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Advances in single-molecule measurement techniques: approaches, applications, and challenges

by

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A note to the reader

When I tell people I study chemistry, I often get a response along the lines of, “oh, so I wouldn’t understand anything you do.” As I love what I do and also love to talk about it, that’s incredibly disappointing for me. My friends and family can attest that I’ll ask for a chance to try and explain – chances are high that the reason you’re reading this is because we’ve had that exact conversation recently. Thank you for trusting me with your time!

A lot of the reason science can be intimidating is because the writing is so dense. Academics use abbreviations and jargon that doesn’t make sense outside of a narrow field, and often we gloss over what has already been studied and publish to get to the new thing we did. That’s out of necessity, to a degree, to prevent us from rewriting textbooks in every single paper. People do this across fields, within their hobbies, and even socially. If you’re talking with your brother about your Aunt Jill, you can assume you both know some background about who she is. To tell a new friend about your aunt, though, you’d be smart to give a little more context.

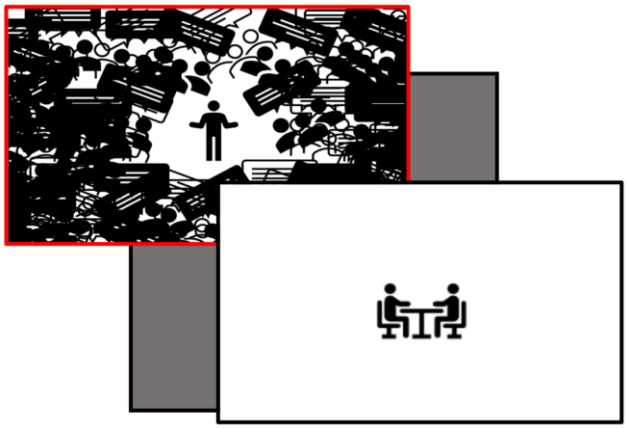
I started graduate school with a background in building small molecules, and over the last six years I’ve worked on five separate projects, three of which I talk about in this thesis. None of them have anything to do with what I studied in college. I spent most of my Ph.D. feeling like the outsider at someone else’s family function. I say all this to make two key points. First, knowing or not knowing the terms of a field is not a measure of some inherent quality “intelligence,” it’s a measure of familiarity. Cut yourself some slack, especially when you’re working on learning something new to you. Second, scientists aren’t trying to be

confusing on purpose. A lot of the time we're talking to people who already know some of the same background.

Society is inseparably intertwined with both science and technology, and so it is the professional responsibility of the scientific community to share findings with every audience. Access to the internet, electricity, and ease of travel around the world are just a few of the technological luxuries many of us cannot imagine living without. Beyond that, research is funded largely by taxpayers, with the expectation that it will have benefits worldwide with its applications to climate change, disease, unequal distributions of resources, and other global challenges. While this thesis certainly does not solve any of those problems on its own, it contributes some small amount to what we, as a world, understand. These little pieces, each a product of hard work, creativity, and a lot of reading, build on each other to support the next world-changing innovation.

Why single-molecule?

Imagine you're in a room packed with people, and everyone is talking at you all at once. In all the chaos and overlapping speech, you wouldn't be able to make out anything anyone was saying. Even if everybody was saying the same thing, if they weren't saying it at the same time or speed, it would be hard for you to pick out the important information. But one on one, you can have a conversation with



someone, ask questions, and get to know them. Chemistry on what we call the “bulk scale,” like a beaker on a lab bench, is like that room full of people. In only a drop of water, there are billions of molecules, all talking over each other. Most of the techniques we have to measure chemical reactions can only take an average of that activity.

Sometimes all the molecules in a reaction are saying the same thing at different times, and sometimes they’re saying something different. For example, if we add something to make a reaction go faster, like we heat it up, we might see the same amount of product form in half the time. Does that mean that all the molecules worked twice as fast? Maybe some were superstars and worked at three times the speed, while others didn’t handle the heat well and their activity was cut in half. These differences in behavior can provide valuable information on what’s actually going on in the reaction, but we can’t see them in the bulk. The development of new single-molecule measurements can add a lot to the understanding chemists have for how reactions work.

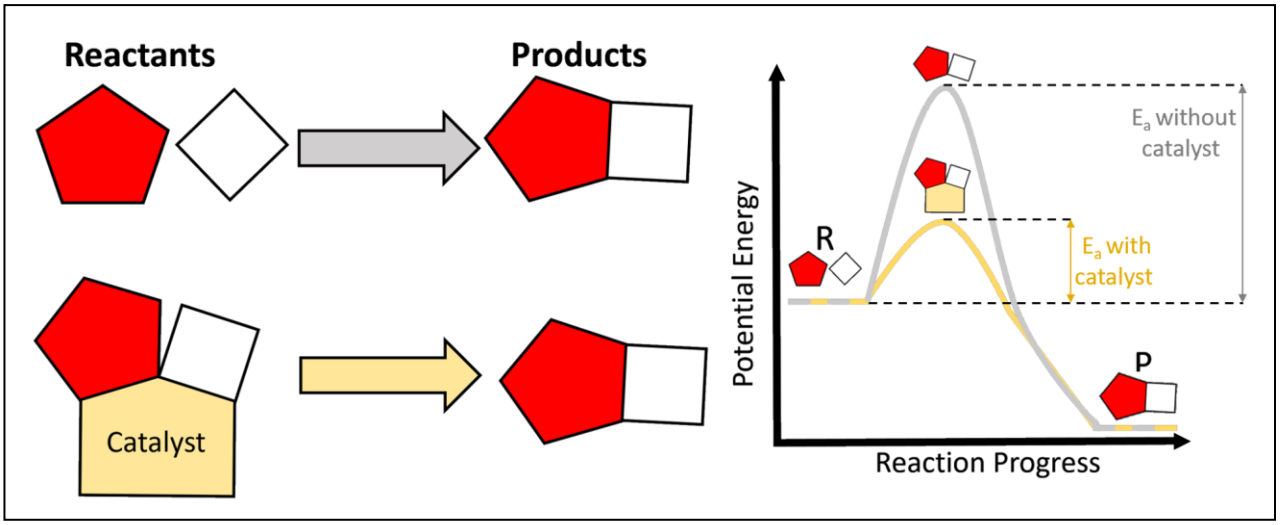
What kinds of molecules are we looking at?

A lot of single-molecule experiments look at biological molecules like DNA or proteins, which are important because they make up our bodies. They also tend to be pretty big for molecules, which makes them a little easier to work with. In this thesis, I applied a lot of techniques people have used to work with biomolecules to different systems.

One important kind of reaction is catalysis, where a molecule called a **catalyst** speeds up a chemical reaction without being consumed. Proteins are biological catalysts, but I study **organometallic** catalysts, molecules with a metal center and organic pieces

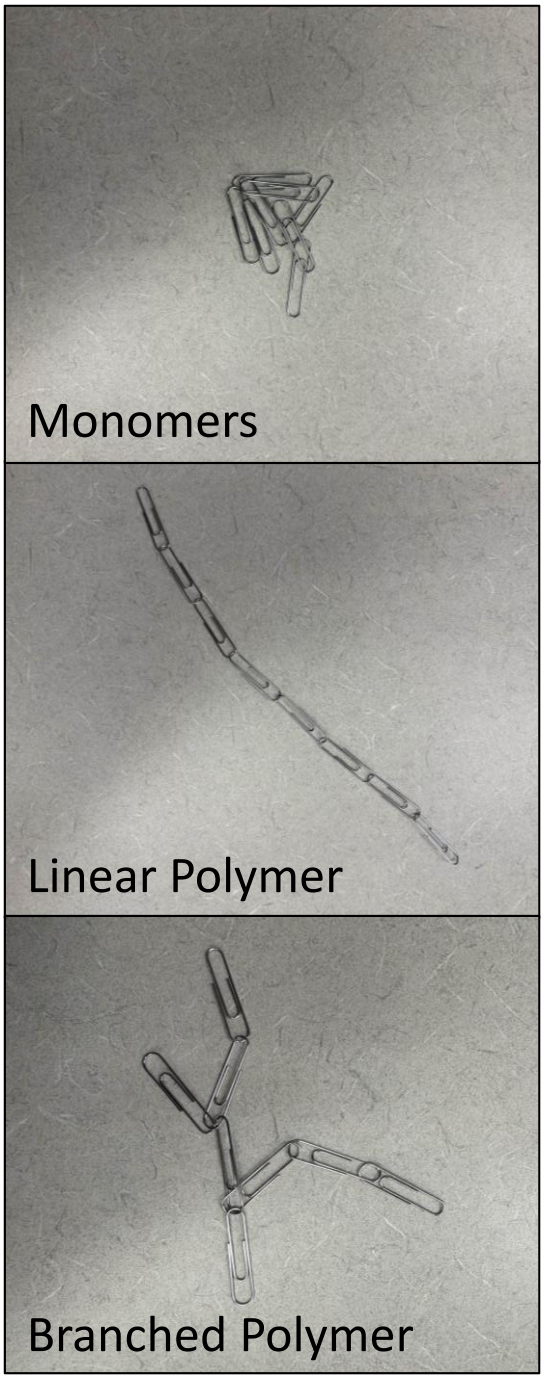
called ligands helping control how the metal interacts with things. In the modern world, more than 80% of manufactured goods involve a catalyst at some point in their production. Without catalysts, many of these reactions would move so slowly they effectively wouldn't happen, at least not on the timescale we need them to.

So, what do we know about how catalysts work? Whenever molecules collide, they have the chance to react, but they will only do so if the collision has enough energy, a value called the **activation energy** of the reaction. Catalysts work by connecting with the other starting materials in a reaction to form a complex, giving a lower activation energy pathway to make the product.



What can single-molecule work teach us about catalysts? While these molecules are regenerated after they help turn over a reaction, they don't work infinitely, or perfectly. Side reactions can lead to unwanted impurities in the collected product, and eventually the catalyst will stop working. Single-catalyst measurements can help researchers see the different behavior of catalyst molecules as they go through these processes, while most traditional bulk scale measurements only measure the overall product made. A better

understanding of how catalysts work can help develop more environmentally sustainable and cost-effective industrial processes.



Polymers are another broad class of molecules that single-molecule work can help understand. A **polymer** is a large molecule made up of many smaller molecules called **monomers** linked together. You can think of a polymer like a long chain of paper clips, with each individual paper clip being a monomer. The paper clips can be joined together in one long chain, or they can branch in different directions. The length and shape of the chain affect the polymer's properties, as do the kind of monomers making it up. Polymer scientists can adjust these variables to give polymers different properties. Plastics are all polymers, for example, but the sturdy plastic of a frisbee is very different than the stretchy plastic of a trash bag.

One thing about polymers that makes them different from other kinds of molecules is how we talk about their **molecular weight**, the mass of one molecule of a compound. It's

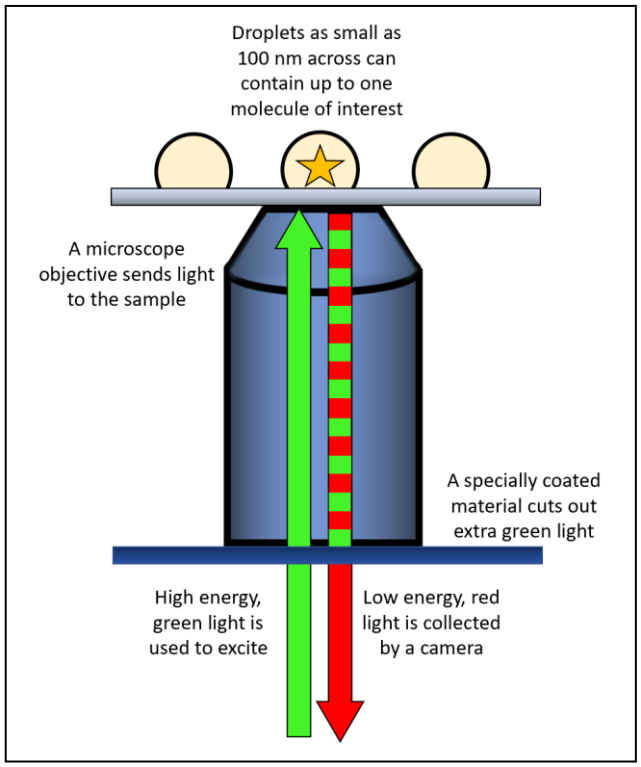
easy to tell the mass of one paper clip, or two, or three. Those first couple links can be separated from each other, and the molecules have different chemical and physical properties, like their reactivity or their melting point. But in a chain of a million monomers, it's hard to separate or tell the difference between molecules with one more or one less link. So instead of talking about the molecular weight of a polymer, we talk about its **molecular weight distribution**.

Most methods of measuring these distributions measure many polymer chains at the same time. The **viscosity** of a dissolved polymer, or how easily it flows, can tell you about this distribution. Another method, **size exclusion**, involves passing the polymer solution through a column with different size pores. Smaller polymers can fit into all the pores, so they have a longer path through the column and take more time to come out the other side. Bigger polymers fit into fewer of the pores, so they come out faster. The time something comes off the column can then be compared to known standards, to determine its weight. A size exclusion column functions kind of like a maze for your polymers. The biggest molecules have a simpler maze, because they can only fit through the biggest pathways. The smaller the molecule, the more possible paths it has to explore, so the slower it comes out.

What can single-molecule measurements tell us about polymer mass distributions? Instead of measuring a group of polymers all at once, single-molecule measurements let us build up the molecular weight distribution of a sample piece by piece. This gives a more accurate measurement of the distribution than bulk techniques do, since each value is recorded independently, not impacting other values.

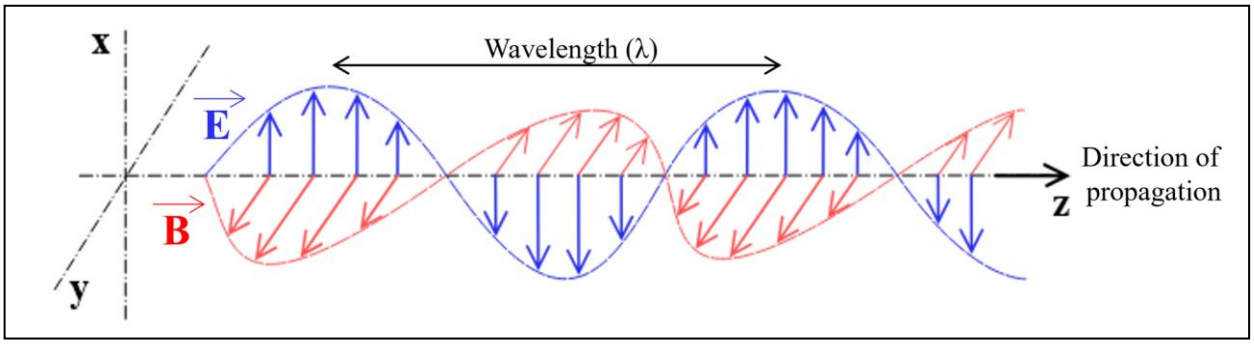
How can you look at one molecule?

Now that we've decided to look at single molecules, we can design an experiment to do so. First, it's important to remember that at a microscopic level, everything in the world is moving. To measure anything useful from our molecule, we have to keep it in one place long enough. There are a lot of ways to do this, from connecting your molecule to a surface to continuously shifting it back into your field of view – or moving your field of view with the molecule! In many of the experiments in my thesis, I used a strategy involving small droplets. With small enough droplets and low enough concentrations of the molecule of interest, most drops should be empty, a few will contain a copy of the molecule we're watching, and a statistically insignificant number will contain more than one. This strategy has a lot of advantages: it's relatively simple to implement and the molecule you're studying can stay in solution, like it normally is during a reaction. There are some disadvantages too, particularly that scientists are still working on understanding how molecules on surfaces align. These droplets have a much higher ratio of surface area to volume than bulk solution, which could affect the chemistry you're trying to measure, depending on what it is. The good news about that is it means there's a lot to learn, and a lot of really interesting experiments we can do with droplets!

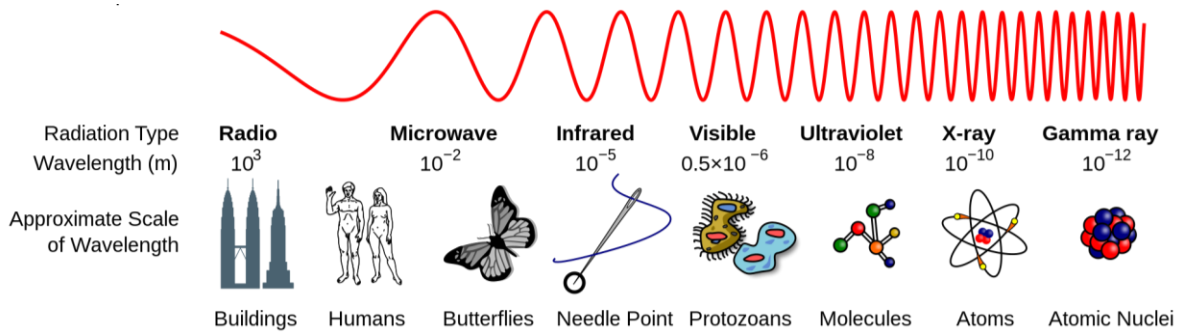


But how can you SEE a single-molecule?

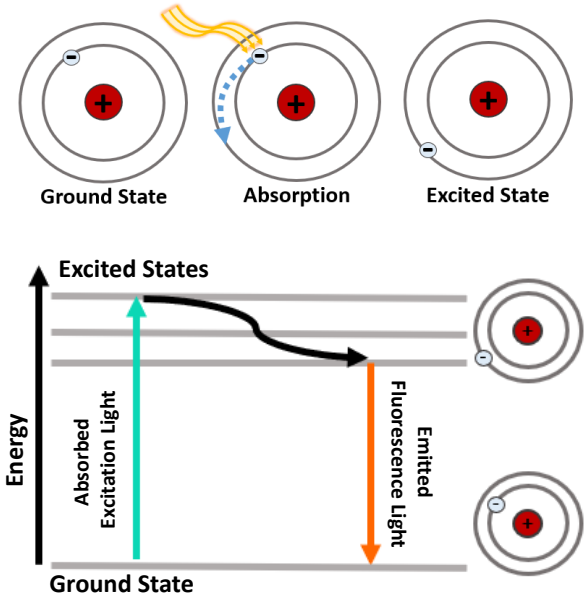
The next challenge is perhaps the most obvious: how can you see something as small as one molecule? To start with, everything we see is due to light, which can bounce off the world around us and to our eyes. Light is a form of energy that behaves like a set of two waves, an electric field (E) and a magnetic field (B) that travel perpendicular to each other in the same direction. The height of these waves, or the amplitude (A), relates to how bright the light is, and the distance between the peaks is known as the **wavelength** of light (λ).



Higher energy light has shorter wavelengths and appears bluer to our eyes, while lower energy light has longer wavelengths and appears redder. The light humans can see is a couple hundred nanometers across, about the size of a virus. In contrast, gamma rays are about a million times smaller - the size of an atomic nucleus - while radio waves are the size of buildings.



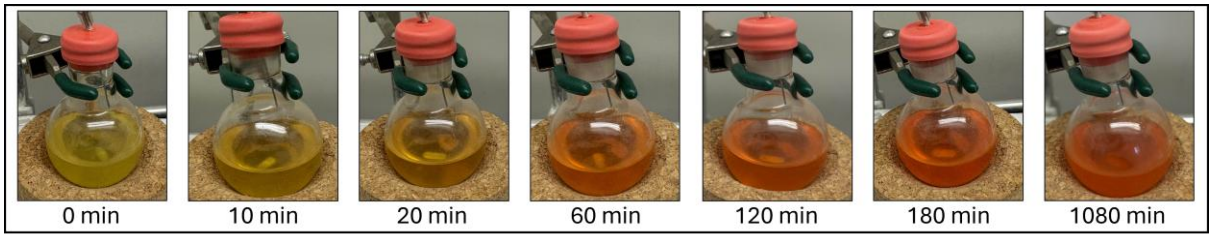
When light hits a molecule, it adds energy. If that energy is just the right amount, the molecule can **absorb** it. When a molecule absorbs light, negatively charged subatomic particles called **electrons** can move further from the center of their atoms. This is a higher energy state because the negatively charged electrons are now further separated from the atomic nucleus, made of positively charged **protons** as well as neutral particles called **neutrons**. When these electrons move back down to their usual **ground state**, extra energy can be released in a process called **fluorescence**. Some energy is lost in this process to heat and molecular motion, so the light emitted is lower energy (redder) than the light that was absorbed. Molecules that can undergo this process are called fluorophores.

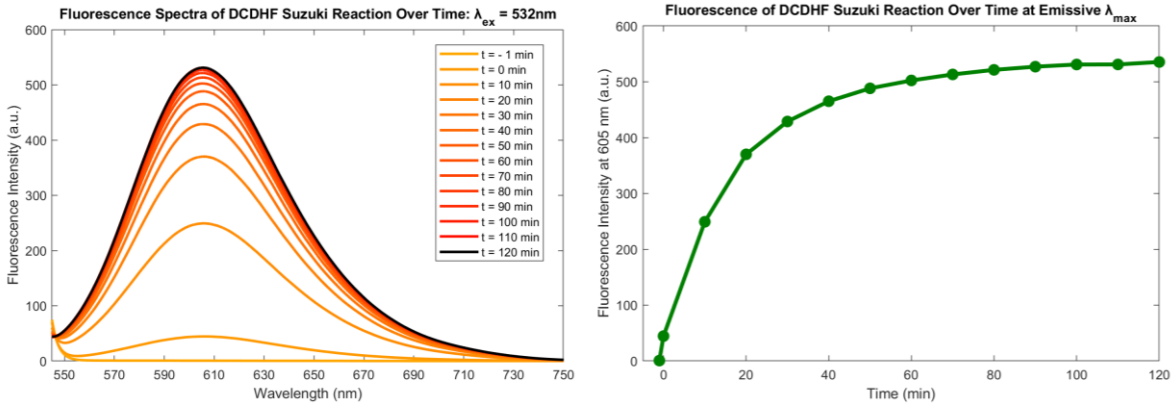


Many single-molecule experiments use fluorescence, as while the molecule itself might be too small to see, the light it gives off is much bigger. We can take pictures of very small amounts of fluorescence on lab cameras, and track how much there is. The amount, position, color, and even direction of light can provide information about what the molecule emitting it is doing.

In one of the projects I worked on, I used a catalyst to join two non-fluorescent molecules into a fluorescent product. In that experiment, the amount of light growing over time told me how far my reaction had proceeded. We first worked on understanding how the

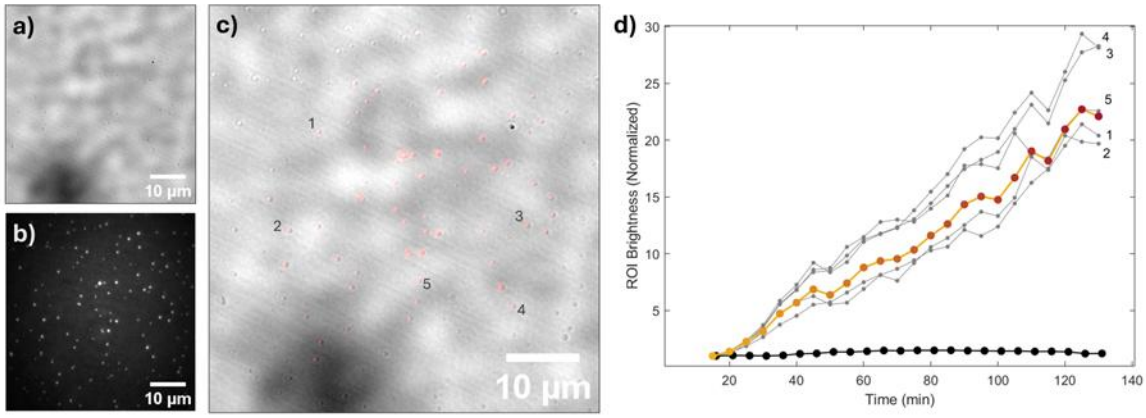
reaction worked on the bulk scale. By eye, the reaction was initially yellow, and it turned red as the product was made. Looking at the fluorescence spectrum over time, we could see a peak rise, getting bigger as the reaction produced more fluorophores. Then we plotted the highest point of that peak over time. You can see the color change (top), the rising peak (bottom left), and its highest point over time (bottom right) here.





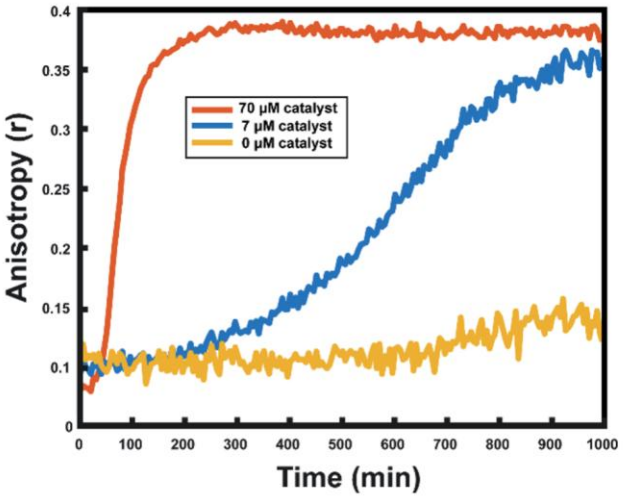
We then moved to the microscope and began trying to perform the reaction in small droplets. The droplets were made with a **vortexer**, which essentially stirs up a mixture of our reaction and water really fast. The reaction solution acts like oil, and doesn't properly mix, leaving small droplets of reaction isolated from one another – like the world's prettiest but worst-tasting salad dressing. This kind of mixture, of two liquids that don't mix suspended in each other, is called an **emulsion**. On the microscope, we were able to see increasing fluorescence from individual droplets like we had hoped. The next figure shows the drops illuminated with white light (a), green light that makes them fluoresce (b), and a mixture of

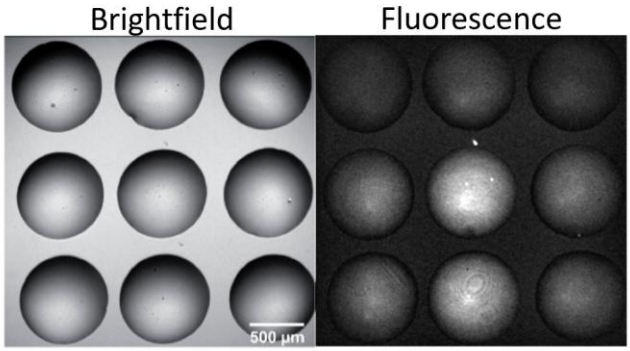
the two images (c). The fluorescence of individual droplets is shown in the thin gray traces of part d, while the average fluorescence of all the drops is the colored line in part d, and a control without catalyst added is the black line that stays at the baseline.



Unfortunately, we couldn't see fluorescence when working at the very low concentrations that would let us say the activity in each drop came from one catalyst making product. To understand why, we investigated the **turnover number** of the catalyst, the number of reactions each catalyst can "turn over" before it stops working. At the concentration used in the last two figures, each catalyst was able to make over five molecules of catalyst. At the concentration we would have needed for single-catalyst studies, the turnover number was less than one. This means that the catalyst we studied likely does not actually work on its own, and instead multiple copies of the molecule work together to drive the reaction. It was also interesting to note that the droplet reaction seemed to progress differently than the bulk reaction. By 60 minutes, the bulk reaction's rise in fluorescence had leveled off, while the fluorescence in the emulsion droplets was still rising linearly. Both of these observations were independently interesting, even if they weren't what we set out to measure.

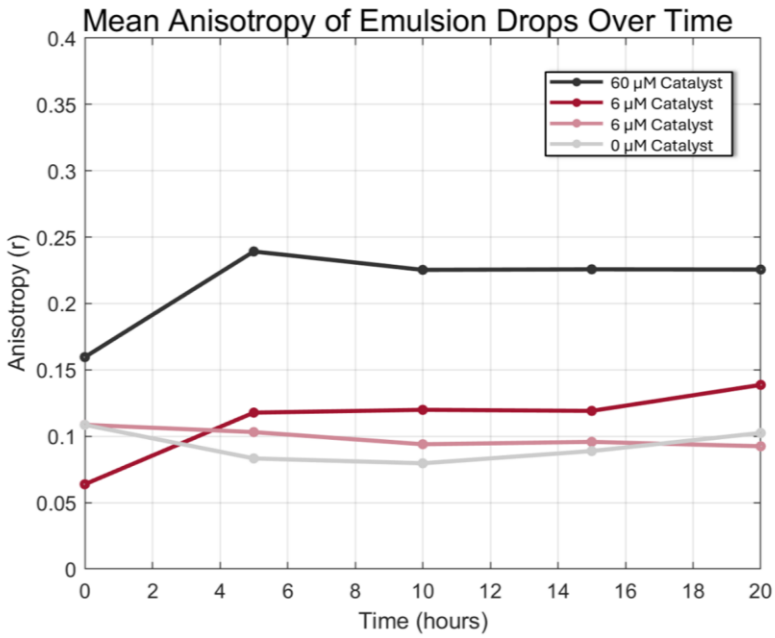
In another set of experiments, I studied a slightly more complicated fluorescent system, where I used specialized optics to sort the light I measure by its **polarization** – the direction the wave of light is moving. That reaction started off with fluorophores moving freely in solution, and the light they emitted going in all directions. As the reaction continued, a catalyst molecule linked the fluorophores together into a polymer. The more polymer that formed, the more rigid the system became, and when the molecules couldn't move as much, they gave off light in one direction more than the other. By comparing the different amounts of light my cameras measure in different directions, a ratio called the reaction's fluorescence **anisotropy**, I was able to tell what stage my reaction was at. Other members of my lab got this technique to measure polymer growth working in relatively large droplets, 500 μm across.

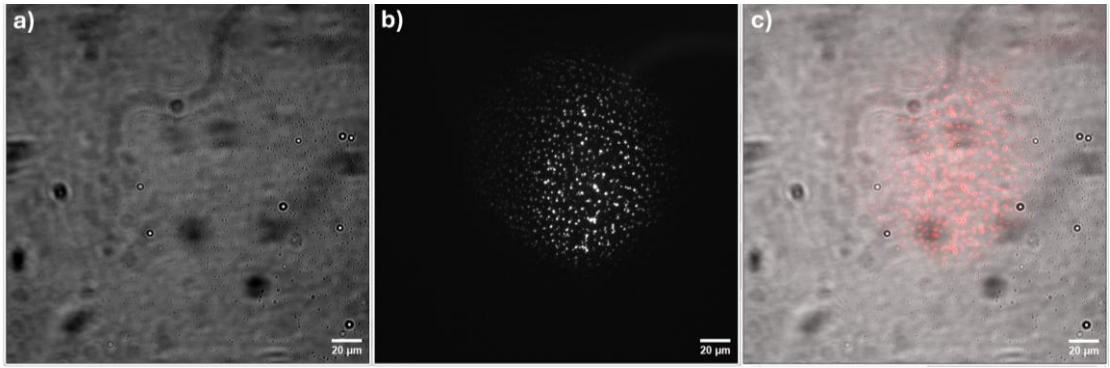




I started working with the same reaction, trying to lower the catalyst concentration and droplet size to the point where a rise in anisotropy in each drop tracked to activity from one molecule of catalyst. Here too, though, we ran into unexpected results. Catalyst of roughly the same concentrations used in my labmates' paper did not cause the same rise in

anisotropy. While the most concentrated catalyst in large drops reached an anisotropy of 0.4 by 2 hours, in my emulsions it did not even reach an anisotropy of 0.25, given 20 hours. More than that, the less concentrated reaction run in my emulsions couldn't even be distinguished from the control run without catalyst.





While the emulsion anisotropy didn't rise in the way that would indicate a reaction, we saw a gel-like solid form in the leftovers of the tube we vortexed things in. Polymer was forming, just not in our droplets. We think part of the reason for this is the constrained geometry of the droplets slowing **diffusion**, or molecular movement. Molecules in solution move around randomly due to their collisions with each other, and on the bulk scale this

effect is from high to low concentration. Imagine two rooms connected by a door, room A with 100 people and room B with 10 people. If the people all flip a coin to decide whether or not to walk to the other room, more people will decide to go from room A to B, simply because more people are starting in A. Eventually, repeating the coin flips enough times, the two rooms should have the same number of people. This is how diffusion is both a random process and simultaneously, diffusing molecules move from areas of high concentration to low concentration.

Now imagine that someone in one of the rooms is directing the people nearest to them to hold hands. This person is the catalyst, and the people joining hands represent monomers linking up to a polymer. As the catalyst starts building a polymer from all the monomers it can reach, it gets harder for new monomers to diffuse into its active range. This isn't that much of a problem in large droplets, so the catalyst can keep adding links as many times as its turnover number allows. In small droplets though, diffusion can have a big impact. Going back to the earlier analogy, the rooms we're talking about in droplets are a lot smaller, so it's harder for people to move around. The catalyst is only going to work for a certain amount of time before it breaks. In droplets, we hypothesize that diffusion slows down enough that the catalyst can't interact with that many molecules before it breaks, so less polymer is formed than in the tube of reaction.

So… did you ever actually see a single molecule?

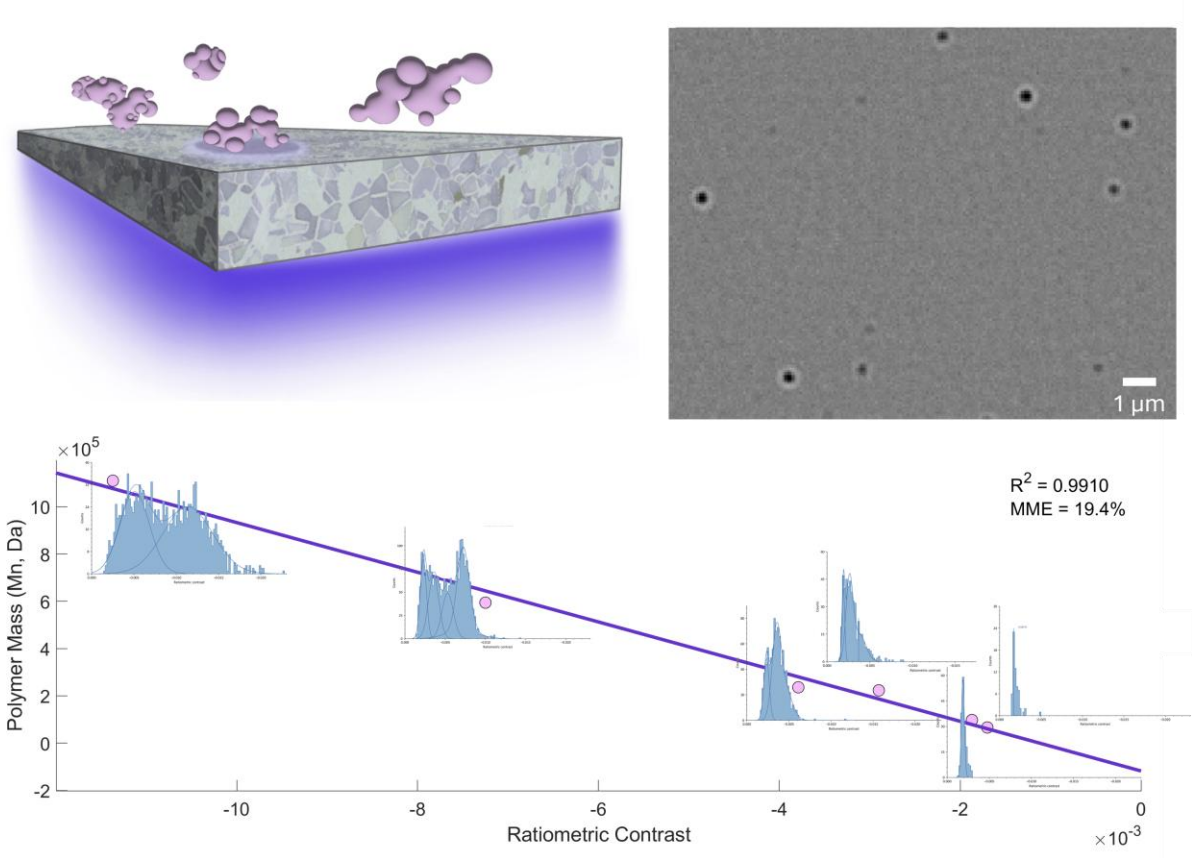
First of all, while the experiments I've described so far didn't actually work on the single-molecule level, the *reasons* they didn't work ended up being interesting themselves.

A lot of times when you're doing science you don't measure the thing you initially set out to. Sometimes what you see instead is useful in its own way, and honestly, even if it isn't, when you're working on developing new measurement techniques it's important to find their limits and weak points. That said, I'm happy to say the answer to the question is yes (though that experiment was a bit different than the others, so hold on for a little more background).

I mentioned in passing that not all molecules fluoresce. Another way molecules can interact with light, particularly bigger molecules like proteins, is by **scattering** it. When light hits these molecules, the electromagnetic field inside the molecule gets disturbed, and its **electrons** begin to move around in response. Electrons are negatively charged particles inside atoms, and the way atoms exchange and share them is what binds atoms into molecules. When they are disturbed, the moving charge creates a new, secondary electromagnetic field, which is scattered light. The more the electrons in a material move in response to light, the more **polarizable** we say the material is.

In a technique called **interferometry**, a microscope records the light reflected from a surface. When molecules land on or depart from the surface, the light they scatter interferes with the reflected light. If the waves line up peak to peak, this interference is **constructive**, and the light's amplitude increases. If they line up peak to trough, the interference is **destructive**, and the amplitude decreases. You can think of interferometry like ripples on a pond made by tossing a pebble in. The pond surface might not be entirely still, the water could be moving, but the pebble's ripples can still be seen interfering with the background if they are big enough. The bigger the pebble, the bigger the ripples it makes. I use a commercialized version of interferometry called **mass photometry** in chapter 2 of my

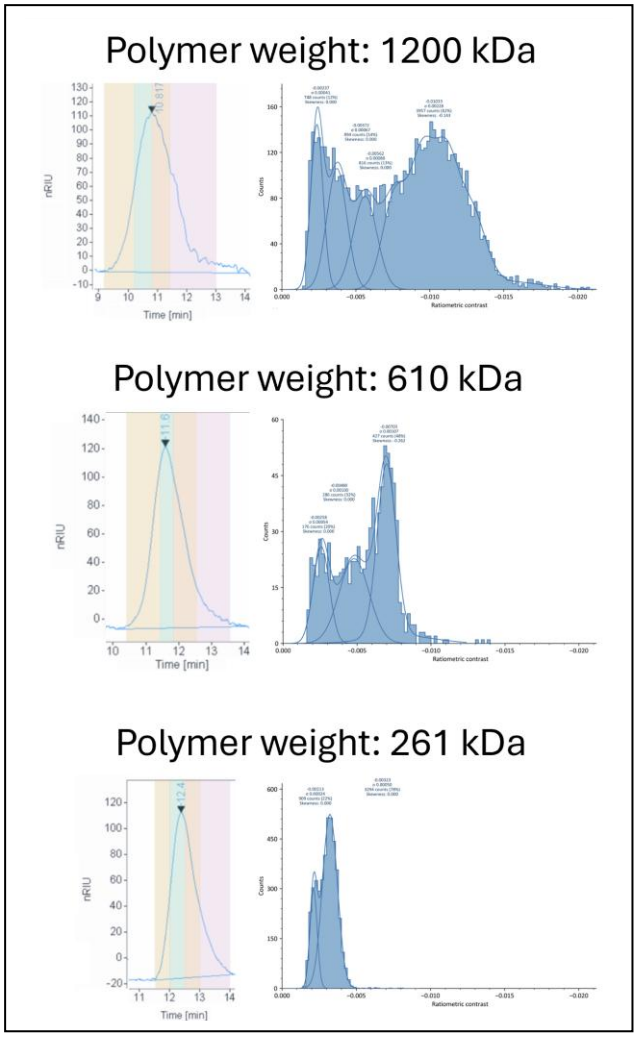
thesis to measure the mass of individual polymer chains. Higher-mass molecules scatter more light, scaling linearly to an optical feature called **ratiometric contrast**. The contrast is ratiometric because the signal from one molecule is hard to see on its own, so the technique relies on the ratio of light in the presence versus the absence of a scatterer.



I used mass photometry to look at a synthetic polymer called **polyethylene oxide**. Because it dissolves in water and is nontoxic, this polymer is used in cosmetic creams and lotions, in drug delivery, and in wastewater treatment. Industrial settings often use large molecules of polyethlene oxide like the ones I studied as a thickening agent or a stabilizer. Besides being interesting as a single-molecule measurement of a polymer's molecular weight distribution, this was also one of the first uses of mass photometry to measure polymers besides biomolecules such as proteins. Compared to polyethylene oxide and

other man-made polymers, biomolecules are very polarizable, so they scatter more light and are easier to detect by mass photometry. Additionally, a protein sample will be a very narrow peak, as multiple copies of a protein follow the same sequence of monomers. In contrast, the broadness of a synthetic polymer's molecular weight distribution means the photometry peaks are more complex. While making these mass photometry measurements I was not only studying the polymer itself. In learning how to apply a powerful technique to a new kind of molecule I was helping develop the world's toolkit of analytical techniques.

In the figure shown here, you can see some of the results from polyethylene oxide mass photometry experiments, with polymers of three different sizes. The measurement on the left is a trace from a size exclusion column, where bigger times mean bigger chains, and the measurement on the right is from mass photometry, where bigger contrast values mean bigger chains. You can see a lot more local behavior in the peak with the photometry measurements, which is really exciting – future work in my lab will focus on figuring out what that means macroscopically, how it impacts the polymer's physical properties.



Conclusions

Single-molecule work is an exciting approach to understanding chemical reactions, and it provides both unique insights and challenges. Not all of the projects I got the chance to work on yielded the results I had expected or even hoped for, but the information I learned is helpful in other ways. As I mentioned earlier, a lot of science is putting out stepping stones for future work, sometimes before you even know where the path leads.

You can keep digging into the theory of any of these concepts and go deeper and deeper to understand more and more precisely. Eventually, though, if you keep asking questions, you'll get to a point where you can't find the answer anywhere. At that point, congratulations! It sounds like you need to start designing some experiments.

Acknowledgements

I am where I am in my career thanks to the many wonderful teachers I've had the privilege to learn from. I'd like to thank my research advisor, Prof. Randy Goldsmith, for his mentorship over the past six years and his support for my science outreach endeavors. Thank you to the Goldsmith lab, a group of excellent writers and speakers I'm lucky to call my coworkers. I'd also like to thank the Wisconsin Institute for Science Literacy for creating this science communication project. In particular, Prof. Bassam Shakashiri and Cayce Osborne have inspired me and challenged me through their steadfast commitment to starting conversations. Thank you also to Elizabeth Reynolds, J.D., whose careful and insightful comments on this chapter transformed it from a rough draft to a publishable piece of work. This research was funded by the National Science Foundation, Schmidt Futures,

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Large droplet anisotropy:
Adapted from Figure 5 and Supplementary Figure 2 of "Optical monitoring of polymerizations in droplets with high temporal dynamic range." A. C. Cavell, V. K. Krasecki, G. Li, A. Sharma, H. Sun, M. P. Thompson, C. J. Forman, S. Y. Guo, R. J. Hickman, K. A. Parrish, A. Aspuru-Guzik, L. Cronin, N. C. Gianneschi and R. H. Goldsmith, *Chem. Sci.*, 2020, **11**, 2647 **DOI:** 10.1039/C9SC05559B