed comparisons to previous data on transcript levels. They note significant disagreement with reference 15 in terms of whether changes were observed with age and in which direction (p. 3644-3645). The authors suggest that fly strain, sex, or temperature of growth could account for some of these differences. [There is also precedent in the literature to suggest that transcript levels and protein levels are not always tightly correlated due to additional regulatory components.] In a few cases, the authors' data on protein levels corresponded to the transcript data. For example, CG15093 and *Punch* were down-regulated with age in both studies, while Cyp28a5 and S-adenosyl-L-homocysteine hydrolase were up-regulated.

In general, the biological trends observed in this study seem reasonable: down-regulation of metabolic components and reproductive proteins with age and changes to proteins involved in defense response (p. 3645-3646).

In-Class Questions

Q1. Consider your answer to out-of-class question 7c. How are tandem MS experiments achieved on the LC-MS-MS system? How does fragmentation occur, and what types of fragments are formed? How do these fragments yield information about the peptides in the sample?

As noted in out-of-class question 7c, the mass analyzer is a quadrupole. Because this is a single quadrupole (rather than a triple quad), the instrument is being operated as a tandem-in-time MS-MS instrument. The precursor ion is selected and fragmented by collision-induced dissociation (CID) in the mass analyzer. The product ions are then scanned out to obtain the MS-MS spectrum. For peptides, CID typically produces *b* and *y* ions. A series of *b* or *y* ions can be used to *de novo* sequence the peptide or matched to a database mass spectrum for identification of the peptide and corresponding protein.

[I spend a class period discussing MS-based proteomics before assigning this article. The article by Ruedi Aebersold & Matthias Mann, "Mass spectrometry-based proteomics," *Nature*, **2003**, *422*, 198-207 is a good resource for this.]

Q2. Proteomics experiments often produce vast quantities of data.

(a) In Figure 4, what is being shown in the panels on the far left of the figure? How does this data relate to the data in the six panels to the right?

The panels on the far left are heat maps showing the LC-IMS results. The false color indicates the peak intensity for each retention time, drift time pair. Each panel on the right is a mass spectrum from the TOF analyzer and corresponds to a single pixel in one of the three left-hand panels. In other words, each pixel in each panel on the right has a corresponding mass spectrum – a huge quantity of data.

(b) In your opinion, should authors be required to share all of the raw data used to construct figures in published articles? Why or why not?

It is often productive to have students discuss this question along with question (c) since few students think that no raw data should be shared. If the discussion is foundering, I find it helpful to point out how little biological interpretation has been done of the results in this paper (considering the huge number of proteins identified). As a results, future targeted biological studies may wish to cross-reference results with proteomics studies like these.

(c) When data is shared, who should bear the cost of the servers, web development and other infrastructure needed to do so?

Some possibilities include individual researchers through their grants, government organizations like the National Science Foundation and National Institutes of Health, the publishers of the journals the data appears in, or other researchers who choose to access the data. You may think of others.

The duration of data availability and the need to make data formats compatible with new software versions or programs are additional considerations. I have sometimes assigned an article by Julie Manoharan, "Thank you for sharing," *Biotechniques*, **2011** as a follow-up reading.

Q3. Quantitation by mass spectrometry in proteomics is challenging because the wide variety of peptide chemistries result in variable MS signal between peptides, and ionization efficiency can also change over the course of an LC run.

(a) Why didn't the authors use internal standards to account for these differences? How were they able to compare the results from the different samples in the absence of an internal standard?

For complex mixtures like these protein digests, numerous internal standards would be needed to accurately reflect all the analytes. Additionally, these internal standards would need to be readily separated from the analytes during the chromatographic or mass spectrometric steps. This would be difficult and likely expensive. [Quantitative mass spectrometry experiments for proteomics often used isotope labeling of some kind to introduce internal standards.]

Although internal standards were not used, the authors were still able to compare results semi-quantitatively using peptide hits and peak integration as proxies for protein abundance. To improve the quality of their data interpretation, the authors only looked at changes in abundance with age for proteins that had a difference of at least 5 peptide hits between time points. The authors also separated the proteins out by the magnitude of change in their peptide hits ($\geq$1.5, $\geq$2.0, or >10) to separate small and large changes in expression. (See p. 3644 for detail.)

(b) In Figure 6, what is the difference between the open symbols and the filled symbols?

The open symbols are from the normalized peak intensities (integrated as described on p. 3640). The closed symbols are the normalized number of peptide hits.

(c) Consider the Figure 6 data and your answer to out-of-class question 13. Do you think the two "semi-quantitative" methods used for analysis are reliable? What limitations do these techniques face?

Based on Figure 6, the semi-quantitative methods agree with one another, which suggests that they are at least reasonably reliable. As discussed in out-of-class question 13, the results from these semi-quantitative method often (but not always) agree with results of more traditional measures of protein expression, further suggesting that the methods are reliable.

The comparisons are limited in that they are semi-quantitative, relative for each protein, and may not capture changes in protein abundance near the detection limit accurately. While imperfect, these methods are practical with current technology and seem to provide useful biological information.

Electrochemistry: Fast Scan Cyclic Voltammetry in Drug-Seeking Rats

Article: P.E.M. Phillips, G.D. Stuber, M.L.A.V. Heien, R.M. Wightman, and R.M. Carelli, "Subsecond dopamine release promotes cocaine seeking," *Nature*, **2003**, *422*, 614-618.

This article describes the results of experiments using fast scan cyclic voltammetry to monitor dopamine release in the brains of drug-seeking rats. While students do not need any specific background in neuroscience to understand the article, they should be reminded to look up unfamiliar biological terms as needed. Additionally, it is important to discuss **Q13** from the out-of-class assignment at the start of the in-class portion to ensure that the students understood the experimental design and key results reported in each figure. **Q1** of the in-class questions assumes that the students have previously encountered common spectroscopic methods and mass analyzers for MS; if this is not the case, this question can be omitted.

Out-of-Class Questions

Many of the out-of-class questions are designed to encourage the students to familiarize themselves with the study and related neuroscience terminology (**Q1-Q4, Q6-Q7, Q9**).

Q1. What is the difference between tonic and phasic dopamine signaling?

From the abstract, tonic signaling occurs on a minute-to-minute timescale, while phasic signaling occurs on a subsecond timescale.

Q2. Look up any other unfamiliar words in the abstract. Then summarize this study in one complete sentence.

Two common terms that students need to research are ventral tegmental area and nucleus accumbens:

ventral tegmental area: a section of the midbrain composed of dopamine-containing neurons that are involved in drug-seeking and reward circuitry

nucleus accumbens: a section of the forebrain involved in reward, pleasure, and laughter as well as aggression and fear

I look for some aspect of the four underlined components of the sentence below when evaluating student summaries of the study:

This study investigated subsecond (or phasic) dopamine signaling in the nucleus accumbens using fast scan cyclic voltammetry (or electrochemical measurements) in rats engaged in drug taking.

Q3. How were the rats taught to self-administer cocaine? How was the cocaine delivered?

As described on p. 617, the rats were trained to self-administer cocaine during 2-h sessions during which they were presented with a lever illuminated by a cue light. When the rats pressed the lever, the cocaine dose was administered through a jugular catheter.

Q4. What stimulus accompanied cocaine delivery?

As described on p. 617, cocaine administration was accompanied by a change in lighting and "a continuous auditory tone".

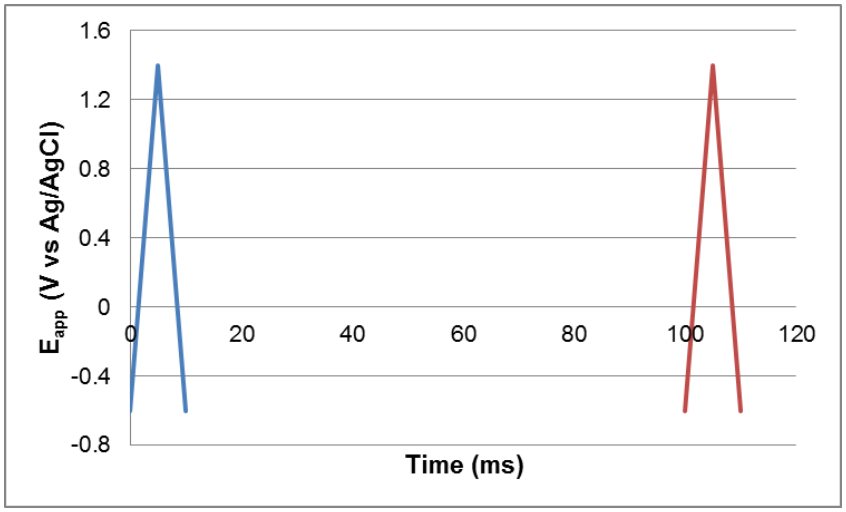
Q5. What were the working and reference electrodes in this study? Why wasn't a counter/auxiliary electrode necessary?

As described on p. 617, the working electrode was a carbon fiber microelectrode and the reference electrode was Ag/AgCl.

The latter question is meant for classes that have discussed the use of microelectrodes previously. For the very small currents that pass through ultramicroelectrodes, ohmic losses are negligible, and a two-electrode system does not pose any problems to accurate measurement.

Q6. Sketch the potential applied to the working electrode as a function of time. Include as much detail in your plot as possible.

Students should label the time for each half-wave and the time between cycles in addition to the applied potentials. Some students may not initially realize that they can calculate these times from the maximum and minimum applied potentials (-0.6 V and +1.4V) and the scan rate (400 V/s), but usually at least one student in a group will identify this calculation. The potential on the electrode between scans is not specified in the manuscript. Students often assume that the potential is held at -0.6 V. The electrode was probably allowed to float (no current sourced or sunk) or may have been held at an unspecified potential relative to the reference. The plot below does not indicate the potential between scans.



Q7. How many cyclic voltammograms (scans) were acquired per second?

Scans were acquired at a frequency of 10 per second (one scan every 100 ms, as described on p. 617).

Q8. How does the scan rate in these experiments compare to a typical CV scan rate? How does scan rate usually affect CV data?

The scan rate for these experiments was 400 V/s. If students have performed a laboratory exercise on cyclic voltammetry, they can refer to the range of scan rates used (e.g., 100 mV/s to 1 V/s). Otherwise, this information may come from a previous lecture or their textbook. For example, p. 737 of the Skoog textbook gives a 50 mV/s scan rate as an example.

For reversible reactions, peak current (i_p) increases linearly with the square root of the scan rate (v), as described by the Randles-Sevcik equation, $i_p = 2.686 \times 10^5 n^{3/2} A C D^{1/2} v^{1/2}$.

Q9. How did the authors correct for current from interferents, movement of the animal, and pH changes in the extracellular space?

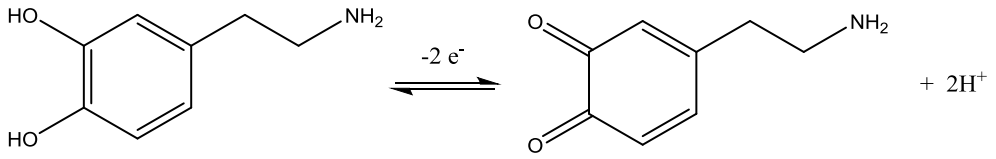
On page 617, in the methods section on fast-scan cyclic voltammetry, the authors note that artifacts from pH change or movement were correct by using a differential measurement was made between currents that occurred for dopamine oxidation at +0.7 V vs Ag/AgCl and another potential at which dopamine oxidation does not occur, but the interfering currents did.

This question, along with **Q10**, should highlight for the students the problem of selectivity at an unmodified electrode. Students should be referred to these questions as needed in their discussions of in-class **Q3**.

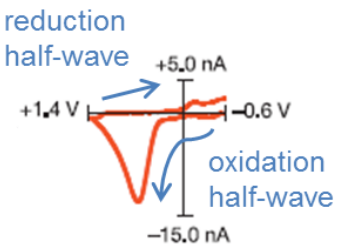
Q10. Complete the table below summarizing the means used to demonstrate that their signal came from dopamine release.

Experiment	How did this demonstrate the signal was from dopamine release?
anatomical	Post-mortem histology was used to verify that the electrode was placed in the nucleus accumbens.
physiological	Electrical pulses were used to stimulate dopamine release and confirm its detection before and after each experiment.
chemical	Cyclic voltammograms recorded in the live animals were compared to CVs from *in vitro* standards.
pharmacological	An MAOI was administered to suppress the potential interferent, DOPAC, and no difference in signal intensity was observed.

Q11. Draw the half-cell reaction for the oxidation of dopamine to dopamine quinone.



Q12. Consider the CV inset in Figure 1a. Label the oxidation and reduction half-waves. Was the oxidation of dopamine reversible? How can you tell?



The oxidation was not reversible, as evidenced by the lack of current on the reduction half-wave.

Q13. Calculate the S/N ratio for the trace in Figure 3a.

This question is an opportunity for students to practice calculating SNR while also encouraging them to examine the data in a paper closely.

Measuring with a ruler, I estimate that the peak-to-peak noise is approximately 3/16" while the peak height is approximately ½". Treating the peak-to-peak noise as roughly five times the rms noise gives

$$\frac{S}{N} = \frac{0.5}{0.2(\frac{3}{16})} = 13$$

Q14. Consider Figures 1-4. In the table, summarize the experimental design used for each experiment and the major results. In the column labeled stimulus/conditions, record what stimulus, if any, was used to elicit dopamine release or drug-seeking behavior. If no stimulus was used, note the conditions of the experiment. In the column labeled dopamine release, estimate the concentration of dopamine released in nM. In the column labeled timing, summarize any important findings about how the timing of the dopamine release related to the timing of the stimulus.

This question is helpful for the in-class discussion since it collects the most relevant points about each figure into one location. A quick check of student answers to this question is helpful before discussions start to ensure that the students are reading the figures correctly.

Figure	Stimulus/Conditions	Dopamine Release (nM)	Timing of Response
1	electrical stimulus train (24 pulses at 60 Hz)	~700 nM	Appears instantaneous, but not quantified in the text
2	no stimulus, rats were seeking and obtaining cocaine by lever press	64.9 ± 16.1 nM (post-response)	7.7 ± 0.6 s before lever press, small peak; 3.7 ±0.5 s before lever press is final approach which starts large peak.

3	audiovisual stimulus (no cocaine)	93.9 ±12.2 nM	peak starts at 0.1 ±0.5 s after stimulus
4	electrical stimulus train (24 pulses at 60 Hz every 120 s)	n/a	animals had highest probability of lever-pressing 5-15 s after stimulus

Notable features of the data summarized in this table are that there was a spike in dopamine before cocaine was actually administered (Figure 2), that a large dopamine response (comparable to the response from cocaine) was observed in the absence of drug simply by recreating the conditions of drug-use (Figure 3) and that dopamine release triggered drug-seeking behavior (Figure 4).

In-Class Questions

Q1. Suggest some advantages of electrochemical detection of dopamine for this application, compared to spectroscopic or mass spectrometric detection.

This question can be omitted in classes that have not covered spectroscopic and mass spectrometric methods. A major advantage is the ability to miniaturize electrodes for electrochemical detection for implantation into the brain of a rat. Because the measurements can be made *in situ* without derivatization or removing samples via microdialysis, the authors are able to achieve the temporal resolution needed for measurements of phasic dopamine signaling. The sensitivity of electrochemical detection, especially when miniaturized, is also an advantage since the dopamine concentrations are in the nanomolar range. Spectroscopic methods are often less amenable to miniaturization because of dependence of signal strength on path length.

Q2. Why was the cyclic voltammetry data not sufficient to identify the signal as coming from dopamine? (Why do the additional experiments that you summarized in out-of-class question #10?)

Refer students to out-of-class questions **Q8** and **Q9**. Students who are unfamiliar with biological experiments may be surprised at the large number of controls needed. Additionally, this question is a useful opportunity to discuss the selectivity of electrochemistry at unmodified electrodes, which is often poor, and the use of controls or chemically modified electrodes to address this.

Q3. In Figure 1, where do the values for the color scale in the bottom panel come from? What plot would you obtain if you took a slice horizontally through the color plot? What would you obtain for a vertical slice?

This question is an opportunity for students to interpret 2D data plots requiring color mapping as a third dimension. Most students readily identify the color scale as representative of the current, but further thought and occasional prompting are required to answer the subsequent questions.

The blue trace above the color map in Figure 1 is a horizontal slice. These slices show the current at a given potential as a function of time. If one chooses the correct potential, i.e., +0.7 V vs Ag/AgCl, this current is correlated with dopamine concentration, yielding a trace that shows the spike and subsequent decline in dopamine concentrations after the stimulus. The red cyclic voltammogram is a vertical slice through the data, folded back on itself. The continuous appearance of the data along the time axis is only possible because the authors used fast-scan cyclic voltammetry and were able to record a CV every 0.1 s (out-of-class **Q6**).

Q4. Suggest one software-based and one hardware-based method to improve the S/N or Figure 3a, and justify your choices.

This question is best suited for an instrumental class that has covered a variety of hardware and software methods to reduce noise. It may be helpful to quickly review hardware and software methods from earlier in the semester before the students begin their discussion of this question. Students can generate a list of hardware methods, such as grounding, shielding, hardware filters, lock-in amplifiers, etc. and a list of software methods, such as ensemble averaging, boxcar averaging, moving average, Fourier transform based filters, etc. In evaluating these possibilities, students should consider the fact that the goal is to collect data with high temporal resolution, which precludes extreme low-pass filtering. Additionally, students should consider that a rat brain is not a static, homogeneous system. Individual rats and even individual stimuli in the same rat may not consistently produce the same response. Instead, individual responses are likely to vary somewhat both in their timing and their magnitude.

Q5. Individuals recovering from drug addiction are often counseled to avoid "triggers", including locations and situations in which they previously used drugs. Do the findings in this study support this advice? Why or why not? Cite a specific figure or figures in your answer.

Encourage students to build a logical progression from one dataset to the next, logically linking stimuli and responses, as shown below. Further conversation could include the limitations of animal models for complex human behaviors.