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JOANNE STUBBE: So the key question is, do these-- and I think this is a general question you can ask, metabolically, inside any cell is do these enzymes that are on different polypeptides cluster. And is there an advantage, kinetically or whatever, is there some kind of an advantage to have clustering inside the cell.

And where have you seen something like this before? Do you remember? Do you remember the section. Where have you see multi enzyme complexes and clustering before? Yeah?

So or the classic one, PKS has been around. But it's completely analogous to fatty acid synthesis, right? And so in bacteria, they're all single polypeptides. In humans, they're all activities that are on single chains.

OK, so that's sort of what's going on here with the purine pathway. We'll see there are ten. This just sort of helps us focus if we get to the data at the end, which I think we will from what we did the last time. Is that you have six enzymes for 10 activities. So that just means you have more than one enzyme per polypeptide, OK?

And so I guess the key thing that I wanted to focus on is do you think it's important to cluster? Here's a pathway. These are the names. We're not going to go through the names. The names really aren't important for what we're doing. There'll be two names that we'll be looking at over and over again.

These are the papers that you guys did, in fact, read. One is the original paper, which got a lot of press. And I just want to show you that there have been there's actually been four papers published in the last six months on this topic. And one of which was published. Yeah. One of which is published with Science, where they are now claiming that this complex is localized to the mitochondria.

OK, so you take pictures. And it's that this is looking at super resolution fluorescence methods. And you can clearly see clumps of blobs focused on the mitochondria. Why would you want it

to be at the mitochondria?

So then you have to ask your question. You might need purines, because that's where you make, through a proton motive force in respiration. Remember, when you convert oxygen to water, you get a huge amount of energy released. You make ATP. But it's going to be made from something. So maybe you would want. That's the way they rationalize it. And they do.

And then they connect it to the other latest hot topic, which is EM torque, which is the major signaling switch for fatty acids and for amino acids. And now in the last two years, purines and pyrimidines, I decided-- I've done a lot of reading about it, decided and believe. I mean, I believe it. But I don't believe the connections yet. So again, this is what you're going to see in the next decade is connecting signaling to primary metabolic pathways, like the purine pathway. That's going to be a big thing and how do you connect them is going to be the key question.

So anybody that wants to do some more reading, this is an updated version. I kept updating this three or four times. And so I think these are the key questions we want to focus on. And so what I'm going to do. Well, define the questions a little bit and whether the things we need to think about just to determine whether this is really important, biologically. Then we'll define fluorescence and what you can do with fluorescence. And then we'll come back and look at the data.

In the paper, we're also going to look. We probably won't get all the way through all of the data. But we will look at some of that data again in either the next lecture or Wednesday's lecture. So you will see it again if we don't get through the data.

So they claim they have a multi enzyme complex. Did you believe that from the data? I mean, they didn't look at all 10 enzymes simultaneously, right? Or six enzymes.

AUDIENCE: Whenever they show an image of the cells, and then they fluorescent, trying to show local sections, it's always so hard for me to figure out--

JOANNE STUBBE: What you see.

AUDIENCE: Yeah.

JOANNE STUBBE: OK, so that was said. We'll look at some of those pictures. But I completely agree with that, that you can't see anything from fluorescence pictures.

So everybody, all chemists or chemical biologists, now have huge numbers of these pictures in their papers. And with Alice's group, I'm always on their case that I can't tell a damn thing. This is on thesis. I can't see anything. And Alice says she can't see anything either. So it's very hard to see things in these pictures. The contrast isn't very good. And what her lab now does is it goes to EM, where you can see things much more clearly. The fluorescence things are tough. So you're not the only one.

And if somebody says it's obvious that this. And you don't see it. Raise your hand and say, I don't see it. Show me what I should be looking at, OK? So that's a good take home message, because everybody and his brother is doing this. And this goes back to knowing how to do it correctly. We're not going to talk about any of that stuff. I mean every one of the methods I'll sort of show you that's out there. You have to really study it to make sure you're handling it correctly.

So I mean I think, to me, this has been a problem that I've been interested in. And I started working on this a long time ago in the purine pathway is not are things sticking together important. Actually, I don't think those are important. You immunoprecipitate all these things. OK, so you say obviously these are talking to each other. But the key thing is the kinetic competence.

And lots of times when you mess around. You get it in a state. And you post-translationally modify it. So it's sitting in this state there probably isn't on the pathway. You need to then show it's on the pathway.

So I think a lot of protein, protein interactions, especially now that we know that proteins move around. And they're in this complex. And they're in that complex. And they're in that complex. The key, I think, is to transient interactions. So why? So this is just my personal take on this. I'm letting you think about this. But is it easy to look at transient interactions? No.

OK, so anyhow, I think people need to start doing a lot more thinking about how to look at that. And one way you could look at transient interactions is if you can fluorescent label something. And they come together on a certain timescale and then move apart. And can you do that inside the cell with the right spatial and time resolution. You might be able to start looking at that. So that the methods that are being developed and continue to be developed are incredibly powerful. And I think will allow us to ask this question happens inside the cell, which

you've all seen pictures in your introductory courses of, man, how complicated the inside of the cell is. That's part of the issue.

So the issue is that you might have a purinosome somewhere in the cell, depending on the growth conditions. But those enzymes might be involved in other things. And so you have only a tiny amount of it, as opposed to trying to make the cell by growth conditions, putting it into all one state. So you can see it.

So the question is, how do you see it? And so that's the key issue. And if you perturb it enough. And you do see it. Then you have to ask the question. And this is a question that you might want to think about in terms of these two papers you were reading. That's what. If you do this, that's what the Marcotte paper said, that the cells were incredibly sick when you take out all the purines.

And in fact, Alice is-- because of this mitochondria connection between the purinosome and Alice's interest in the mitochondria, she's had people trying to repeat this. And Vicki Hung worked on this and couldn't repeat it.

So she didn't spend that much time on it. But all I'm saying is it's not a slam dunk to be able to do this.

But that being said, I think this has been an issue that people have been thinking about for decades. And it's just really hard to test experimentally inside the cell. This is where we need chemical biologists to figure out new ways of being able to look at this, so that you can actually make a measurement that's interesting.

So I guess the question I want to start with, before we researched looking at fluorescence, is why do you think it would be important to do this. Or do you think it would be important to have a complex. What's the advantage of doing that? Yeah.

AUDIENCE: You were saying in lecture that you want to increase the effective molarity. And so by having all these things right next to each other, there's-- obviously you're going to have more interactions per second.

JOANNE STUBBE: Well, you may or may-- you may not. It depends. So I think this is the key question. Is diffusion fast inside the cell?

AUDIENCE: Yeah.

JOANNE STUBBE: Yeah. It's still very fast. For small molecules, it's incredibly fast. Even for proteins, it's incredibly fast. So even if this guy is over here. If you're turning over here at a much slower rate, and you have enough of them so you can interact at diffusion control, do you need this organization? There are a lot of smart people who think you don't need that. There are a lot of smart people who think you do need that. But this is the question I want to raise.

However, so catalytic efficiency is absolutely it. But where might you really need catalytic efficiency. And so that goes back. There are places where you really need this.

So if you look at the first intermediate in the pathway, this guy, what do you think about that guy? Do you think he's stable?

So if you look at the first intermediate in the pathway, which we'll talk about next time. So this is amino phosphoribosine-- phospho-- I'm drawing a complete-- I think I'm tired.

Anyhow it's the amino analogue of ribose 5-phosphate. Phosphoribosylamine, that's what it's called, PRA.

OK, do you think that's stable, as chemists? So what do you think that could do?

AUDIENCE: Could you release the amine?

JOANNE STUBBE: Yeah, so how would you do that?

AUDIENCE: So if it's protonated, and then the ring opens till--

JOANNE STUBBE: OK, so that would be one way. You want to release it that way. OK, so it would have to be under conditions. We could do that under neutral conditions. What else can happen to this ring. That doesn't happen.

There are lots of ways this molecule can break down. OK, it depends on the details of the environment. How else could this molecule ring open?

You wouldn't need to ring open here. You just go through an oxocarbenium ion and have water attack. So what if it opens that way. So that's the way you form aldehydes. All sugars are in equilibrium with aldehydes. These things are in equilibrium. So you have a ring open species. But then what happens if a ring closes? It can ring close from the top face or the bottom face.

You have an imine. What can happen to the imine?

It can hydrolyze. This molecule, and this is a molecule my lab worked on decades ago, has a half life in solution of 10 seconds. So is 10 seconds short or long, biologically?

What do you think?

AUDIENCE: I'm going to say short. But I don't know

JOANNE STUBBE: Yeah. I think it's amazingly long inside the cell. So I think as a chemist, nobody could ever.

Nobody ever saw this intermediate. My lab was the first one that figured out how to look at it. And I won't go through that. But the fact is that 10 seconds is a long time inside the cell, if you think about how small the cell is and how fast diffusion is.

OK, so one place, though, where you might want to have organization is if you have something chemically really unstable. OK, because then when you generate it, it could potentially be passed off, or as you say in the immediate vicinity, it's a competition. But if it's right there, you're effective molarity, that would get into that first question, the effective molarity. It would be high enough to get passed on. It would get high enough to get passed on to the next guy in the pathway.

So that would be one thing is instability.

And in the purine pathway. We will go through this a little bit. But really, that's one of the things that's most amazing about Buchanan's elucidations of the pathway is only intermediates are unstable. Nobody, still, if you're looking at omics, looking at nucleotides, nobody knows how to deal with these molecules. They're all chemically unstable. And they don't get that they're chemically unstable. They don't ever see them. The reason they don't see them is because they don't know how to handle them to keep them alive during the analysis part of the project

OK, so you have this instability problem. And in the purine pathway, the instability problem is a real problem for not just this guy. This guy is obvious. But for other guys. OK, so then the next question is where else. And if you're thinking about metabolism in general, where else might you want to have organization of your enzymes?

You might want to have it if you generate an intermediate in the pathway. And then there. It's a branch point for other metabolic pathways. OK, so there's an intermediate in this pathway that can go to thiamine biosynthesis to histidine to tryptophan in biosynthesis. I'm not going to go

through that. But that would be another place that I think it's obvious that you could sequester, under a different set of conditions, and prevent the other pathways from happening.

So if you have an intermediate branch point, you can prevent other pathways.

So those two things I think are important. One of the questions is, do you increase the flux through the pathway?

OK, so there's been a lot of engineering people. People really care about this in terms of engineering. If you want to engineer a metabolic pathway, should you be linking all your proteins together? And there have been a lot of papers published. If you look at bioengineering papers, where they link all the pathways, all of the enzymes together in a way, because they want them to cluster, because they think they're increasing the flux through the pathway.

And so there are some people that do calculations that show you increase the flux. Other people do calculations so you don't increase the flux. So I think, again, this is an area that I think is very active. And it's pertinent, because everybody and his brother is trying to make biofuels. You'd need to do a lot of engineering from a lot of enzymes from different places, putting them together. How do you make them efficient?

OK, so we asked the question about flux. And I think, mathematically, people are looking at that. You need to know a lot about the kinetics of your system. These systems, there's a lot known about the kinetics. So and then this goes to the question of how, what is unstable. And you need to think about diffusion. I think this is not so easy to think about this. But we do need to think about flux through the pathway.

And then the other thing that's interesting in terms of regulation is it turns out in eukaryotes, where things are much more regulated than in prokaryotes, because of the increased complexity of everything. Almost all of these pathways are organized on multiple activities on one polypeptide. That's telling us something, I think, since we see this over and over and over again. So there must be some reason to do that.

So for all of these reasons in terms of the purine pathway this has been sort of a target for people for a long time. That's one of the reasons I decided to talk about it, because this was one of the first papers where people were excited that they thought they had evidence for this kind of organization in the cell. Not in the animal. But in the cell. OK. Let's see what I want to

say next. I'm trying to keep this on some kind of a schedule.

OK, so this is the hypothesis. The hypothesis is that these things are organized in some way. And this was taken out of-- probably it was a review paper. It wasn't taken out of a paper you had to read. Here's the cell. That's the nucleus of the cell. And what do you see. I think you can see this right. You see these little dots which they call punctate staining.

So what else do you need to know that they don't have in this picture that's really sort of key to thinking about this model. So here they've just have a bunch of enzymes stuck together and all in a little ball. OK, so if you read the paper there was a couple of things.

AUDIENCE: How you're getting the fluorescence?

JOANNE STUBBE: How you're getting them?

AUDIENCE: If it's by effusion or fluorescence.

JOANNE STUBBE: Yeah, so how you're getting the fluorescence becomes key. OK, so we're going to talk about that. How did what was a major way they got the data. We'll talk about this in a minute in more detail but. But whenever you're going to use fluorescence, you have to figure out how to get a probe onto your protein. So that's like a major focus. And this again is where chemical biology needs to play a role. We still need better ways to be able to do this. You've seen over the course of the semester. I think in a lot of ways you could potentially do this. We'll come back to that in a minute.

But if you look at this, what's missing? And this is something that drove me crazy when I reviewed the original paper.

AUDIENCE: I just noticed, so you're getting that. But they didn't stain the membranes really. There's not a good-- I mean, you can kind of see the shape of the cell. But it would be nice to have a clear sort of--

JOANNE STUBBE: OK, so they might have done that. Did you look at the supplementary material? They might have stained the membrane. OK, so I think everybody would believe you see little blobs. OK, so what do you need to think about in terms of the little blob.

AUDIENCE: The size.

JOANNE STUBBE: The size. Right. Yeah, so that's one thing. They don't ever they don't ever talk about this. They

might in some of the very later papers. But if we know this, we have structures of all the enzymes in the pathway. So you could make a guesstimate about how big these blobs should be, if you had one of each of these. And these things are huge.

So this would tell you that you would have many, many of these. This is one thing that I think they need to do some more thinking about that they could have many, many of these things. And then the question is, why would you want many, many of these things. And how were they organize? Are they just sort of randomly organized or are they really organized in something like that with this big huge protein in the middle. That's one of the ones they look at. FGAM synthase, that has a molecular weight of 150,000, which is huge for an enzyme.

And so for a long time-- and the catalytic activity-- my lab has studied that-- is way over here. And so you have a lot. Could it be a scaffold. OK, so that's where that idea actually came from. So but the hypothesis is that these guys are organized. And they're under certain growth conditions. That's the key. And we'll look at those pictures that come together if they do this when you need to make purines. And then they can go apart.

OK, so the key thing, I think, is. And I wanted to just remind you why we're spending this time looking at fluorescence. And we probably should have spent two or three recitations on fluorescence methods. But we didn't. Is that we've seen this many times before. We've seen stopped-flow fluorescence in the Rodnina paper, where we were looking at the kinetics of fidelity of EF-Tu. And somehow they put a fluorescent probe onto the piece of tRNA. That was not trivial. How you got the probe there. And that probe could-- and we'll talk about this in a minute. But it could change. It changes when it's in different environments.

And so you can use it as a way to monitor changes. So reactive oxygen species, we just looked at this. And I decided to put this up, since we didn't have the structures up last time. Fluorescein is one of the dyes that. This is fluorescein that people use. This is a version of fluorescein. But we talked about how do you know that epidermal growth factor is generating hydrogen peroxide?

OK, so what we need is a sensor of hydrogen peroxide. So we talked about that last time. And this is the sensor that people use. Why did they use it. We talked about it. But we didn't have the structure. So they use the dye acetate of this molecule. This one they use, the triacetate. The one that they use in paper was the diacetate. Anyhow, you need to get the fluorescent probe into the cell. So that's something you're going to have to deal with.

And so if you acetylate it, you don't have phenols or phenolates which might not get through the membrane, which apparently they don't. So then when they get in the cell, what do they do? They hydrolyze, OK. So what happens is when they hydrolyze, they are now-- you have these hydroxylated compounds that are able to be oxidized by an oxidant. And one of the oxidants that can do this. And there are others that can do it as well, is hydrogen peroxide. So people use this as an indicator of hydrogen peroxide. But it's not specific. Yeah?

AUDIENCE: So are they also trapped after that esterase, like from diffusing that out to the--

JOANNE STUBBE: No. I mean, I don't think they diffuse back out, because I think they're the phenolates. So I think the diffusion out, like with many of these things, like if you use-- lots of times you esterify phosphates to get them into the cells. Once they hydrolyze, they charge. They don't get back out. So I don't really know. But that's what I would guess.

So I guess the key thing and the basis for some comments that I made in class was that we don't really have. We don't know that this is specific for one reactive oxygen species. And so there are lots of people in the chemistry, biology interface trying to make specific sensors. OK, that's not easy to do. The hydrogen peroxide, they're getting better. In fact, Ting's APEX, which is a peroxidase, sort of similar to what we talked about with peroxidations in the myeloperoxidase can actually function as a hydrogen peroxide sensor.

So anyhow, what happens is that when it gets oxidized, it becomes fluorescent. So it's not fluorescent. It becomes fluorescent. So it just gets turned on. And you can see something, OK? So that's something we talked about.

In Liz's part of the course, we talked about the fact that we can watch protein unfolding in the *E. coli* proteasome. OK. And what did you look at in the proteasome, clip X clip P? You looked at titin. That had a little tryptophan on it. And tryptophan can absorb. It's not a very good thing, because it absorbs in the UV. But tryptophan fluorescence is used a lot. There are lots of tryptophans, so it's also really hard to use. But titin was this little tiny protein. And it was the only tryptophan.

And they also did experiments with green fluorescent protein, which is what we're using in this paper. We remember. They pull on it. And you pull and you pull when you pull and then all of a sudden it unfolds. And you lose your chromophore. So you go from the on state to the off state.

So all of these things. Binding measurements. You talked about. You had one problem set. I don't know whether you guys did that problem set or not. But there was a-- what was the calcium sensor? Does anybody remember? Anyhow, there was the calcium sensor, where you were asked in the problem set for a something or other that you asked to measure the KD for. And you can do binding assays.

So fluorescence is an incredibly powerful tool as is the take home message. And we've seen it throughout the course. We just haven't talked about it.

So now the key thing. And we're going to talk a little bit about fluorescence at probably a freshman level. Many of you guys, who were the undergraduates. You guys, have you done fluorescence experiments? You haven't done in the lab? I thought we had two Eureka labs that were fluorescence oriented.

AUDIENCE: [INAUDIBLE]?

JOANNE STUBBE: No?

AUDIENCE: Yeah, yeah. so we--

[INTERPOSING VOICES]

JOANNE STUBBE: So doesn't Tim's? He does sensors to sniff. I don't know what to sniff, but to sniff something, TNT or--

AUDIENCE: Right. but we didn't use fluorescence with that.

JOANNE STUBBE: You didn't use fluorescence for that. OK, or the Tokmakoff lab?

AUDIENCE: The one experiment we did in lab is we labeled a protein, the green absorbing dye. And it used laser anisotropy to measure KD rotations. And so the--

JOANNE STUBBE: OK, so you guys are experts, then, on fluorescence. Well, hopefully you-- anyhow, so one of the questions is we need to ultimately the key thing for any of this is we're going to have to have a fluorophore.

So that's it. So we need the key starting point is a fluorophore And what are fluorophores. So you want something that's usually aromatic and large. It could be-- it could have a lot of nitrogens in it.

Oh, I knew I forgot something. So there's a book called, Molecular Probes. OK so I gave you a handout on fluorescence. I forgot to bring the book, if anybody wants to see it. This book is worth its weight in gold if you're a chemical biologist. Because this has everything in the world you need to know about fluorescence. It's described in a thoughtful way.

They sell all the probes. If you want to do something to tweak something, they'll help you do all of that. So this book, this is molecular probes book. I think it's online now. I have a copy that's five years old. I use it a lot. It's a really important book. And I got this out of the book. And it just shows you in the book, they have all these pictures of these fluorophores. So they're just big, huge, greasy molecules. You have to worry about solubility a lot of the time. So you have to stick sulfates, or something that ends up making it soluble.

So that's going to be a key thing.

So we need to have a fluorophore. And we have many options that we can buy these things. OK, so what's this? OK, so what we want to think about is this. So in your, the latest version of your handouts, I've written down what I'm going to say. But it's pretty simple for-- I'm talking about this in a pretty simplified viewpoint. But what we're going to see is these fluorophores are going to allow us to. They allow us to do assays. I'm going to show you a quick example of that.

That is you can have something that is. You can have a molecule that is quenched, so you have a quencher on one side. I'll show you. And I'll show you the way the quenching comes from, something fluorescent on the other side. You can't see anything. You cut it in half. It could be a protease. It could be a nuclease. The quencher goes away. And you see fluorescence.

You could have a sensor for metal binding, which Liz talked about. So you have two fluorophores. OK, you've got to figure out what the right fluorophores are. Something binds. They change confirmation. And they change confirmation in some way that you can actually detect a shift in the wavelength.

And then you're looking. In our case, we're just sticking something on the end to see something. You were making a protein fluorescent. That's all we're doing.

So you can use it for assays. You can use it for FRET. And in the current-- so you can measure distances. We're not going to go into that. But any of you that are interested in the

current version of the handout, I have sort of short tutorial on what FRET is and where you should go to look this up.

And then we just basically have a fluorescent tag. OK, and we'll come back and talk about the tag. We already talked about the fact that we have green fluorescent protein, red fluorescent protein tags. But we'll come back and talk about the other tags.

So we have a fluorophore And so what does that mean in terms of what's going on. So you have your molecule. And your molecule has a ground state, which we'll call this S_0 . This is the ground state. And you have many vibrational modes. And you have this big huge fluorophore that can absorb your electron. And your fluorophore can't absorb a photon.

And so what happens is. So we're going to have excitation with a photon in a certain way, in a wavelength that can be absorbed by the electron in your molecule to the excited state, which they call S_1 . And so you can have your electron going to an excited state. And we have a wavelength of light when that happens. And that depends on the structure of your molecule.

So you don't want to be in the UV region. You want to be out in the region where you have less interference. And so that's the key game you have to play to get into that region in the visible. You really have to put a lot of stuff on here. You just can't make a small little molecule that absorbs at 600 nanometers. So that's part of the problem. So you're making big things of necessity, so you can actually see something happen.

And so then what happens under those conditions. So we're going to have the excitation wavelength of light at a certain λ_{max} . You absorb. It's just like absorption. You have a certain wavelength that it absorbs more frequently. Then what happens in the excited state on a very fast timescale, you lose energy. OK, so under these conditions, you're doing a relaxation. And then we'll see in a minute. I'll talk about what are the mechanisms of relaxation. But that can tell you. You can use those relaxation mechanisms in a different way to design your fluorescent experiments.

So what you see in this cartoon is that you're relaxing on a very fast timescale. And physical chemistry has told us that to see fluorescence, it needs to go down. So these are the vibrational modes. So you're exciting your electron electronically and vibrationally. And then you need to go down in vibrations. You're losing energy somehow.

What happens to that energy? OK, we can talk about what can happen to that energy. And

when it gets to the lowest level of the excited state, you have fluorescence. OK, and so that also happens on a pretty fast timescale.

So the key thing here. So when you get to the lowest. So this is the lowest level, it fluoresces. And so this is where the photon emits. OK, so the photon wavelength for emission or $h\nu$ emission. And the key thing that you've probably heard about, again, when you were introduced to fluorescence is because you're losing energy here, what happens to the energy? You're going to a longer wavelength. OK, so the excitation and the emission wavelengths are distinct. And that's called the stokes shift. So it's the wavelength of excitation vs. the wavelength of emissions.

So you have a stokes shift, which is the wavelength of excitation minus the wavelength of emission. And so you need to look at molecules. People have spent a lot of time. You saw those 25 lists of things where people have designed things that actually work quite effectively.

OK, and so then the question is you losing energy. You are always going to be at longer wavelengths. OK, so that's good, that makes it easier to see, because there aren't that many things inside the cell that give you a background, which is what you need to worry about in all of the experiments you're doing inside the cell. The brightness, we'll come back to that in a minute.

So what kinds of models can give you. What kinds of mechanisms are there for relaxation of the excited state.

And so there are a number of mechanisms that can be involved. And one is, again, non-radiative relaxation. And how does that happen? So you're changing vibrational modes. And when you're in the excited state, if you're in solution, you have interactions with solvent or other molecules, all of which can affect this kind of transition. If you're in the active site, there can be other things. So the key here is the environment.

And again, it could be solvent. It could be protein. And the only way you can tell is by actually looking at the fluorophore on your molecule to end up seeing what you end up seeing.

OK, so a second way that you can see. And you probably saw this in your introductory. Yeah?

AUDIENCE:

So what would be an example? Like, if a unit of the energy being released is a photon in one case for non-radiative, what's the unit of energy?

JOANNE STUBBE: What is the unit of energy? So energy, heat is one way that you lose all of this. So it's vibrational energy. I would say, it's mostly heat. So you're changing excitation levels somehow. And the beauty of fluorescence. And this is the key to the sensitivity is you're not doing anything to your molecule. So your electrons got excited. They give off a little heat or whatever. They somehow change a little bit. And then they go back down to the ground state again.

So what can you do? You can excite them again. So this can happen over and over and over again, unless the molecule in the excited state becomes destroyed. So that's called photo bleaching. So the key thing here. And this is, I think, this ability to recycle is the key to sensitivity. But again, I haven't used fluorescence inside the cell. I've never done this myself, experimentally. So I don't really know. But you hear about photo bleaching all the time. So I think this is not a trivial thing that you can just blow off. It would be nice. But what you're doing is you're using the same excitation. And then loss and excitation and loss over and over and over again.

And so it provides a much more sensitive assay than what you normally see for something like absorption.

OK, so let's see. There was one other thing.

Oh, so we talked about this mechanism, non radiative relaxation. How else could you relax? You can go from a singlet state to a triplet state. OK, I'm not going to talk. But intersystem crossing, yeah. So you can go from the singlet excited state to the triplet state. I'm not going to talk about this. But the triplet state then can phosphoresce.

We're not going to be discussing that at all. But that's one possibility.

We just talked about the fact that you can have something in there that quenches the fluorescence. It interacts with something in a distance dependent fashion. And that, again, affects the intensity of your fluorescence.

So you also have reaction with the second molecule. And that can become. And it could be good or bad. If it reacts with oxygen, what happens is oxygen, the energy is immediately transferred to the oxygen. So that's why in many fluorescence experiments, you remove oxygen from all of your samples. It acts as a quencher. So you have. And it could be oxygen, which acts as a quencher. Or it could be another fluorophore. In which case. and if everything

is set up correctly, you can get the energy to shift the energy of emission can get shifted to longer wavelengths. So that's what FRET is all about.

OK, so it could not. A second molecule could be another fluorophore.

OK, so those are sort of ways that you can relax. And then you can set up different kinds of experiments, depending upon what the objective is of using fluorescence.

So I've written this out in more detail. And for those of you who want to look at FRET, I've defined FRET. I've given you the equations. And people use this quite a bit inside the cell. You need to study this. There are a lot of issues associated with it that you need to think about.

And I'll come back. You need to think about. It's not. There are a lot of constants that determine the rate constant for your FRET, OK. And so you just you need to think about all these constants to be able to interpret the data in a thoughtful way. And I've given you a tutorial that I felt was pretty good that I get off the web that just shows what FRET is.

And that we have many, many dyes that we can measure distances from 10 to 100 Angstroms using FRET. That's not in this paper. So I didn't. And this just sort of is a cartoon of what I was just telling you. So here, you might have an interaction. But if you cut it, the interaction could be gone. Here, you might have no interaction. But when some small molecule binds, you see an interaction. And you can pick this up using fluorescence changes.

OK, so people do these kinds of experiments all the time. And this kind of an assay is extremely-- there are two kinds of assays that one does. So if you work in a pharmaceutical company, people do this all the time. They want a very sensitive assay. Everybody uses fluorescence. They might use an assay like this, where you go from nothing to something. OK, so you have high sensitivity.

And the other thing they use is, which I gave you in your handout, is fluorescence polarization, which I'm not going to be talking about. But those are the two major methods that people develop assays around in the pharmaceutical industry.

So fluorescence is here to stay. We still need better tools. It can be quantitative. You can measure a quantum efficiency of the electron, light, that's involved in the excitation and the photon that's involved in the emission. If it's 100 percent efficient, then you're quantum efficiency is 1 anyhow. So you have a whole range of quantum efficiencies.

OK, so now what I want to do is we're late. But we'll at least get to the other sources telling you what I just told you. OK, so I want to just introduce to you some of the issues that we're going to be facing. And we are going to talk about this in class, probably Monday or on Wednesday morning, OK. So I'll extend this in class.

But they've attached green fluorescent protein to all of these things. So this is issue number one. What should they have done in these papers that they didn't do? If you read the paper carefully. I mean, it's hard to read a science paper, because all the key pieces of data are in supplementary information.

So they made a few. In all of these, I can't remember what they made. But they made fusion proteins, right? So here, you have a purine enzyme. And here we have some kind of fluorescent protein. So that's the probe they're using.

OK, so what's wrong with that, with the way they did their experiments? Can anybody look at the details of what's going on?

So what, if you made this fusion, what would be the first thing you would do with a fusion protein?

AUDIENCE: My first thought would be, if it changes the activity of the original protein. GFP's a very large [INAUDIBLE].

JOANNE STUBBE: Right. Exactly. GFP I'm going to show you in a second. I think I can show you this in a second. These are just the ways they were looking. But you have all these probes. GFP, it's over here. These are the organic dyes. Here's an antibody. We'll come back to that. So GFP is big.

So does it change activity? They didn't assay that. To me, that's mind boggling. OK, because I've dealt with these. I know these proteins, that one protein there. So two of them they're dealing with. One of them is a trifunctional protein. The other one's 150 kilodaltons. These proteins are not easy to deal with, OK.

So to me, this is a key thing. So this goes back to the Marcotte paper, where he's saying, well, I mean, maybe these things don't express very well. And they aggregate. They don't fold. We saw how complicated the folding process is.

What is the second thing? How did they get the proteins into the cell? How did they get? They don't get proteins into the cell. How did they get? Yeah, how did they get GFP constructs into

the cell?

AUDIENCE: Transient transfection?

JOANNE STUBBE: Yeah, transient transfection, what is the issue there? Without going into details, but what's the issue?

AUDIENCE: Like, when the cell's normal mechanism, like the cell's own enzymes maybe--

JOANNE STUBBE: So you do have a normal-- you do have the normal enzyme. They didn't make any effort to knock out the purine enzymes. OK, but I think the key thing with transient transfection is the levels.

First of all, a lot of cells don't have anything. But then you don't care about that, because you don't look at them, because they're not fluorescent. OK, but do you think the levels are important.

I think the levels are incredibly important. So the question is 100-fold, 1,000 fold over the endogenous levels. And so to me, the first experiments I would have done before I did any of these other experiments is I would have looked at. You might have chosen the trifunctional protein, which they did, because it has activities 2, 3, and 5. And this other big huge protein, which-- so these are the proteins they focus on is activity 4. So 4 is huge. You might think it could function as a scaffolding protein to interact with activities 2, 3, 5. All of that's totally reasonable.

OK, but they didn't deal with those issues. So you need to figure out how to attach something that's fluorescent. So one way is genetically. OK, and we've seen this. So we're just fusing GFP onto the protein of interest. Another way in this paper, also, and you mentioned that, is they were using endogenous antibodies.

OK, so antibodies can't get into cells. So how do you assay this? So these are also tough experiments. So somehow you fix the cells. So they aren't falling apart when you're trying to perturb the cell to allow the antibodies to get in. And then you permeabilize the cells. Have any of you ever done that?

I've done it in yeast. In yeast, it's brutal. I mean, it works. But it's the conditions are like it's a witch's brew. Anyhow, so then you get the antibody in. And that's what you're looking at. And if you look in the-- I have. We're not going to get that far. But I have pictures of-- So when they

compared the transient transfection with the endogenous levels, that might give them some feeling for what levels, the levels of expression actually are.

And of course, the way that people really want to attach things is using small things, whatever these lists of dyes are that we have. And what are the methods that you guys have learned about to attach these fluorophores. So instead of using a genetic fusion, which is probably. That's a really good way, except the protein, the green fluorescent protein is big.

Green fluorescent protein is also a dimer. So people have spent a lot of time engineering green fluorescent protein to be a monomer. So the ones you buy commercially now are all monomers. That would add complexity to everything on top of this.

How would you attach some of these things? So we know what the structures of these things are.

AUDIENCE: You can do like a halo tag.

JOANNE STUBBE: So you could do a halo tag. Have you talked-- we haven't talked about that. So give me another method. Give me a method we've talked about.

AUDIENCE: A handle, [INAUDIBLE] handle to attach?

JOANNE STUBBE: Yeah, but how would you do that? How do you attach these handles? You want to attach a fluorophore. OK, so it turns out that all of these things here, which you can't see. But these little aromatic things have been synthesized. So click it on. If they could have a settling there. But then it needs to be clicked to something.

AUDIENCE: [INAUDIBLE].

JOANNE STUBBE: So you can't just. So how do you click it?

AUDIENCE: [INAUDIBLE].

JOANNE STUBBE: So but is that easy to do inside the cell? No. And in mammalian cells, it's impossible. OK, so you can't use unnatural amino acids inside the cell. The technology is not there at this stage.

So the question of how you attach this. You could make your. If you could make your protein outside the cell. You might be able to do that. But then you have the problem of getting your protein inside the cell. So getting a probe that's fluorescently, you're labeling the protein of

interest is not easy. And Alice Ting's lab, again, has spent a lot of time, not that successfully. But using ligases that you can then incorporate into the cells that can then react with things you put onto your protein to attach fluorophores.

But this is an area that's really important, because in my opinion, looking at regulation inside the cell, we don't really want to perturb. We don't want to be at very high levels. And we want to be able to see something to understand regulation. So I think. So anyhow, the issue is that we want to be as small as possible. We don't want to be Brad's lab. What is Brad's lab? Does he use these nanobodies that are antibodies?

AUDIENCE: Like an [INAUDIBLE]?

JOANNE STUBBE: No. They have all these. They have things called the nanobodies now. So I think they are like the little guys you make on your solid phase peptide synthesizer. But they are specific. They specifically bind to proteins. So there are only five examples that I've seen in the literature. So they act like antibodies. But they're-- huh?

AUDIENCE: Like [INAUDIBLE], like little--

JOANNE STUBBE: They're little tiny proteins that are maybe. I don't know. 50 amino acids that somehow, some guy at the University of Chicago-- not Kent-- developed these things. And they specifically. They act like an antibody. They can specifically interact with a protein of interest. And then you attach a green fluorescent protein onto it. So again, what you have something smaller. So because with these antibodies. What you see is the non-specific, right? I mean, we've seen that. And with fluorescence, that means you have fluorescence background in everything you do.

So anyhow, I think we're not that. So that's just you're using fluorescence microscopy. This tells you why you're interested in fluorescence microscopy. And we'll just close here. And we're going to come back and talk about this in class. But this is sort of the example of the data that you need to think about. So what we hear is in the presence of purines, you don't see any of these little dots. You remove the purines.

OK, so this is not so easy either, because the way we grow cells, we don't have defined media, right? I mean we're using. I don't know what you guys use now, but fetal calf serum or something. It's got all this stuff in it that we don't really know what it is. We don't use defined media. And apparently, when they-- the Marcotte paper-- when they were describing this, said

it was not so easy to remove the purines. And the method they used to remove the purines also removed other stuff, OK. So you're stressing the cell. That was the take home message.

So under those conditions, you see something different. OK, and so then they did another experiment, because they were worried about levels. Here, they are they have an antibody to the trifunctional protein. And so this is what they see under low purine conditions. Does this look like this? I don't know.

So you can't tell by looking at one picture. OK, so you've got to do statistical analysis of all these things.

So I think this sort of-- we'll come back and talk about this in class. But I think this is the first example where people are trying to look at this. The data is interesting. But we've already raised issues of what some of the problems are. And hopefully, you can think about more of the problems.