Penn State RET in Interdisciplinary Materials

Teacher's Preparatory Guide

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Distinct aqueous phases without a membrane, complex coacervates, create a different sort of phase diagram

Purpose This lab is designed to help students understand the following Big Ideas from the AP Chemistry course description:

- Charge concentration (an extension on stoichiometry) (AP Chemistry Unit 4)
- Intermolecular Forces, solubility, organic functional groups influence on IMFs (AP Chemistry Unit 3)
- Synthetic polymers, biopolymers (AP Chemistry Unit 2, 3)
- Organic acid deprotonation (AP Chemistry Unit 8)
- Phases, phase diagrams between aqueous phases (AP Chemistry Unit 3)
- Energetic Favorability, enthalpy and entropy (AP Chemistry Unit 6, 9)
- Spectroscopy, Beer's Law, turbidity (AP Chemistry Unit 3)

Objectives Students will:

Main Lab:

- Create a crude physical phase diagram of a liquid-liquid two aqueous phase system with turbid coacervates in a bulk aqueous phase
    - Along one axis of a 96-well plate, the ratio of concentrations of the two polymers that coacervate when mixed will change
    - Along the other, the concentration of NaCl will change
    - Explain why coacervates form and the effect NaCl has on coacervates using structural diagrams and intermolecular forces
- Interpret a photo of their data by eye or with ImageJ to determine the degree of coacervation
- Create a graph of their data to refine their phase diagram

Extensions and demos:

- Use aqueous coacervates to separate a dye without a separatory funnel and organic solvents and explain the partitioning using intermolecular forces from the structural diagrams
- Explain why coacervates dissolve in the bulk aqueous phase at low pH using structural diagrams and intermolecular forces
- Explore the temperature dependency of coacervates by warming and cooling them to cause phase transitions. Explain in terms of thermodynamics of an entropically-driven process.

Time required The main lab requires 45-50 min but with extensions and demos, the full lab requires three times that.

Developed by Steven Schulz and Dr. Christine Keating
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Level High school AP Chemistry, first year college general chemistry

Pennsylvania Integrated Standards for Science and Ecology, Grades 9-12

Physical Science

Structure and Properties of Matter

2. Plan and conduct an investigation to gather evidence to compare the structure of substances at the bulk scale to infer the strength of electrical forces between particles.

4. Communicate scientific and technical information about why the molecular-level structure is important in the functioning of designed materials.

Chemical Reactions

1. Construct and revise an explanation for the outcome of a simple chemical reaction based on the outermost electron states of atoms, trends in the periodic table, and knowledge of the patterns of chemical properties.

2. Develop a model to illustrate that the release or absorption of energy from a chemical reaction system depends upon the changes in total bond energy.

4. Refine the design of a chemical system by specifying a change in conditions that would produce increased amounts of products at equilibrium.

Energy

5. Develop and use a model of two objects interacting through electric or magnetic fields to illustrate the forces between objects and the changes in energy of the objects due to the interaction.

Life Science

Structure and Function

2. Develop and use a model to illustrate the hierarchical organization of interacting systems that provide specific functions within multicellular organisms.

3. Plan and conduct an investigation to provide evidence that feedback mechanisms maintain homeostasis.

Engineering, Technology, and Applications of Science

Engineering Design (Define Problems, Develop Solutions and Improve Designs)

2. Design a solution to a complex real-world problem by breaking it down into smaller, more manageable problems that can be solved through engineering.

Teacher Background

Ordinarily in high school and first-year college courses, students are exposed to phase separation between hydrophobic, oily liquids and hydrophilic, aqueous layers. This is explained by intermolecular forces and enthalpy, and eventually entropy, changes necessary for solutions to form.

Perhaps students hear of salting out alcohol from aqueous solution and consider IMFs influencing an organic molecule's solubility to affect a separation that would be otherwise impossible.

In the last couple of decades, separation of aqueous phases has become more widely studied as it has been determined to vitally impact cell function within the cytoplasm and nucleus as well as being useful for industrial separations. Large biopolymers such as nucleic acids and proteins can associate strongly with one another creating a polymer-rich aqueous phase with a membraneless interface called a coacervate distinguishing it from the polymer-poor surrounding aqueous phase. We will model this with the synthetic polymers poly(diallyldimethylammonium chloride), *PDADMAC*, and poly(acrylic acid), *PAA*, that form coacervates in low sodium chloride concentrations. While studying this system, students will create a phase diagram by mixing solutions of different polymer concentrations with different concentrations of sodium chloride.

The boundary between solutions that for coacervates and are therefore turbid and appear cloudy and those with insufficient polymer concentrations or excessive NaCl concentrations will be found.

Salt ions interfere with attractions between charged polymers causing them to no longer phase separate but to form one transparent aqueous phase with the bulk water. Rather than "salting out" alcohol, the polymers are "salted into" the bulk phase and the separate coacervate phase is gone.

Materials for each lab group, see preparation below

- A 96, 8x12, microwell plate. https://www.amazon.com/Advangene-U-bottom-treated-sterile-culture/dp/B077T1G2KV/ref=sr_1_14?keywords=well+plate&qid=1657275560&sr=8-14
- 6 dropper pipettes, we drew down these droppers by pulling the stems and cutting off the remaining unnarrowed part to produce smaller drops. If you pull too hard, they'll snap so it takes some practice. https://www.sisweb.com/part/GS138040
- A source of pure water, we used commercially available distilled but fewer ions is better.
- 16 mL poly(diallyldimethylammonium chloride), *PDADMAC*, solution. See preparation below. https://www.sigmaaldrich.com/US/en/product/aldrich/522376
- 16 mL poly(acrylic acid), *PAA*, solution. See preparation below.
- https://www.sigmaaldrich.com/US/en/product/aldrich/323667
- 10 mL 1.5 M NaCl. See preparation below.
- Commercially available food coloring
- Protamine sulfate. https://www.sigmaaldrich.com/US/en/product/sigma/p4020

Advance Preparation, solution preparation for 12 groups

A. *PDADMAC*, solution prepared by diluting molecular weight <100K, 35% wt polymer solution to 9.4% with water and NaOH for pH buffering.

1. Dilute 8.0 mL of 35% wt solution with 150 mL of the purest water available. Stir vigorously, vortex if available.
2. Test the pH with a pH probe or pH paper. It will be acidic and must be pH adjusted to as close to pH 7 as possible (our range 6.5 to 7.5 but high is better than low) by adding one drop at a time 0.1 M NaOH and retesting the pH after vigorously stirring in each drop.
3. Add water to reach a total volume of 200. mL. Stir and check the final pH.

B. *PAA*, solution prepared by diluting molecular weight 1.8K solid polymer in water and NaOH for pH buffering.

1. Dissolve 1.35 g *PAA* in 150 mL of the purest water available. Stir vigorously, vortex if available.
2. Test the pH with a pH probe or pH paper. It will be acidic and must be pH adjusted to as close to pH 7 as possible (our range 6.5 to 7.5 but high is better than low) by adding one drop at a time 0.1 M NaOH and retesting the pH after vigorously stirring in each drop. It should take much more NaOH to pH adjust *PAA* to 7 than it did for *PDADMAC*. If necessary, use a higher concentration of NaOH or for a very high volume, NaOH pellets to bring the pH to about 6 and then finish with adding 0.1 M NaOH to reach pH 7.
3. Add water to reach a total volume of 200. mL. Stir and check the final pH.

C. 1.5M sodium chloride solution prepared by dissolving 99% pure NaCl in water and NaOH for pH buffering.

1. Dissolve 13.9 g NaCl in 150 mL of the purest water available. Stir vigorously, vortex if available.
2. Test the pH with a pH probe or pH paper. If acidic it must be pH adjusted to as close to pH 7 as possible (our range 6.5 to 7.5 but a little high is better than low) by adding one drop at a time 0.1 M NaOH and retesting the pH after vigorously stirring in each drop.
3. Add water to reach a total volume of 160.0 mL.

Safety Information (See SDS for each chemical)

While the polymers are generally safe and easy to work with, note that disposal of *PDADMAC* requires care in the SDS. Also, protamine for the optional Temperature dependence should be refrigerated if stored and made as fresh as possible. Heating the container the protamine as high as 70 ^{o}C will ensure it has not clung to the sides of the container after refrigeration.

Teaching Strategies

This lab is designed for pairs of students. If students enter their data into a shared spreadsheet that is programed to average and graph it, they can create a more accurate phase diagram than the one they would create alone.

Resources: You may wish to use these resources either as background or as a resource for students to use in their inquiry-based design.
https://en.wikipedia.org/wiki/Coacervate
https://en.wikipedia.org/wiki/PolyDADMAC
https://en.wikipedia.org/wiki/Polyacrylic_acid
https://en.wikipedia.org/wiki/Protamine_sulfate

Directions for the activities

The main lab would take about 45 minutes to perform but should be preceded with pre-lab preparation and followed with post-lab data analysis. The extensions could be done in another 45 minute lab period or done as teacher demos during following class periods.

Procedure [Include the procedure here with more detailed background information for the teacher. Include pictures, and/or diagrams.]

Cleanup

Consult local disposal requirements especially for *PDADMAC* as it is a flocculating agent.

Example Student Worksheet or Guide

Distinct aqueous phases without a membrane, complex coacervates create a different sort of phase diagram
(with Answers in Red)

Introduction

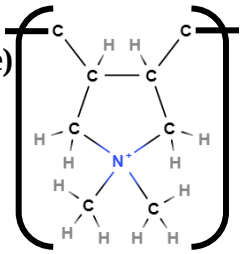
Like polarities dissolve like, or so the saying goes. In this lab, we will see that separate aqueous phases can be made without a separating membrane. These coacervate systems form when oppositely charged polymers attract to one another with water molecules in a polymer-rich phase leaving the bulk aqueous phase with far fewer polymers. The separate phase appears cloudy, or turbid. Added salt ions also have charges and attract to the polymers.

Materials [list]

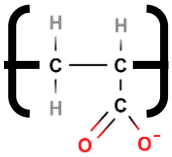
- A 96-wellplate
- 4 plastic dropper pipets
- PDADMA solution
- PAA solution
- Distilled water
- NaCl solution
- A device with a camera

Predict what will happen when these two polymers mix to form coacervates.

Poly(diallyldimethylammonium chloride)



Poly(acrylic acid)



The cationic polymer will attract to the anionic polymer by opposite charges and, along with water molecules, form a polymer-dense aqueous phase.

Predict what will happen when salt is added to coacervates.

The cloudy coacervates will dissolve into the bulk phase and the solution will be clear.

Procedure

0. Put on goggles and follow all safety guidelines given by your teacher.

1. You will be using columns 1-9 and rows A-H of a clean, dry 96-wellplate. Fill the wells with drops of the four solutions according to the spreadsheet below. Count carefully and be sure to use one dropper for each solution. Notice the salt content increases as you go down the columns and the ratio of polymers favors *PAA* as you go across the row.

		1	2	3	4	5	6	7	8	9
A	**Water**	7	7	7	7	7	7	7	7	7
0 mM	**Salt**	0	0	0	0	0	0	0	0	0
	PDADMAC	8	7	6	5	4	3	2	1	0
	PAA	0	1	2	3	4	5	6	7	8
B	**Water**	6	6	6	6	6	6	6	6	6
100 mM	**Salt**	1	1	1	1	1	1	1	1	1
	PDADMAC	8	7	6	5	4	3	2	1	0
	PAA	0	1	2	3	4	5	6	7	8
C	**Water**	5	5	5	5	5	5	5	5	5
200 mM	**Salt**	2	2	2	2	2	2	2	2	2
	PDADMAC	8	7	6	5	4	3	2	1	0
	PAA	0	1	2	3	4	5	6	7	8
D	**Water**	4	4	4	4	4	4	4	4	4
300 mM	**Salt**	3	3	3	3	3	3	3	3	3
	PDADMAC	8	7	6	5	4	3	2	1	0
	PAA	0	1	2	3	4	5	6	7	8
E	**Water**	3	3	3	3	3	3	3	3	3
400 mM	**Salt**	4	4	4	4	4	4	4	4	4
	PDADMAC	8	7	6	5	4	3	2	1	0
	PAA	0	1	2	3	4	5	6	7	8
F	**Water**	2	2	2	2	2	2	2	2	2
500 mM	**Salt**	5	5	5	5	5	5	5	5	5
	PDADMAC	8	7	6	5	4	3	2	1	0
	PAA	0	1	2	3	4	5	6	7	8
G	**Water**	1	1	1	1	1	1	1	1	1
600 mM	**Salt**	6	6	6	6	6	6	6	6	6
	PDADMAC	8	7	6	5	4	3	2	1	0
	PAA	0	1	2	3	4	5	6	7	8
H	**Water**	0	0	0	0	0	0	0	0	0
700 mM	**Salt**	7	7	7	7	7	7	7	7	7
	PDADMAC	8	7	6	5	4	3	2	1	0
	PAA	0	1	2	3	4	5	6	7	8

2. Take your wellplate to the photographing station with consistent light and take a picture from above showing as much of the bottom of each well as you can.

3. Fill out the observation chart below with your determination of the turbidity by eye.

Record Your Visual Observations, **Paste your photo of the array of wells below**

0 = no turbidity, 1 = some turbidity, 2 = turbid

	1	2	3	4	5	6	7	8	9
A									
B									
C									
D									
E									
F									
G									
H									

Analyze the Results

Draw a phase diagram on your visible observation chart above by drawing the line above which coacervate made the solution turbid and above which the coacervates were dissolved. Be sure to get all the 2's above the line and 0's below. The 1's can go on either side to make a continuous smooth curve. If you turn your paper around, the x-axis will be increasing *PDADMAC* and the y-axis will be increasing NaCl.

Complete the sentence: When the concentration of NaCl increased, the coacervates eventually…

If directed by your teacher, open your photo in ImageJ and enter the values in a shared spreadsheet. Create a graph of the data, yours or the shared data, whichever seems most meaningful.

Revisit your predictions. Were they correct?

Draw Conclusions

1. What happens to the entropy of the coacervate system when salts dissolve them? Think in terms of the number of separated particles. *Forming the independent polymer chains increases the entropy, but so does releasing the ions that were formerly associated with them. Thus it is entropically favorable to coacervate. At high [NaCl], it becomes less entropically favorable to release the ions and the coacervates dissolve when the individual polymer chains reform.*

2. Were there anomalies in your data? Attempt to explain them. *Yes, there are always anomalies. Reasons vary.*

3. Give sources of error for this lab. *Drop sizes vary, counting is hard, coacervates can settle over time or attract to the sides of the glass or plastic container and clear the well…*

Enhancing understanding Cover this section *after* the activity.
Give further context to the findings that the students made and the purpose of the lab.

Review the findings with students:

Students should note that they have created a phase diagram showing the Salt sensitivity of the coacervates. They might note that there is some asymmetry in the curve with smaller concentrations of *PDADMAC* and larger concentrations of *PAA* ratios are least sensitive to NaCl.

Going Further Students who have a good grasp of the content of the lab can be further challenged with these extensions:

1. Our *PDADMAC* and *PAA* concentrations were equal, not as we would traditionally understand equal molarities by number of moles of particles per liter of solution but by number of moles of charges per liter of solution. Each polymer is a long chain of either positively charged diallyldimethylammonium chloride (see structure above) in the case of the cationic *PDADMAC* or negatively charged acrylic acid (structure also above) in the case of the anionic *PAA*. We will assume at pH 7, all monomers on both polymers hold their charge. This is particularly important for *PAA* because, at low pH, the carboxylic acid groups begin to protonate, it loses its charge, the attractions become weak, and the coacervate do not form (see number 4 below). Here is a sample calculation of 93.75 mM charge concentration used to create the *PDADMAC* solution in this lab from the 35% wt *PDADMAC* source (D = 1.09 g/ml). 8 mL of *PDADMAC* source solution was diluted to 200 mL after pH adjustment.

$$\frac{8\ mL\ PDADMAC\ source}{200\ mL\ dilute\ solution}\ x\ \frac{1090\ g\ PDADMAC\ source}{1000\ mL\ PDADMAC\ source}\ x\ \frac{35\ g\ PDADMAC}{100\ g\ PDADMAC\ source}\ x\ \frac{1\ mol\ DADMAC}{162\ g\ DADMAC}$$

$$= \frac{0.019\ mol\ DADMAC\ monomers}{200\ mL\ dilute\ solution}$$

$$\frac{19\ mmol\ DADMACmonomers}{0.200\ L\ solution}\ x\ \frac{1\ positive\ charge}{DADMAC\ monomer} = \frac{94\ mmol\ charge}{1\ L\ dilute\ solution} = 94\ mM\ charge\ PDADMAC$$

Once diluted in the wells, the concentrations of *PDADMAC* ranged from 50 mM charge to 0 mM charge and the *PAA* from 0 to 50 so the total charge in each well was 50 mM. Charge matching is an attempt to pair up the polymers so they coacervate well. It was noted, as predicted, that a higher concentration of *PAA* created more volume of coacervates and greater turbidity. This is because the shorter polymer chain (1.8K in *PAA* vs. 100K in *PDADMAC*) allowed more freedom for them to crosslink the larger *PDADMAC* chains. Simply matching the number of polymer chains would have not been useful.

2. If available, concentration of coacervates can also be inferred quantitatively using the turbidity

feature on a UV-VIS Spectrometer. Droplets scatter all wavelengths of visible light so an optimal wavelength can be found and used to note the difference in transmission through a blank and the samples.

3. Allow coacervates to sit overnight in capped tubes or centrifuge to separate the two phases. Is the coacervate phase on the top or bottom after the droplets coalesce? *With the high polymer density, we expect the coacervate phase to be on the bottom.* Compare the thickness of the coacervate layer with the bulk layer after settling for several concentrations of coacervates with different turbidities. What would you expect? *For the same given volume of solution, the more turbid the initial solution, the thicker the coacervate layer after coalescing.*

4. Create a 3x6 array in columns I-K of the wellplate with drops of each *PDADMA* and *PAA* in each well. In column I add from 0-5 drops of 0.1 M HCl from the top down. Do the same in column J with NaCl and column K with NaOH. How does pH affect coacervate stability? *Coacervates dissolve at low pH. This is due to the carboxylic acid groups on the PAA protonating making the polymer neutral and no longer attractive to the unchanged positive PDADMA. If the concentration of NaCl were to increase above 0.3 M, coacervates would melt (as we just showed above), but this is impossible by adding 0.1 M to neutral polymer solutions. NaOH would also dissolve similarly to NaCl but not because the pH affects protonation or deprotonation of the functional groups but because the ions from the base also affect the coacervates for the same reasons as the NaCl. Adding a drop of higher concentration of NaCl and NaOH to the well with none added can show this.*

5. Partitioning red food color in coacervates. Red food coloring has a negative 2 charge and will preferentially dissolve in the coacervate. Add a drop of $1/40^{th}$ diluted commercially available red food coloring to a well with 4 drops *PDADMA* and 4 drops *PAA*. Pipet stir. Mount a drop on a glass slide or plastic petri dish with a glass coverslip and view under 40x magnification. Draw the droplets. *Students should see the droplets on the slide with far greater pink color than the bulk aqueous phase. They may even see grains of solid aggregates in the droplets since the concentration rose to the point of saturating the dye and forming a precipitate. This is an industrially significant application of coacervates that allow separations without organic solvents. This is also how cells use coacervate-like aqueous phase separation to concentrate substrates and enzymes, for example, to control reaction rates.*

6. Teacher: Create a bulk solution of 198.7 mg of protamine in 40 mL of distilled water. Put 50 mL of this solution in an Erlenmeyer flask and add 2 mL of the *PAA* solution used above and 20 mL of distilled water. Coacervates should be seen at room temperature. Demonstrate that the turbidity has increased for students with a red laser pointer. Heat the flask in a flame until the coacervates dissolve (the solution goes clear) or the solution begins to boil. Show the students that the turbidity has decreased. Put the flask in ice and leave for some time. Coacervates should reappear and can be demonstrated again with the laser pointer. Cycling from high to low T should continue to show the same result. This system is T dependent but not as NaCl dependent.

Assessment and rubrics:

As well as the questions and discussions above, students should be able to apply the following concepts to this lab:

Developed by Steven Schulz and Dr. Christine Keating
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*Development funded by the **National Science Foundation***

1. Polymers, covalent bonding, VSEPR, organic functional groups
2. Equilibrium
3. Intermolecular Forces and solubility
4. Thermodynamics (these systems are entropically driven)
5. Phase diagrams
6. Acid Base
7. Biochem (protamine is a protein from salmon semen
8. Colloids, turbidity, Tyndall effect

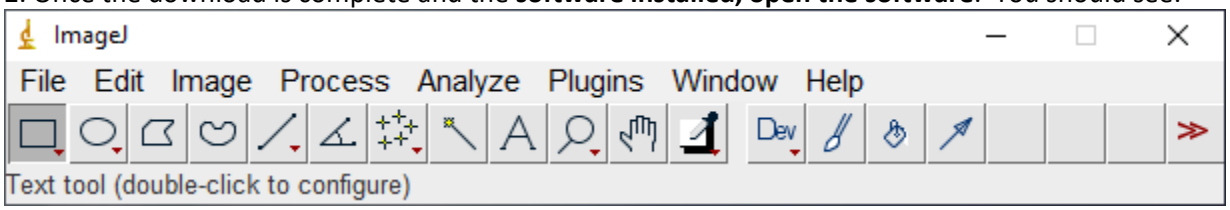
Installing and Using ImageJ Software

ImageJ is a free, easily downloadable software that measures the brightness of selected pixels in a photo. The software is used in actual research publications. We will use ImageJ to determine the degree of turbidity in wells of the 96-well plate and thus infer the concentration of the coacervates.

This is, of course, not without challenges. Glare, differences between the sides and bottom of the wells, and coacervates settling or adhering on the plastic all change the brightness of the well independent of the concentration of the coacervates. However, it is remarkably reliable for the simple tool that it is. Multiple samples from the same well will generally be close to the same value even if sampling is deliberately sloppy.

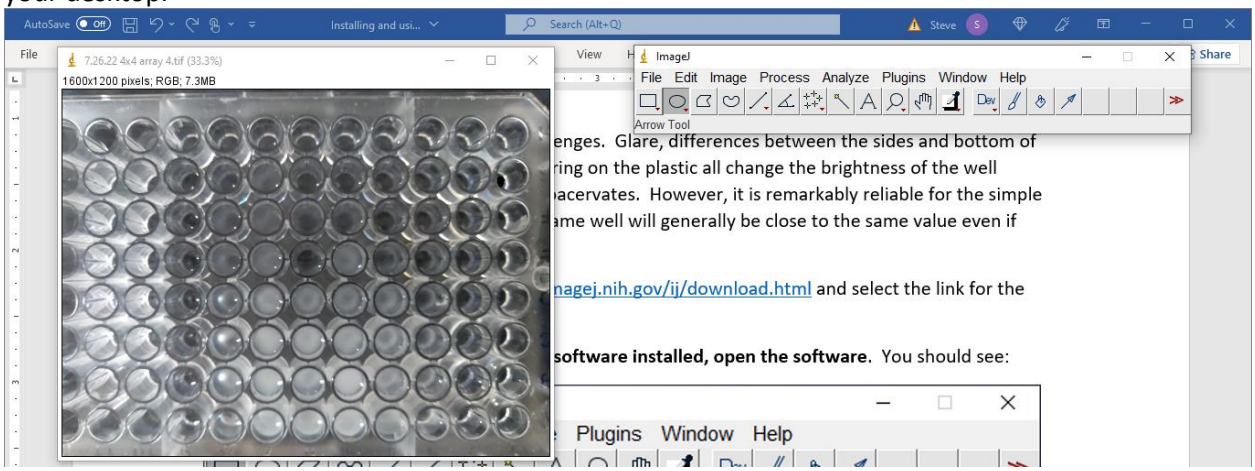
1. **Download the software**. Go to https://imagej.nih.gov/ij/download.html and select the link for the correct operating system.

2. Once the download is complete and the **software installed, open the software**. You should see:

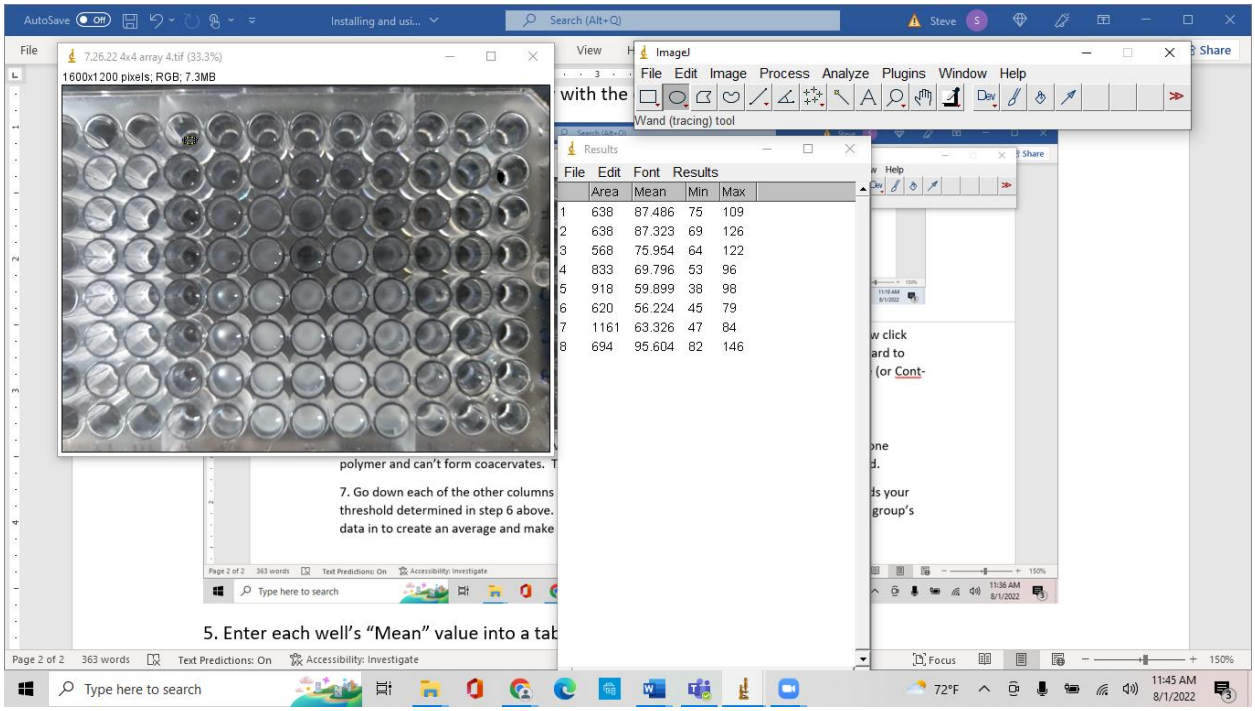


Pro-tip: Move this window to the top-right corner of your desktop so it does not become covered by other windows.

3. Go to File>**Open** and find the photo to analyze. Move the window with the photo to the top-left corner of your desktop.

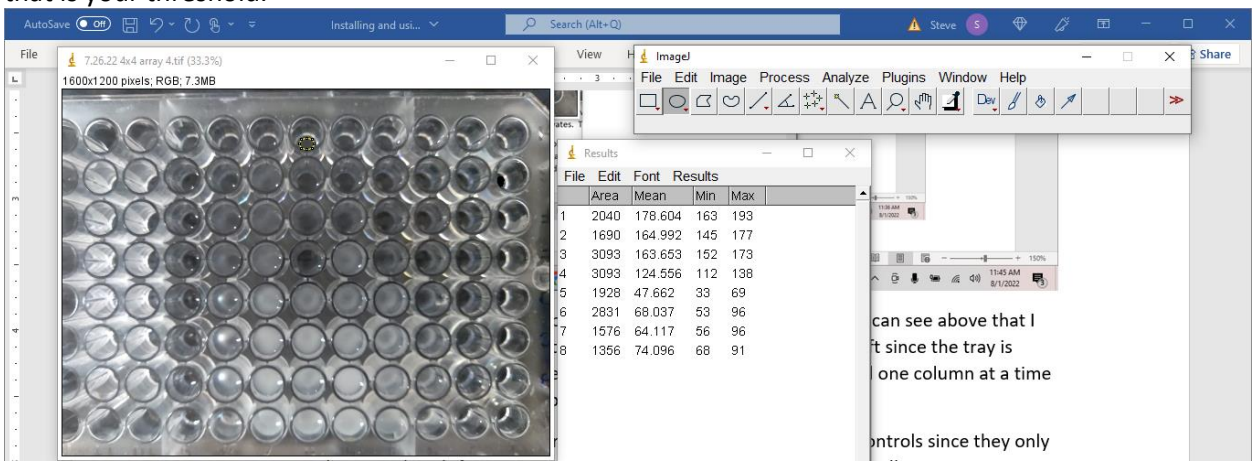


4. In the ImageJ controls window, click the second to left button with the oval in it. You can now click and drag to select oval-shaped samples of pixels to analyze at the bottom of each well. If it is hard to see, you can zoom into the well. Now return to the ImageJ controls and click Analyze>Measure (or Cont-M). This will open a new "Results" window with the collected data.



5. Enter each well's "Mean" value into a table organized by row and column. You can see above that I have collected all the values for column 9, the farthest occupied column on the left since the tray is upside down with [PAA] increasing to the left and [NaCl] increasing up. I collected one column at a time and then transferred it's values into the table.

6. Look at the 8:0 and 0:8 columns (number 1 and 9) of well's values. These are controls since they only contain one polymer and can't form coacervates. The highest values of these 16 wells is now your threshold. For column 9 above, the max is 96 because of glare. If the values in column 1 don't exceed 96, that is your threshold.



7. Go down each of the other columns (2-8) and record the last well to have a value that exceeds your threshold determined in step 6 above. For example, column 6 is analyzed above and the last well to exceed my threshold (96) is well #4 which has three drops of NaCl or is 0.3 M NaCl. Note: Well #1 has zero NaCl so 1 => 0 in the table. If available, a shared spreadsheet is perfect to put each group's data in to create an average and make your own class phase diagram.