Introduction to Cell Biology
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Introduction to Microscopy – Part 3

Professor Nagaraj: Let us begin with Arnav. Arnav Mishra, can you hear me?

Arnav: Yes, sir. I had the query related to the basics of microscopy. Sir, in the initial part, you told about the diffraction barrier. And why it affects the clarity of the image we are trying to observe. Sir, can you re-explain a bit in short how exactly that diffraction barrier creates some difficulty.

Professor Nagaraj: So, see one of the challenges here is the amount of light that is actually, because you are looking at very small objects. Even though the microscopy slide in which, for example, you have put something to look at has enough light. At that time magnification or that focus, the amount of light that is getting into your objective has a significant impact on the quality of the image that is generated.

Now, the light source that you are using, in most of our cases its light microscopes, for light microscopes the way the light diffracts after it hits a surface, also means that the amount of light that is getting into the objective is affected by this diffraction. So, you can alter that diffraction by adding like a layer of water or a layer of oil. Oil affects the diffraction significantly more and reduces the diffraction in such a way that there is stuff that is being brought into the objective more completely. And this allows for more light to make it into the objective, which means that you are now able to see things brighter. And that allows you to see things in a way that you would not have been able to otherwise.

Arnav: And sir I had the second question related deconvolution. How exactly mathematical algorithm is helping us to clarify?

Professor Nagaraj: So, the, as I said, but to put it in a very simple way, if you have a light source, where light is being emitted from a fluorescence image, and suppose that image inside the cell where the fluorophore is sitting is like this, is where my finger is. When you record the image, even though you are recording using a confocal, the light does not, is not restricted only to that

particular region. I will see light in the neighbouring regions as well. And that may come from the fact that there is a limit in how visualization can happen or in the way the microscope is set up and how it is recording the image.

What deconvolution does is this algorithm looks at the intensity of light that is coming, and now determines, it makes a prediction actually. It does not really know whether it is correct or not. It makes a prediction based on the fact that this region is actually the real source of the light, that everything that is around this, and that is based on how exactly the light is distributed in the recorded image. And it essentially removes those as background. Leaving you with this particular stretch of fluorescent image, which is my finger as the final image.

So, when you see this image in the regular confocal, you will not only see this, but you will see all the light that is around it, and it will be like a fuzzy structure. You will have a region that is brighter than the rest, but it is still fuzzy. And confocal deconvolution will clean all that and leave you with a more crisp image. Now, this is a very oversimplified way of describing this. Maybe we will go to the next question and then come back to a Achyusman. Deep, can you go on with your query?

Deep: Sir, I was asking in the deconvolution as it said that it makes a prediction. So, are there error rates in this?

Professor Nagaraj: Yes, yes, yes, yes. Yes, yes, of course, of course, there are, right. So, the quality of deconvolution is, lies in the power of the algorithm and how it calculates, what it should think as the specific image and what it should consider as the background. And that is why deconvolution algorithms that are written companies that write them or teams that write them claim or tell you what exactly is the mechanism or the method that they are using to calculate this, and why a method is better than the other one.

But they are fraught with errors, obviously, but they also evaluate this based on different aspects, particularly, for example, what should be the bleed through, how many molecules should be present at a particular point to be able to get a certain amount of bleed through. So, there is a certain set of calculations that are made with real images also to evaluate the nature of deconvolution and the quality of the outcome it generates.

But you are right, what you are seeing is eventually a modified version of the parent image. And there is a certain confidence in saying that this is closer to the real thing. But we do not know. Now, there are tools for example, and I did not get into that here, where you can do, you can combine a fluorescence image with something like an electron microscopy image, and I, we did not talk about electron microscopy at all here.

And electron microscopy clearly is probably the benchmark for a lot of the imaging that you want because of the quality of the image that you get. And so you can kind of take a fluorescence image and compare it to an electron microscopy image. You can take the same fluorescence image, deconvoluted it compare it to the electron microscopy image of the same thing and see what actually looks closer to the real thing. And so, evaluations like that have been done to say why deconvolution by a particular algorithm actually is good or not.

Deep: Sir, I wanted to know about the different contrasts of fluorescence images that you showed. What is their significance, like we use them to see different properties of the, of what we see or there is something different.

Professor Nagaraj: Good question. So, you can do both. You can use fluorescence microscopy to largely pinpoint a particular thing inside the cell. It could be, for example, there is a dye called DAPI that goes and labels the entire nucleus because it actually binds nucleic acid and it will fluoresce very brightly in the nucleus, and this can be used to kind of visualize the nucleus.

Now, depending upon the resolution you have, you can, and the kind of staining that you use, you can also use the DAPI to go see structures inside the nucleus if you decide to. Similarly, there are dyes that go to the plasma membrane, that go to the Golgi to other organelles. The thing to also consider here is fluorescent dyes have been modified in such a way and there are tools like FRET and I did not talk about this, which essentially takes two fluorophores and allows them to come together in such a way that when a protein molecules conformation changes, these two come close, and now this emits light that this absorbs, and then this emits light in a completely different wavelength.

So, you are able to see activate fluorescence with this wavelength, and detect fluorescence of this, which lets you see how close these two things are, which then lets you see the conformation of the molecule that they are attached to. So, if them coming close together makes, means the

molecule is folded in such a way that it has become active, then now this imaging will let you see the activation status of the molecule. So, you are able to do that as well using fluorescence microscopy. Harsh, your question.

Deep: Sir, I have another doubt that, what is the full form of DIC in that confocal?

Professor Nagaraj: So, I will, let me pull this up. It is differential interference contrast, which is essentially a technique that is a way to introduce contrast in the image. And again it uses a set of condensers and objectives to be able to do it. Harsh, your query next.

Harsh: I had a doubt in confocal microscopy. In that how can we see the interior of a cell?

Professor Nagaraj: So that you are not actually seeing the interior of a cell. You are actually seeing a plane inside the cell. So, if this is your entire cell, as long as you illuminate one plane, the fluorescence light is coming and hitting that plane. It will see whatever is in that plane. And in that plane if there are fluorescent things, it will be able to see everything that is in that plane.

Now, it shoots another image, which is the neighboring plane, which are right next to each other. Now, this plane, again, it will illuminate, it will record everything that is there and it will keep doing this for different sections. So, what you are seeing is a section in that particular plane. Now, if that plane is inside the cell, then you will see what is inside. If the plane is like, if this is the cell and you are illuminating the surface of the cell, you will be able to see what is on the surface. So, it is essentially recording image in a particular plane. And that plane, depending upon where it sits, will let you see what is in that plane.

Harsh: But sir if the plane is inside a cell, then how can a light pass through the cell?

Professor Nagaraj: Light can go through cells. A laser can easily penetrate a cell, which is one more reason why some of the microscopes that are now being developed, part of the challenge is when you use a very powerful laser to go illuminate a cell, you actually damage the cell because of the light going into the cell and acting on that particular plane. So, things like a confocal actually give you visualization capability. But it also limits you by the fact that you may actually harm the cell.

You cannot keep the cell alive for significant periods of time after confocal microscopy. In many cases, we do confocal microscopy to begin with in fixed cells, cells that have actually been treated with something such that they are no longer alive. But confocal microscopy can now be done on live cells as well. And there are more advanced tools which need less intensity lasers to illuminate a plane, and also have better recording systems. That means even a small amount of light is captured better.

And so you are able to use less light to kind of shine on a particular plane to be able to see it better. And so you are able to do live cell recordings without actually harming the cell. So, in studies with embryo, there are labs in IISER, for example, that look at Drosophila embryo. You are able to visualize live events that are happening in entire embryos because of these capabilities. But you can see inside the cell, if that is a query.

So, right now, to the best of my knowledge, there is not a method that uses radioactive tagging to visualize stuff, simply because one, there are significant challenges in being able to radio tag something. But as long as you have a detector that collects the radioactive content, you should be able to visualize it. So, technically it is possible. But is there a microscope that actually does this, probably not.

The other challenge with radioactive tagging is that it may actually harm the cell. And this happens even with something like a laser that we shine on cells. And so, if you are able to, if it damages the cell too much, you being able to visualize it becomes really challenging to do. So that could be one of the limitations in being able to radio probe stuff. But as long as you have a detector, technically it is doable, in my opinion.

Where is radioactive tagging used? So, radioactive tagging is actually used to label cells for certain specific signatures, and this could be activation of something, for example, and then cells are lysed. And then you can either pull down the protein or you can take the entire lysate and do a reading of the radioactive content to ask how much of the probe has been incorporated. And the amount of probe that is incorporated will allow you to say, okay, there is activation of a certain pathway or a process depending upon the radioactive probe that you use. So, that is probably where something like this becomes useful. And there are many places that radioactive probing is still used, is still being used.