Cellular Biophysics
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Experimental Techniques to Quantify Cells

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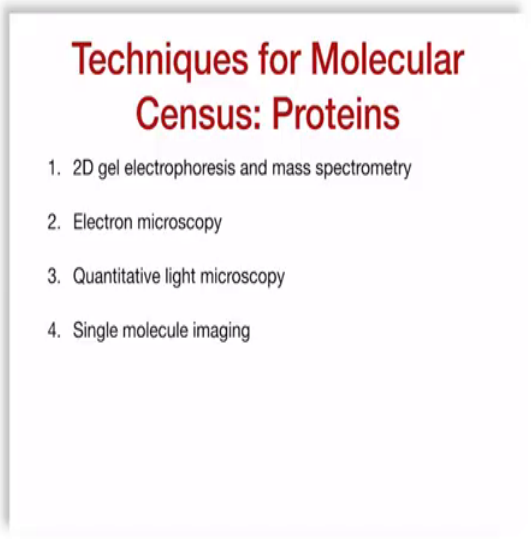

So, as we were saying we want techniques that allow us to compare our order of magnitude estimates, the qualitative picture, the approximation with some precise numbers and for molecular census, meaning numbers about the number of proteins, lipids, biomolecules, et cetera we want methodologies that allow us to accurately quantify these.

Now, one of the numbers that we talked at length about is the protein abundance in a cell in *E. coli* and the most powerful technique for examining protein abundance was 2D gel electrophoresis followed by mass spectrometry. So, we are going to go to that first.

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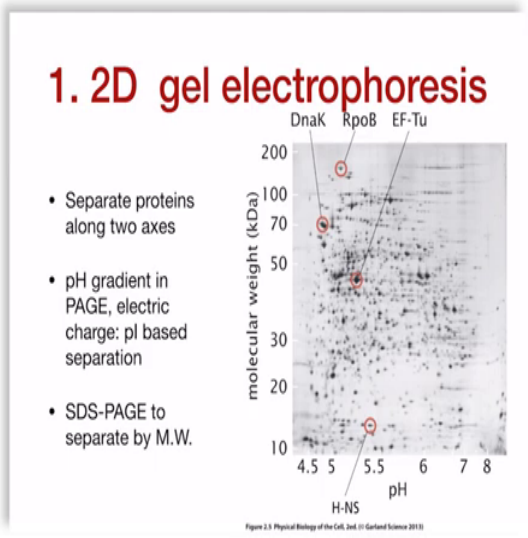

What is 2D gel electrophoresis? So, hopefully many of you at least have come across, as this page as it is called sodium dodecyl sulfate, polyacrylamine gel electrophoresis. This is a one-dimensional gel electrolysis. So, what is 2D over here? It is 2 dimensions, the first dimension is a pH gradient using the isoelectric point of an amino acid to separate it.

After that is done, this is done with usually a slab of gel material, so you separate along one axis, you turn that axis around and layer that gel onto a denaturant gel which is nothing but SDS-PAGE that we refer to earlier and separate now along the second axis. The first one isolates according to the pi at the isoelectric point.

The second dimension isolates according to molecular weight. This leads you to have this kind of a picture that looks complex at first sight, but either through mass spectrometry or through antibody staining or many other methods you can identify the exact nature of these proteins, that is how these circles have been drawn. Identifying DnaK, RpoB, EF-Tu and H-NS as examples of four proteins from *E coli*.

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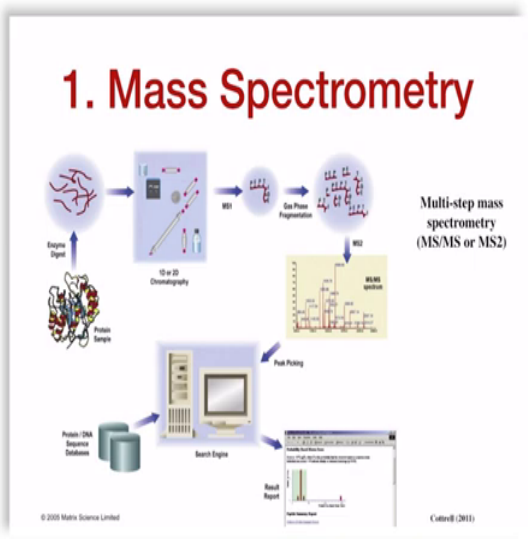

What about mass spectrometry? So, in terms of methodology for protein isolation mass spectrometry involves taking a protein sample, proteolytically digesting it, typically it would be trypsin performing a 1 or 2 dimensional chromatography to separate the fragments and run a mass spectrum on it.

Gas phase fragmentation then allows us to see these beautiful peaks that you see in the right part of this figure which in turn signatures of what polypeptides are present. The methodology of using these spectral peaks is based on optimizing the enzyme digestion so that the typical size is a few amino acids long of each fragment. This is sometimes referred to as a fingerprint, this spectrum because it is characteristic for a given protein.

But we do not end here. If you now feed it into a computer which is connected to a search engine of a DNA protein database then the software typically designed for these kind of problems is capable of doing peak picking meaning identifying the peaks, matching them with the database and predicting a result that is your expected protein sequence, this is essentially nothing but identifying the proteins.

You can reverse this method and use this for a protein of a fixed band to also do protein sequencing different from admin degradation for example. So, this is how we get proteins and their identity. By quantifying the amount of protein isolated you can quantify its amount and therefore its concentration also and therefore its abundance in molar units.

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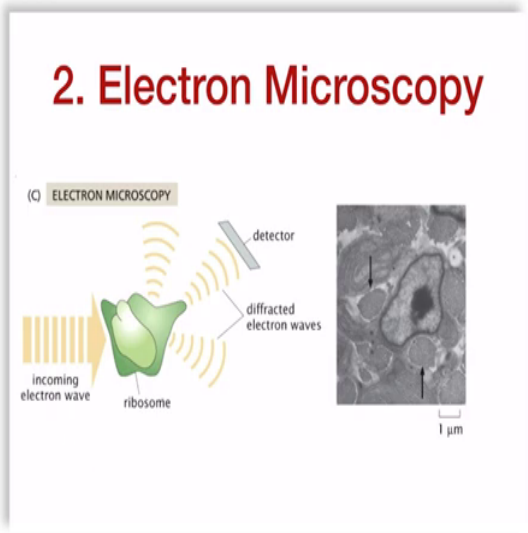

Electron microscopy on the other hand which is again invented out of a curiosity exercise for discovery involves shining an electron beam on objects and detecting the refractive electron waves, these are reconstructed into an image, this image shows you electron dense and electron transparent regions and these are then interpreted spatially. The advantage is that it is far better resolved in nanometer scale than light microscopy.

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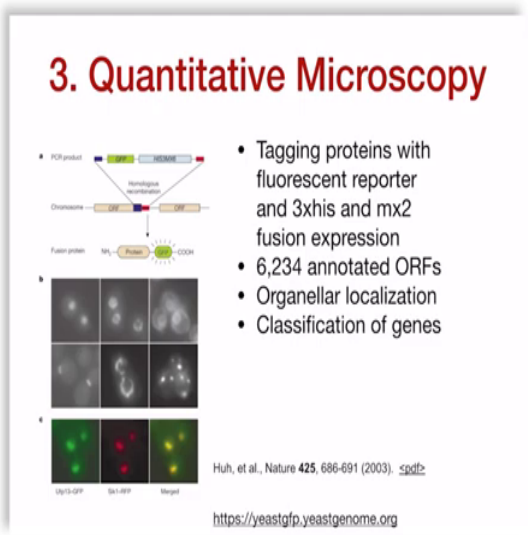

And that brings us of course to quantitative microscopy. This is a very popular method partly because you get not just identity of proteins, for example here, but also their location, not just location but even their dynamics. This is very powerful as you imagine because unlike in the

case of 2D gel electrophoresis in electron microscopy these are fixed snapshots there from a specific time point, whereas this sort of microscopy can give you a whole time series or a spatio temporal map of the molecule of entries.

Typically this has been engineered using a tool which was developed after the discovery of the green fluorescent protein, we have spoken very briefly about green GFP as a typical protein and the idea is to tag an existing gene in the open reading frame you remember we talked about open reading frames as the coding sequences with a another gene resulting in a transcription and followed by translation of a protein that is a fusion protein as it is called.

In this case the protein is tagged with GFP, here at its C terminal or COOH terminal. By using both 3 histidine and mx2 fusion expressions in this group study published in 2003 the group of the authors Huh and company managed to annotate in other words identify that localization and perhaps speculate about the function of 6234 ORFs in terms of open reading frames. This was done of course by introducing this genetic tagging method on the chromosome, on the yeast chromosome.

The organellar localization and classification of genes then becomes very easy because you just look and you can find out. So, for example in this image here, the gene of interest is tagged with GFP, the organelle of interest is tagged in RFP and the co-localization shows that the two are exactly co-localized. You can see more about yeast genome engineering and quantitative microscopy on this website of yeast gfp dot yeast genome dot org.

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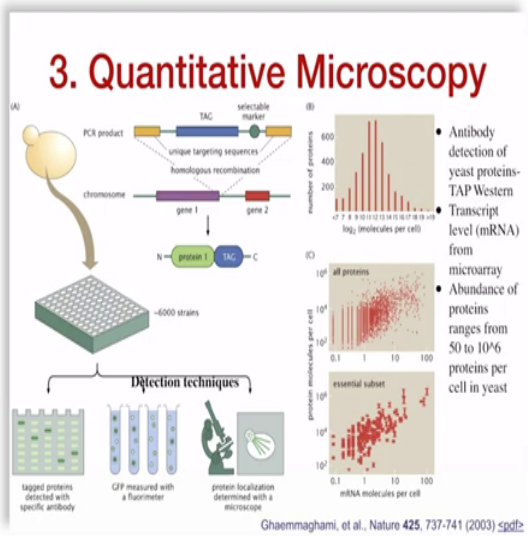

This kind of tagging besides giving you qualitative picture where things are localized can also give you a quantitative picture. So, the idea is to as earlier make a PCR product with unique targeting sequences, the tag and the selectable marker on a sequence such that the unique targeting sequences go to a specific region downstream for example here of the gene.

By homologous recombination they integrate with the genome and because we have chosen the start and stop codons correctly they can produce a protein which is tagged. To do this with about 6000 strains, detect whether the specific tag proteins are actually by antibody, measure with the JP Fluorimeter the fluorescence and localize the position of, localization of the molecule by microscopy.

But we can also look at the number of proteins as a function of the molecules per se. Meaning to say, we get an idea of the abundance. So, the most common abundance is 2^{12}, which is 4096 roughly 4000 molecules per cell. The correlation in fact can also be done, has been done by Ghaemmaghami et al. in 2003 where all proteins were quantified and their abundance correlated with the abundance of MRNA.

So, the MRNA molecules per cell seem largely to linearly correlate and saturate for higher values with respect to proteins. The idea of all proteins versus essential subset is that if all proteins are put however you see an initial flat line, no change with RNA value and then an increase.

Suggesting that there are some proteins for whom the copy number of RNA molecules increase does not result in increase in protein number could be either due to down regulation or lack of correlation, whereas for a subset the essential proteins as they are sometimes called without which the cells would not function divide and go this is a linear relation meaning as much RNA there is those much those many proteins are off..

The abundance meaning to say number of cell, number of proteins per cell ranges between 50 to 10^6 proteins per cell in yeast. The method used for quantification was TAP tag method. The transcript level was estimated using microarrays these are also sometimes called DNA chips or expression arrays. So, you see that the combination of microscopy and microarrays can give us abundances as well as correlations.

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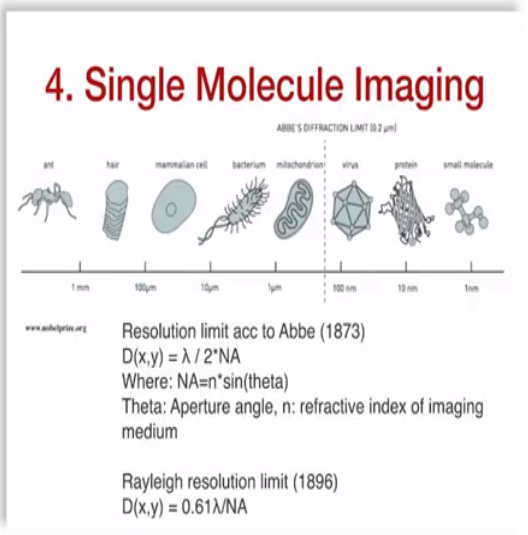

Lastly I want to touch on a method of microscopy that is referred to as single molecule imaging. As the word suggests, single molecule imaging is based on microscopy using light at a scale of a single micro molecule, 1 protein or less and we discussed that an average protein's diameter is about 2 nanometers so about 5 nanometers is the diameter of the protein.

Just to give you an order comparison of the size scales involved the order of magnitude scale on this in this image points to millimeter scale for animals, small animals, small insects through hair lengths, through mammalian cells, mitochondria, viruses at 100 nanometers, proteins and small molecules.

This dashed line here is referred to as Abbe's diffraction, limiter is about 0.2 micrometers or 200 nanometers. Abbe's diffraction limited, limit is an, is a number that was arrived at through physical derivation by Ernst Karl Abbe in 1873 and it states that the distance in x, y between two molecules, two point sources of light that can be successively, successfully resolved is equal λ by 2 times NA, where lambda is the wavelength.

And NA is the numerical aperture of the microscopic setup, NA is also equal to n times $sin\theta$ and θis the aperture angle, n is the refractive index of imaging medium. Rayleigh modified this and came up with a simplification perhaps at this resolving distance D is equal to 0.6 times lambda by NA.

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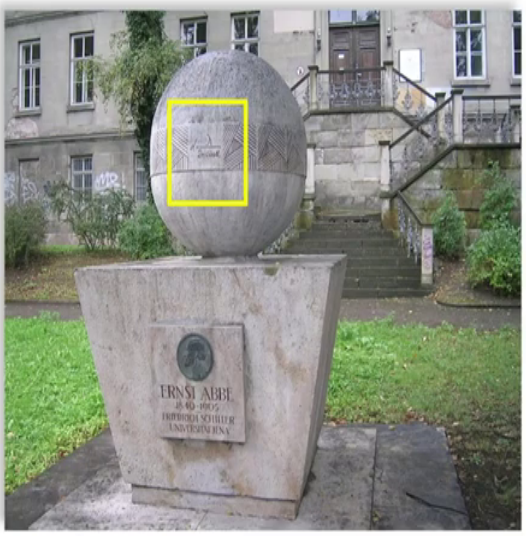

If you ever go to Jena, as it is written in English, I urge you to visit the grave of Ernst Abbe a father of modern biological microscopy and a very important figure in identifying the limit to light resolution, it is very fitting also that his grave stone "Qabristan, Qabar" has the equation that he invented on it, you would always want to have something beautiful like this, your memorial should have the equation you invented.

So, in terms of single molecule imaging a couple of, so the point we were trying to make here is that the physics of light do not permit us to distinguish between two objects smaller than 0.2 microns apart approximately, which means that if two molecules which are only 10 nanometers in size are 10 nanometers apart you will only see a big blob, you will not see two molecules that is what is the meaning of diffraction limit. So, if you want to go beyond the diffraction limit you need to do something.

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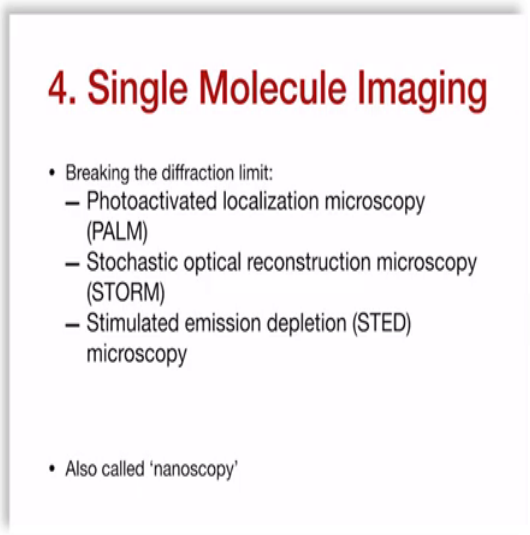

These 4 methods photoactivated localization microscopy PALM, stochastic optical reconstruction microscopy STORM, and stimulated emission depletion microscopy STED, these 3 methods are also collectively called 'nanoscopy' because unlike microscopy they are able to look at things at nanometer scale and separate between.

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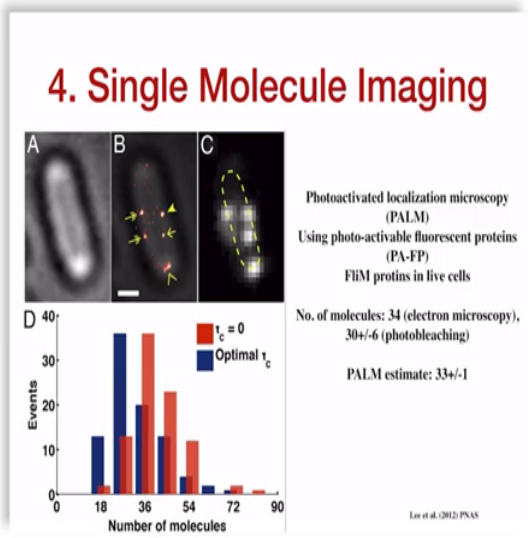

So single molecule imaging as such can be performed for example using photoactivated localization microscopy PALM for which we need not a normal fluorescent protein like green fluorescent protein but a photo activable fluorescent proteins. Photo activable fluorescent proteins are those that with a specific wavelength of light that is not the excitation wavelength they are activated otherwise they are in an inactive state.

And as they stay in the active state if you image them they will give you signal. After the passage of sometime these molecules will revert to their dark state, this dark state means you cannot see them, you cannot see them means they do not show up in a microscopic image which means they are not there. The molecule is there but the light is not there.

And by cyclically activating and inactivating or activating and waiting for the cell spontaneous inactivation of molecules, you can sample a region and get around part of the diffraction limit this was a very clever innovation by Eric Betzig and Jennifer Lippincott-Schwartz, Eric Betzig was given the Nobel Prize for Chemistry along with Stefan Hell.

What you are looking at in the image here is a single bacterial cell with the photoactivated fluorescent proteins showing the number of molecules that could be identified and with the number of molecules being counted based on the PALM estimate compared to electron microscopy.

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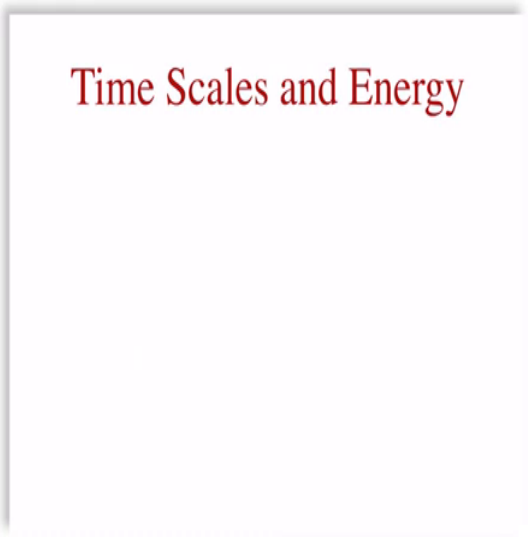

So, in the next segment we are going to talk about time scales.