

20.320, notes for 9/13

Thursday, September 13, 2012
9:36 AM

Coming Assignments

1st part of design project coming up, assigned 9/14 (tomorrow) and due 9/21.

Last time

We covered ITC and mentioned Mass Spec.

Co-Immunoprecipitation (Co-IP) and Mass Spectrometry (MS)

Co-IP, as the name implies, is about precipitating a particular protein by attaching it to antibodies, and then looking to see what else gets dragged down with it. It's a way to see what other proteins the first one reacts with. The purpose of Co-IP is discovery of a particular protein's interactions, not finding the particular affinity with which any of those interactions happens.

This is usually done in conjunction with Western Blotting, where additional antibodies are used to detect the presence of Co-precipitated proteins. This is a targeted technique, where you have to be looking for a particular protein.

MS, on the other hand, is not targeted. It's a technique for discovery, that allows you to find out what got Co-IP'd with no previous expectations or bias about it. The path to protein discovery, then, is:

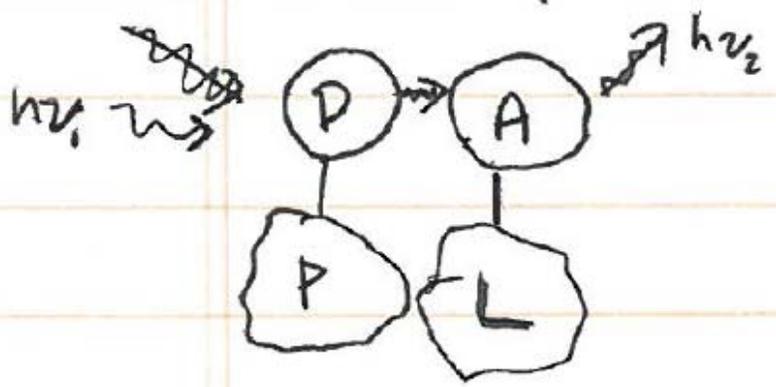
1. IP
2. Digestion with trypsin (an enzyme that cuts proteins into peptides)
3. Analyze peptides with liquid chromatography (LC) and MS.

MS can give you the masses of unknown peptides by fragmenting and measuring the charge-to-mass (m/z) ratio of all the fragments. With some computational analysis, you can identify the proteins. MS fragmentation gives you the sequence. Note that it does not show you function, K_d , or protein structure, but it's still extremely useful for identification. (Note that it *is* possible to get some structural information by crosslinking the protein together to form stable bonds other than those of the primary amino acid ribbon).

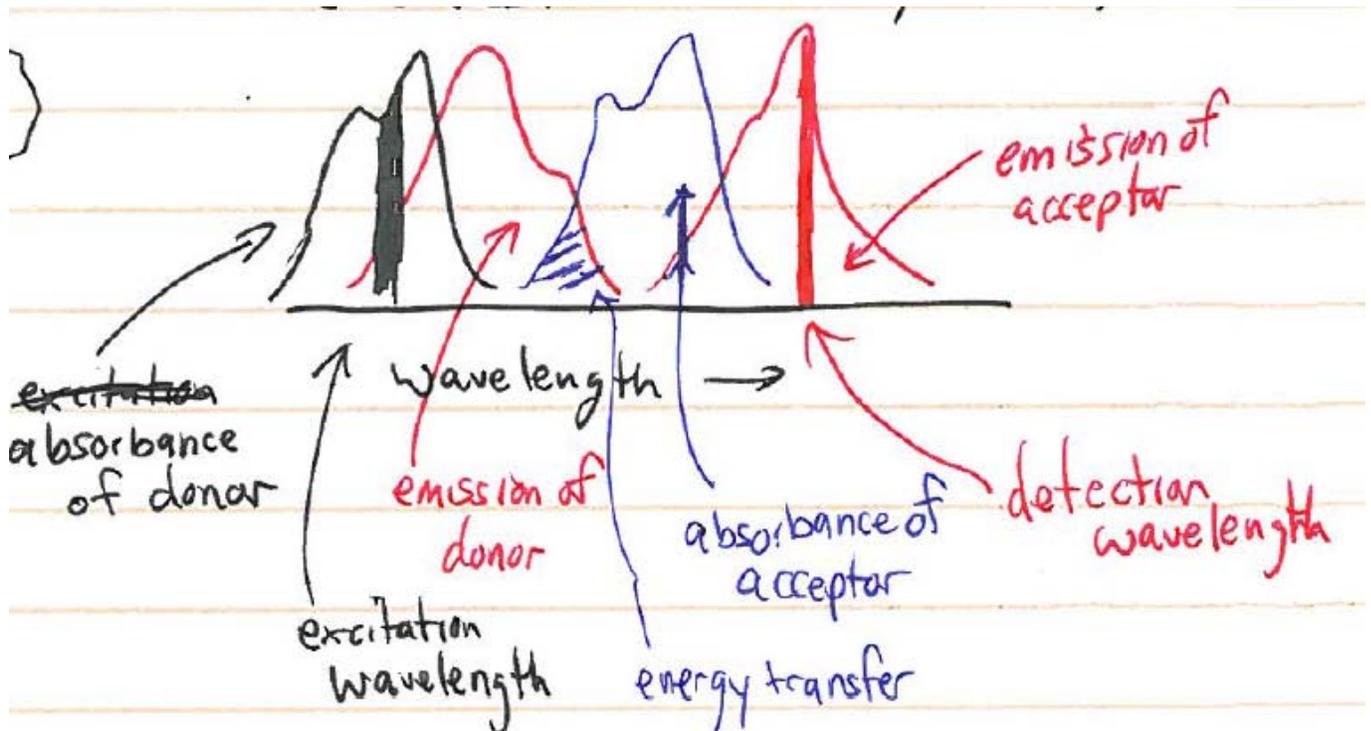
Combined with Co-IP, MS can give you evidence of interaction. Proteins that are associated will precipitate together, but note that they may be only indirectly associated. That, you'll also miss some protein complexes if they're not stable enough to undergo the MS process. Also, finally, remember that this is not a real-time analysis. Still, it's one of the most powerful techniques we have.

FRET: Förster Resonance Energy Transfer

FRET is the molecular ruler, a way to determine whether two things interact (or ever come close to each other) in living cells. It's based on two fluorophores which normally fluoresce one way, but that will change their pattern of fluorescence when brought to a certain, very close proximity. Rather than fluoresce on its own, a donor will transfer its excitation energy to the donor, which will then fluoresce at a wavelength that otherwise doesn't show up. Donor and acceptor FRET probes have to be stuck onto protein and ligand.



You need to choose fluorophores such that the emission spectrum for one overlaps with the excitation spectrum of the other.



Limitations

A big one is relatively **low sensitivity**. It's limited by the overlap in adsorption spectra between donor and acceptor. The donor must emit within the excitation spectrum of the acceptor, and whatever energy is emitted outside that overlap will be lost. Choose your fluorophores to have the right overlap.

It's also possible for good probes to not interact well; **location of the probe is critical**. If you put them both on the far side of P and L, away from the interaction site, the probes may not come close enough to work. On the other hand, the probes may accidentally be placed on the interaction site itself, thus messing up the effect you hoped to see.

Another issue is the **size of the probes** themselves. Since in many cases they are GFP or other fluorescent proteins (~30kDa), they can sometimes be even larger than the P or L we want to analyze. You need to genetically encode fusion proteins, and then make sure that expression and/or localization isn't fundamentally changed.

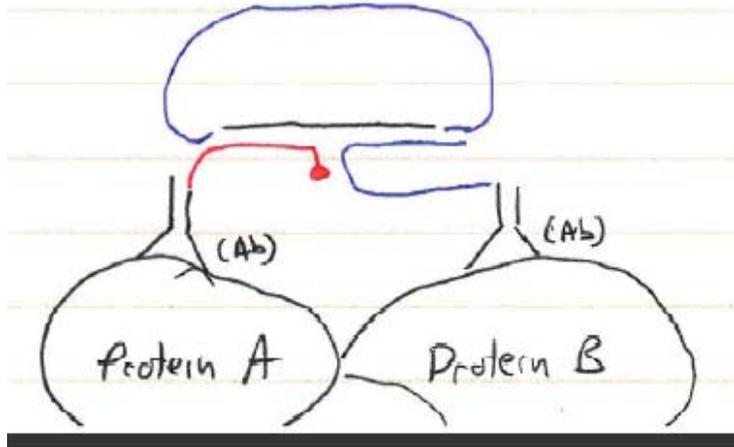
Mostly, it's a pain.

Strengths

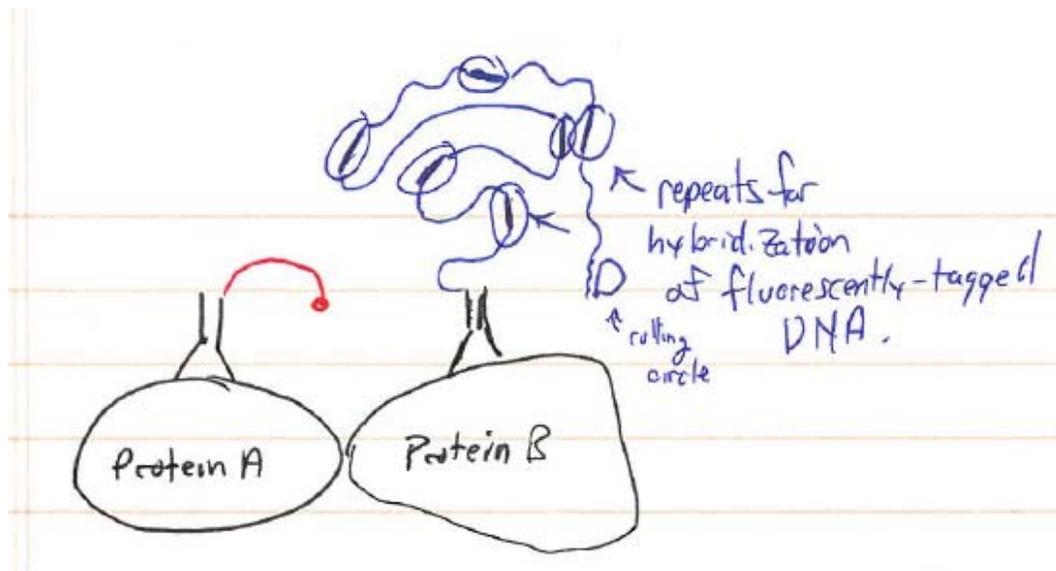
Live-cell imaging
Transient information

Primary Ligation Assay, PLA

This is an alternative to FRET, which we can do on fixed and permeabilized cells (dead on a microscope slide, but retaining their structure).



1. Fix cells
2. Permeabilize to allow access by DNA and antibodies
3. Add antibodies with single-stranded DNA (ssDNA) probes
4. Add complementary DNA that bind to probes in proximity
5. Add more DNA that circularizes the previous, creating a circular template for Rolling Circle Amplification (RCA)
6. Allow Phi29 DNA Polymerase to carry out RCA, creating a long repetitive copy of the circularizing probe.
7. Add fluorescent DNA that is targeted for the repeats above, and now you get a massively amplified fluorescent signal.



Benefits

Great amplification. 1000's of fluorophores per interaction.

Limitations

Requires antibodies to your targets.

Not real-time, though you can take lots of snapshots and compare them.

Surface Plasmon Resonance (SPR)

We have techniques for detecting the existence of interactions. We don't yet, however, know K_d . Why do we care so much about it, and how can we measure it? Remember that the components of K_d are k_{on} and k_{off} .

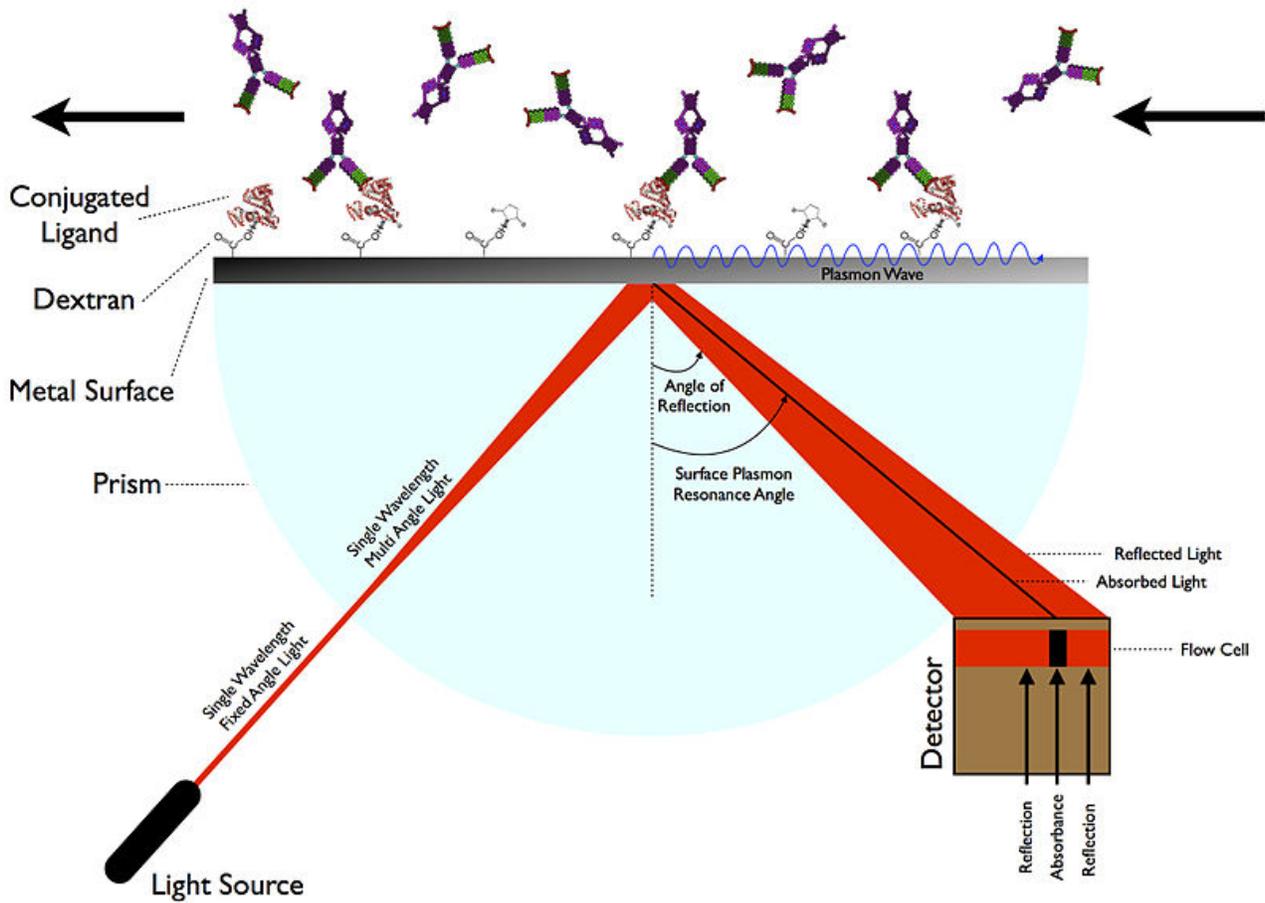
k_{on}

This constant is the on-rate, the rate at which the complex forms. It's typically governed by diffusion, because the proteins need to find themselves first and it's hard to get them to do that faster than diffusion.

k_{off}

This constant is mostly governed by interactions within the complex itself.

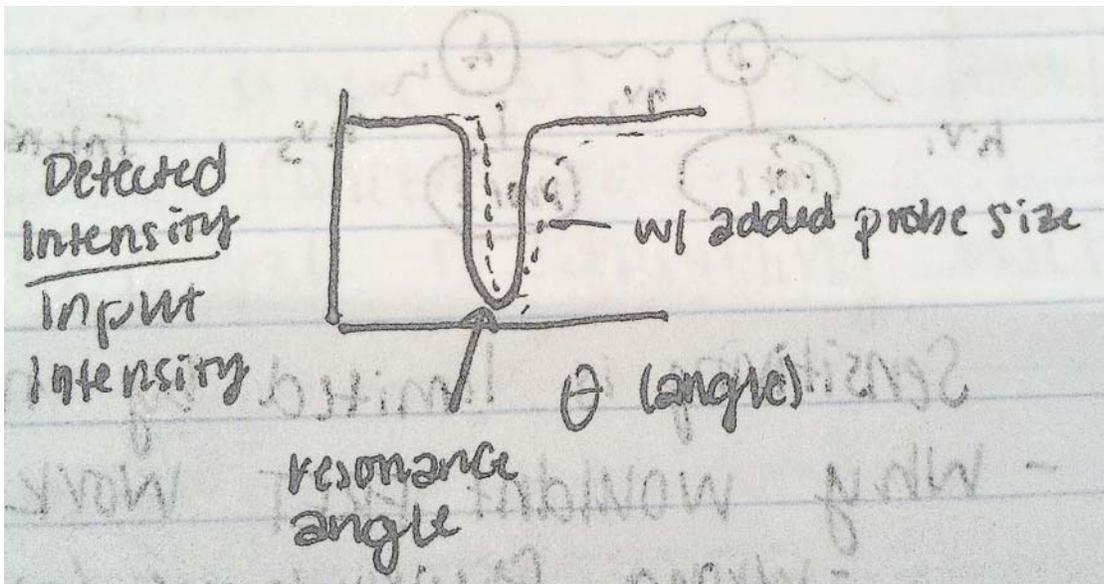
Biacore is the name of the company that dominated the SPR market, and so SPR is something called that also. The technique is based on oscillations in a thin film of material excited by incident light. Surface plasmons are surface electromagnetic waves that move along the boundary between the metal (a thin gold film) and the external medium (an aqueous solution in our case). These waves are very sensitive to the mass of particles attached to the surface, and are thus used to detect the absorptions of molecules onto it.



Courtesy of [Sari Sabban](#). Used with permission.

Source: S. Sabban, "Development of an in Vitro Model System for Studying the Interaction of Equus Caballus IgE with Its High-Affinity FcεRI Receptor," Ph.D. Thesis, The University of Sheffield, Sheffield, 2011.

Onto this surface we can covalently attach a bunch of proteins. If we then wash over a second protein, we will be able to detect interactions by changes in the mass of attached groups. It's all measured by resonance units of the SPR resonance angle, which can give us a very precise indication.



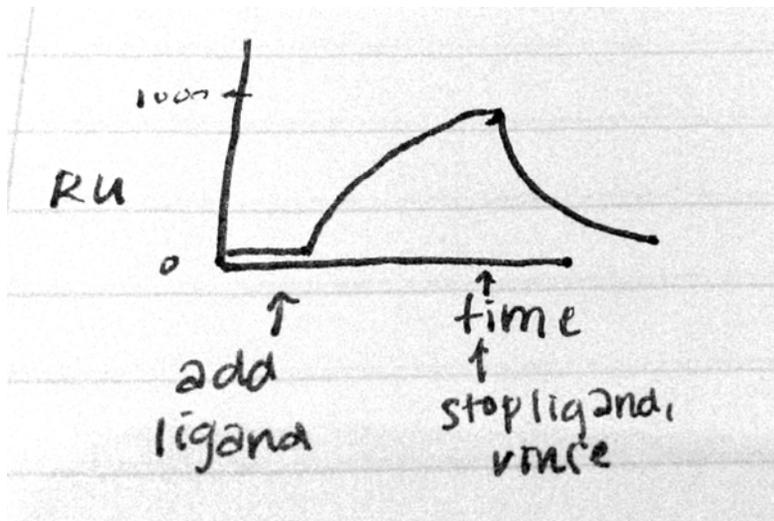
1 Resonance Unit (RU) = 0.0001° (fraction of resonance angle) = 1pg/mm².

Out of simplicity, we'll call the attached stuff ligand and the flowing stuff protein.

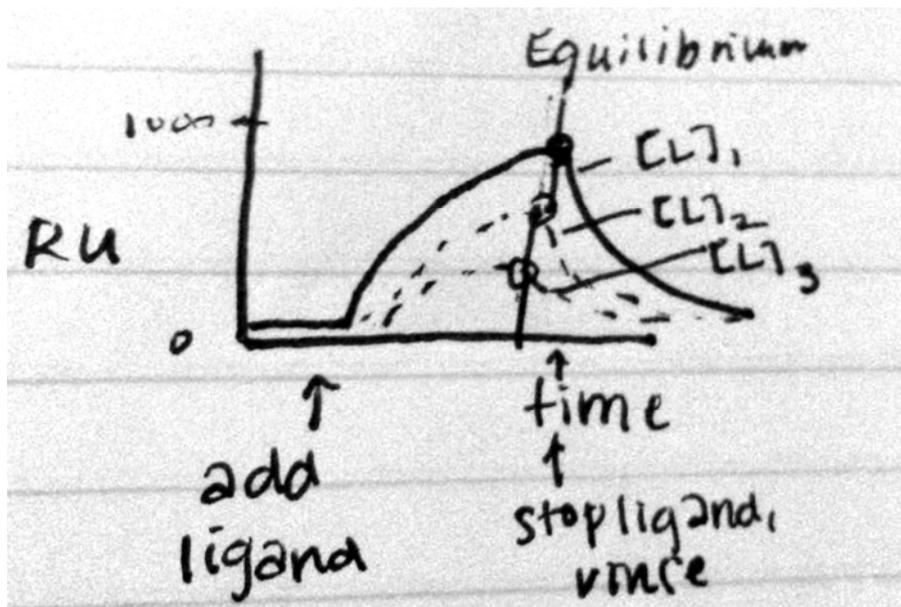
It works best when you attach a small molecule to the surface and flow a larger protein on top. Since what you measure is a change in mass, attaching the larger molecule to start with will make your measurement much more difficult. If you bind small ligands, about 10^7 proteins is the lower detection limit. If we were to bind large ligands and try to detect very small proteins, we might need as many as 10^{10} .

Graph interpretation

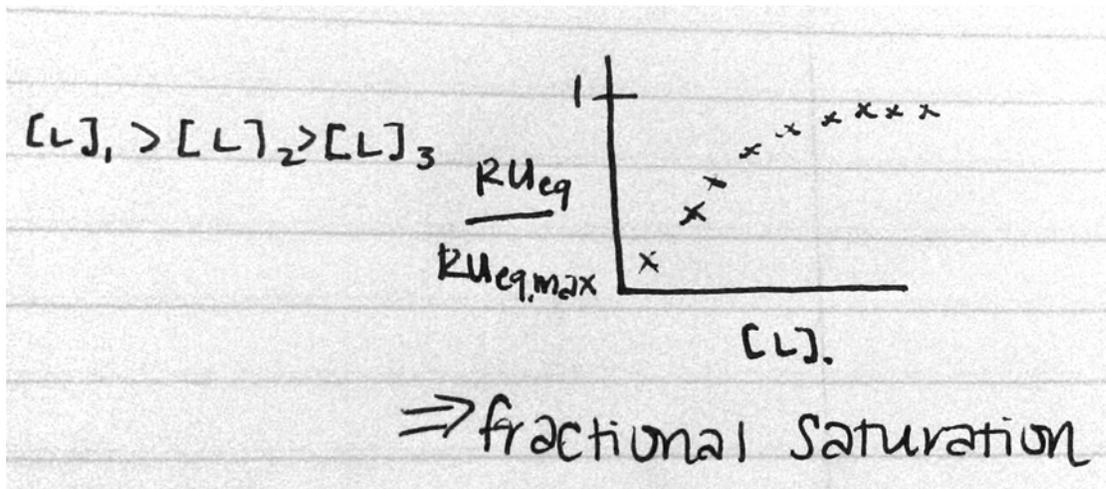
During SPR, we look at a graph of RU over time.



Higher concentrations of the same ligand will lead to a higher RU count.



And we can plot these concentrations against the effect that they have.



Look familiar? It's the same principle as with our previous titration analysis. **When ligand is in excess**, we can use the same approximation as before. Fractional saturation, Y , becomes RU_{eq}/RU_{max} .

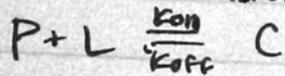
Relating to fractional saturation:

$$\frac{RU_{eq}}{RU_{max}} = \frac{[L]_0}{[L]_0 + K_d}$$

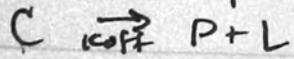
Obtaining our parameters

One of the advantages of SPR is that we can get measurements that more directly relate to the parameters we want, k_{on} and k_{off} . If we look at the dissociation phase and the change of complex concentration (which, again, is related to the RU count), we can measure the dissociation constant. It's related to the half-life of the complex.

Association Phase:



Dissociation phase:



Assumption: once unbound, doesn't re-bind

$$\frac{d[C]}{dt} = -k_{off} [C]$$

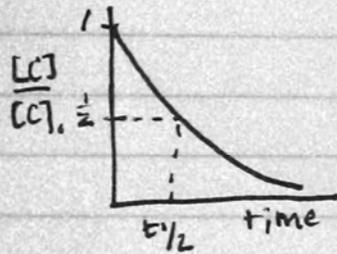
$$\Rightarrow \frac{d[C]}{[C]} = -k_{off} dt$$

integrate

$$\ln[C] = \ln[C]_0 - k_{off} t$$

$$[C] = [C]_0 e^{-k_{off} t}$$

$$t_{1/2} = \frac{\ln 2}{k_{off}}$$



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